ANTIBODY-BASED DETECTION AND QUANTIFICATION OF
PECTOBACTERIUM CAROTOVORUM SSP. CAROTOVORUM

A Thesis
Presented to
The Faculty of Graduate Studies
of
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By
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for the degree of
Master of Science

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ABSTRACT

ANTIBODY-BASED DETECTION AND QUANTIFICATION OF
PECTOBACTERIUM CAROTOVORUM SSP. CAROTOVORUM

Melissa Maria Ivana Bassoriello
University of Guelph, 2010

Advisors:
Professor J. Christopher Hall
Professor Theo J. Blom

Pectobacterium carotovorum ssp. carotovorum (Pcc) is implicated in the destruction of ornamental plants in greenhouse recirculating systems. PCR-based detection and quantification of Pcc requires expensive instrumentation and knowledgeable users. This thesis describes the production of polyclonal antibodies and a single-domain antibody fragment (V_{H}{H}) against Pcc lipopolysaccharide (LPS), and the development of user-friendly diagnostic assays for detection and quantification of the pathogen. Polyclonal ELISAs against heat-killed (HK) Pcc (limit of detection (LOD) = 81 CFU/ml; limit of quantitation (LOQ) = 216 CFU/ml) and Pcc LPS (LOD = 23 ng/ml; LOQ = 76 ng/ml) were developed. A preliminary user-friendly dipstick assay was also developed ($\geq 10^5$ CFU/ml). A phage display library was constructed ($6.0 \times 10^5$ clones/ml), yielding one unique anti-Pcc LPS V_{H}{H}. Using the Pcc LPS-specific V_{H}{H} to produce affordable, user-friendly diagnostic assays is feasible since antibody fragments can be produced on a large scale through expression in Escherichia coli or Piccia pastoris.
ACKNOWLEDGEMENTS

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This thesis would not have been possible without the support of my family and friends. To my parents Nick and Angela for their encouragement, advice and financial support, to my sister Rosanna, brother Franco and brother-in-law Glenn, I am grateful for everything you have provided me with even if I have not immediately realized or acknowledged it. Special mention to my niece Alisha, I am very proud of you and wish you only the best in life and throughout your future endeavours. You all mean so much to me.

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“Science is not plannable but an unexpected thing”

-Dr. Heiko Heerklotz

Associate Professor and CRC in Lipid Science and Technology, Faculty of Pharmacy, University of Toronto
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<th>Explanation</th>
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<tr>
<td>A/A&lt;sub&gt;o&lt;/sub&gt;</td>
<td>Ratio of absorbance and maximum absorbance</td>
</tr>
<tr>
<td>A&lt;sub&gt;o&lt;/sub&gt;</td>
<td>Absorbance at zero competition (mean blank)</td>
</tr>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>Ab&lt;sub&gt;1&lt;/sub&gt;</td>
<td>Primary antibody</td>
</tr>
<tr>
<td>Ab&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Secondary antibody (enzyme-conjugated)</td>
</tr>
<tr>
<td>Ag</td>
<td>Antigen</td>
</tr>
<tr>
<td>AMP</td>
<td>Ampicillin</td>
</tr>
<tr>
<td>APCs</td>
<td>Antigen-presenting cells</td>
</tr>
<tr>
<td>BCA</td>
<td>Biological control agent</td>
</tr>
<tr>
<td>BCA-Assay</td>
<td>Bicinchoninic acid protein assay</td>
</tr>
<tr>
<td>BLAST</td>
<td>Basic local alignment and search tool</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CDR</td>
<td>Complementarity determining region</td>
</tr>
<tr>
<td>Cel</td>
<td>Cellulose</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming units</td>
</tr>
<tr>
<td>C&lt;sub&gt;H&lt;/sub&gt;</td>
<td>Constant domain of heavy chain of Ig</td>
</tr>
<tr>
<td>CI-ELISA</td>
<td>Competition/Inhibition ELISA</td>
</tr>
<tr>
<td>C&lt;sub&gt;L&lt;/sub&gt;</td>
<td>Constant domain of light chain of Ig</td>
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ConvIgG  Conventional chain IgG
DMEM   Dulbecco’s modified Eagle’s medium
DMSO   Dimethyl sulfoxide
DNA    Deoxyribonucleic acid
DTT    Dithiothreitol
E. coli Escherichia coli
EDTA   Ethylene diamine tetracetic acid
ELISA  Enzyme-linked immunosorbent assay
ESP    Extracellular polysaccharides
ExPASy Expert protein analysis system
f1     Strain of filamentous phage
Fab    Antigen binding fragment of immunoglobulins
F(ab’)2 Bivalent antigen binding fragment of immunoglobulins
Fc     Crystallizable fragment of immunoglobulins
fd     Strain of filamentous phage
FPLC   Fast protein liquid chromatography
FR     Framework region
Fv     Variable domain binding fragment of conventional immunoglobulins
gIIIp (g3p) Coat protein 3 of filamentous phage (gene 3 protein)
gVIIIp (g8p) Coat protein 8 of filamentous phage (gene 8 protein)
GAR    Goat anti-rabbit
GAR-HRP Goat anti-rabbit horseradish peroxidase
GAL-HRP Goat anti-llama horseradish peroxidase
<table>
<thead>
<tr>
<th>Term</th>
<th>Description</th>
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<tbody>
<tr>
<td>gpIII</td>
<td>Phage minor coat protein gene III</td>
</tr>
<tr>
<td>GRAVY</td>
<td>Grand average hydropathicity</td>
</tr>
<tr>
<td>HA</td>
<td>Hemagglutinin</td>
</tr>
<tr>
<td>HAT</td>
<td>Hypoxanthine, aminopterin, and thymidine media</td>
</tr>
<tr>
<td>HcIgG (HcAb)</td>
<td>Heavy chain IgG from camlid family</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid</td>
</tr>
<tr>
<td>HF</td>
<td>Hi-Flow Nitrocellulose membranes</td>
</tr>
<tr>
<td>HIS</td>
<td>Histidine</td>
</tr>
<tr>
<td>HK</td>
<td>Heat-killed</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>HV</td>
<td>Hypervariable regions</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Inhibitor concentration required to reduce A&lt;sub&gt;0&lt;/sub&gt; by 50%</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin (e.g. IgG)</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IMAC A</td>
<td>10 mM Hepes &amp; 500 mM NaCl buffer</td>
</tr>
<tr>
<td>IMAC B</td>
<td>10 mM Hepes, 500 mM NaCl &amp; 500 mM Immidazole</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl β-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>KAN</td>
<td>Kanamycin</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodaltons</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani media / lysogeny broth (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, pH 7.5)</td>
</tr>
<tr>
<td>LF</td>
<td>Lateral flow</td>
</tr>
<tr>
<td>LOD</td>
<td>Limit of detection</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>LOQ</td>
<td>Limit of quantitation</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>M13</td>
<td>Strain of filamentous phage</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MCS</td>
<td>Multiple cloning site</td>
</tr>
<tr>
<td>MDx</td>
<td>Millenia Diagnostics, Inc</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>Magnesium chloride</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MPBS</td>
<td>Skim milk &amp; phosphate buffered saline</td>
</tr>
<tr>
<td>MT</td>
<td>Melting temperature</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>NB</td>
<td>Nutrient broth</td>
</tr>
<tr>
<td>NEB</td>
<td>New England Biolabs</td>
</tr>
<tr>
<td>o/n</td>
<td>Overnight; 12-16 h</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>OD₄₅₀</td>
<td>Optical density absorbed at wavelength of 450 nm</td>
</tr>
<tr>
<td>PaNie</td>
<td><em>Pythium aphanidermatum</em> Necrosis inducing elicitor</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline (1 x PBS; 8 g NaCl, 1.44 g Na₂HPO₄, 0.24 g KH₂PO₄, 0.2 g KCl in 1 L dH₂O; pH 7.4)</td>
</tr>
<tr>
<td>PBST</td>
<td>Phosphate buffered saline + 0.05% v/v Tween-20</td>
</tr>
<tr>
<td>Pcc</td>
<td><em>Pectobacterium carotovorum</em> subsp. <em>carotovorum</em></td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>Pel</td>
<td>Pectate lyase</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
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<td>------</td>
<td>------------</td>
</tr>
<tr>
<td><strong>PEG</strong></td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td><strong>PEG-NaCl</strong></td>
<td>Polyethylene glycol-sodium chloride</td>
</tr>
<tr>
<td><strong>Peh</strong></td>
<td>Polygalacturonase</td>
</tr>
<tr>
<td><strong>PFU</strong></td>
<td>Plaque forming unit</td>
</tr>
<tr>
<td><strong>Pnl</strong></td>
<td>Pectin lyase</td>
</tr>
<tr>
<td><strong>Prt</strong></td>
<td>Protease</td>
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<td><strong>rDNA</strong></td>
<td>Recombinant DNA technology</td>
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<td><strong>RNA</strong></td>
<td>Ribonucleic acid</td>
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<td><strong>RT</strong></td>
<td>Room temperature; 24-26°C</td>
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<tr>
<td><strong>RT-PCR</strong></td>
<td>Reverse transcription followed by PCR</td>
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<tr>
<td><strong>scFv</strong></td>
<td>Single chain Fv</td>
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<td><strong>SDS-PAGE</strong></td>
<td>Sodium dodecyl sulphate-polyacrylamide gel electrophoresis</td>
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<td><strong>SOC medium</strong></td>
<td>Super optimal catabolite repression medium (20 g/L tryptone, 5 g/L yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgSO₄, 10 mM MgCl₂, 20 mM glucose, pH 7.0)</td>
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<td><strong>Tₐ</strong></td>
<td>Annealing temperature</td>
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<td><strong>TₜH</strong></td>
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<td>3,3′,5,5′-tetramethyl benzidine</td>
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<td>2-Amino-2-hydroxymethyl-propane-1,3-diol</td>
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<td><strong>Tween 20</strong></td>
<td>Polyoxyethylene (20) sorbitan monolaurate</td>
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<td><strong>VₜH</strong></td>
<td>Variable domain of heavy chain of Ig</td>
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<td><strong>V&lt;sub&gt;H&lt;/sub&gt;H</strong></td>
<td>Variable heavy domain from HcIgG (single chain Ab)</td>
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<td>Weight/volume (g/ml expressed in %)</td>
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<tr>
<td><strong>YT</strong></td>
<td>5 g/L yeast extract, 8 g/L tryptone, 2.5 g/L NaCl, pH 7.2</td>
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1. GENERAL INTRODUCTION, RATIONALE AND RESEARCH OBJECTIVES

1.1. Introduction

The gram-negative bacterium *Pectobacterium carotovorum* ssp. *carotovorum* Jones (*Pcc*) is the causal agent of soft rot diseases of a variety of ornamental and vegetable species in greenhouses (Pérombelon and Kelman, 1980; Wright, 1998; Ravensdale, 2007). As recently as 2009, the Ontario greenhouse industry was valued over $1.9 billion, and soft rots were responsible for an estimated loss of up to 20% for several crops (OMAFRA, 2009).

A significant part of the greenhouse industry routinely recirculates nutrient solutions, which ultimately increases the incidence and severity of disease due to *Pcc* and other pathogens such as *Pythium* and *Fusarium* (Schuerger, 1998; Blom, 2003). Consequently, there is a need to reduce the amount of inoculum circulating in hydroponic systems and, therefore, reduce the amount of disease caused by *Pcc*. A rapid, user-friendly, pathogen-specific and quantitative tool for capturing and detecting *Pcc* in greenhouse hydroponic systems would be extremely beneficial to the greenhouse industry.

Current methods for controlling *Pcc* are labor and cost prohibitive, and rarely provide adequate control of the pathogen, especially when dealing with nutrient re-circulation (Howard *et al.*, 1994). Stringent sanitation, including purging of re-circulated solutions and the use of excessive chlorine can lead to high environmental impacts. An inexpensive, user-friendly paper-based detection and quantification tool could allow growers to act immediately in controlling the harmful pathogen and to make decisions if further control measures are necessary (i.e., chemical, biological, or cultural). This
would reduce the risk of the harmful bacteria and in turn lower the environmental impact, by allowing growers to use preventative measures only when necessary.

Current detection and quantification methods for microorganisms including \textit{Pcc} are labor-intensive, costly and/or inaccurate. Polymerase chain reaction (PCR) has been the commonly practiced method for detecting \textit{Pectobacterium} spp., which can detect extremely low levels of the bacteria (i.e., $\leq 4$ CFU/ml; Kang \textit{et al.}, 2003); however, PCR is labor-intensive and generally requires users with advanced knowledge of the methods. Immunoblot and enzyme-linked immunosorbent assay (ELISA)-based detection systems, using pathogen specific monoclonal antibodies for various \textit{Pectobacterium} ssp., are commercially available with a low range of detection (i.e., $10^3$ CFU/ml). Traditionally, ELISA is one of the most sensitive and frequently used methods for estimation of pathogens (i.e. bacteria) and lipopolysaccharide (LPS) molecules (Hill and Matsen, 1983; Freudenberg \textit{et al.}, 1989). Consequently, specific antibodies can be used to capture and quantify pathogens and their toxins in greenhouse hydroponic solutions. Antibodies (Ab) have historically proven to be excellent diagnostic and therapeutic tools due to their small size, ability to withstand high temperatures and ability to remain stable in aqueous solutions. Animal immunization has provided a wealth of valuable antisera and antibodies as research agents (Arbabi-Gharoudi \textit{et al.}, 1997; Frenken \textit{et al.}, 2000; Van der Linden \textit{et al.}, 2000; Jobling \textit{et al.}, 2003).

Polyclonal antibodies are a combination of immunoglobulin (Ig) molecules produced against specific antigens (Ag) with each Ig recognizing a different epitope on the Ag. A monoclonal antibody (mAb) is a specific Ab (clone) with affinity for a unique and specific antigen (epitope). Conventional hybridoma techniques have been
traditionally used to generate mAbs, allowing for the selection of high affinity antibodies against antigens of choice. Camelidae sp. (e.g., llamas), unlike humans, produce conventional antibodies and rare heavy chain antibodies (HcIgG/HcAb), which have been used to generate mAb fragments known as single chain antibody fragments (V<sub>H</sub>H). These V<sub>H</sub>H molecules, composed of the variable heavy chain domain of HcAbs, are very small (ca. 15 kDa) antibody fragments. Due to their small size, V<sub>H</sub>Hs have, in some cases, an increased binding capacity in that they have the ability to recognize typically inaccessible epitopes; they are also extremely stable at high temperatures (Harmsen et al., 2009; Lauwereys et al., 1998).

Whole bacterial cells (i.e., Pcc) and lipopolysaccharide (LPS), the major surface component of gram-negative bacteria specifically implicated in plant pathogenesis, are major antigens capable of initiating an immune response in various animal hosts. Thus, whole heat-killed (HK) Pcc cells (with LPS molecules), as well as purified Pcc LPS molecules, appear to be appropriate candidates for the detection and quantification of the Pcc pathogen or Pcc LPS in greenhouse hydroponic solutions. In theory, the shear abundance of LPS (ca. 3.5 million molecules) on the surface of each Pcc cell makes these molecules excellent candidates for use in aqueous detection applications, likely increasing the sensitivity of Pcc (LPS) detection and quantification.

1.2. Research Objectives

The research conducted for this thesis encompassed the overall goal of developing rapid user-friendly assays for the detection and quantification of Pcc (LPS) in greenhouse hydroponic solutions, using animal-derived polyclonal and single chain antibody
fragments (V_H). Since specific microorganisms (whole bacterial cells and bacterial components) can initiate immunological responses in animals, it was hypothesized that i) high affinity polyclonal serum could be developed and characterized against HK Pcc and Pcc LPS (chapter 3) and ii) high affinity V_Hs against Pcc LPS could be isolated from an immune library (chapter 4); with one or both types of Abs being used to develop a specific, rapid, user-friendly assay. To investigate these hypotheses, along with creating a reliable detection tool for Pcc, several research objectives were established and addressed in chapters 3 (i-v) and 4 (vi & vii) of this thesis. These objectives are:

i. Development and characterization of polyclonal rabbit serum raised against heat-killed Pcc and Pcc LPS to determine which elicits a more specific response to Pcc;

ii. To determine whether cross-reactivity exists among antibodies specific to Pcc and Pectobacterium spp. or other microorganisms that may be present in greenhouse hydroponic solutions;

iii. Development of polyclonal rabbit serum-based ELISA for detection and quantification of Pcc;

iv. Development and optimization of a rapid, user-friendly colorimetric dipstick assay using polyclonal rabbit serum antibodies against HK Pcc and Pcc LPS;

v. Assessment of ELISA and a dipstick assay using various greenhouse hydroponic solutions;

vi. Construction of an immune V_H library from a llama hyper-immunized with heat-killed Pcc and Pcc LPS;
vii. Selection (panning) of high-affinity and specific antibody clones (V\textsubscript{H}H) to \textit{Pcc} LPS through phage-display technology.
2. **LITERATURE REVIEW**

2.1 **Context**

The gram-negative bacteria *Pectobacterium carotovorum* ssp. *carotovorum* (*Pcc*) is implicated in the damage and loss of a number of ornamental plants and vegetable species in greenhouse-based hydroponic systems, resulting in worldwide economic loss. This pathogenic bacterium, which is implicated in soft rot disease, causes the maceration of host tissues through the activity of cell wall degrading enzymes (Pérombelon and Kelman, 1980; Wright, 1998). Soft rots caused by *Pcc* amount to an estimated annual loss of 12% to 20% of several crops for greenhouse growers in Ontario, Canada alone (Ravensdale, 2007; OMAFRA, 2009).

2.2 **The genus *Erwinia***

First described in 1917, the genus *Erwinia* incorporated all members of the family Enterobacteriaceae that causes disease on plants, a designation given irrespective of their relatedness to other members of the family (Kõiv and Mäe, 2001; Toth *et al.*, 2003). This inclusive grouping of species has caused many nomenclatural difficulties and has led to the relocation of various species into other genera (Toth *et al.*, 2003). The genus *Erwinia* now consists of 18 species falling into two main groups, the necrogenic, or *Amylovora*, group and the soft rot, or *Carotovora*, group. Within the soft rot group, *Erwinia carotovora* and *Erwinia chrysanthemi* are the most commercially important soft rot pathogens. Until the early 1980s, *E. carotovora* contained two subspecies, *Erwinia carotovora* ssp. *carotovora* (*Ecc*) and *Erwinia carotovora* ssp. *atroseptica* (*Eca*) (causing blackleg and storage rot of potato plants). More recently, *Erwinia carotovora* ssp.
betavasculorum (causing soft rot in sugar beet), *Erwinia carotovora* ssp. *wasabiae* (causing soft rot in various vegetables), and *Erwinia carotovora* ssp. *odorifera* (causing soft rot in leek and celery) were included in the *Carotovora* group (Toth *et al.*, 2003).

Harbaugh *et al.* (1998) suggested that the soft rot *Erwinias*, primarily *E. carotovora*, *E. atroseptica* and *E. chrysanthemi*, be reclassified into the genus *Pectobacterium*, based on 16S ribosomal DNA (rDNA) sequence analysis (Chatterjee and Starr, 1980; Harbaugh *et al.*, 1998; Pérombelon, 2002; Toth, 2003; Smadja *et al.*, 2004). The proposed species within this new genus would include *Pectobacterium carotovora* ssp. *atroseptica* (*Pca*), *Pectobacterium carotovorum* ssp. *carotovorum* (*Pcc*) and *Pectobacterium chrysanthemi*. Interestingly, the name *Pectobacterium* delineates from the main pathogenic feature of soft rot *Erwinias*, the breakdown of host pectins by hydrolytic enzymes, pectinases (Avrova *et al.*, 2002). Given its devastating effects on greenhouse crops in Ontario, *Pcc* will be further discussed in detail.

2.2.1 Physiology

*Pectobacterium carotovora* ssp. *carotovora* (*Pcc*) is a gram-negative plant pathogenic bacterium measuring 0.5 to 0.8 µm by 1.0 to 3.0 µm (Howard *et al.*, 1994; Raven *et al.*, 1999). *Pcc* has a rod-shape consistent with other gram-negative bacteria, along with peritrichous flagella and fimbriae that allow for motility and adherence to host tissues (Raven *et al.*, 1999; **Figure 1**). Furthermore, these facultative anaerobes exhibit both respiratory and fermentative metabolisms (Avrova *et al.*, 2002).
The main body of *Pcc* consists of a rigid peptidoglycan layer overlaid by an outer membrane. Lipopolysaccharide (LPS), phospholipids, porons and lipoproteins are the antigenic constituents of the external layer of the cell envelope or outer membrane.

**Figure 1.** Characteristic shape of gram-negative bacteria. Gram-negative bacteria, such as *Pcc*, have rod-shaped cellular structures with flagella (pili) and fimbriae attached. Flagella and fimbriae allow for bacterial motility and adherence to host tissues, respectively (Source: University of Newcastle, 2005).

(Yi and Hackett, 2000). LPS or endotoxin is the most immunoreactive component of the organism’s surface, with the ability to activate a variety of biological responses including plant pathogenesis (Raven *et al.*, 1999; **Figure 2**). Along with this ability to activate pathogenesis, LPS also has a role in membrane permeability, resistance to phagocytosis,
Figure 2. Bacterial membrane structure and components. The outer membrane of *Pce* contains the immunoreactive LPS, proposed to activate pathogenesis (Source: University of Ottawa, 2002).

and as a receptor for adsorption of bacteriophages (Chatterjee and Starr, 1980; Darveau and Hancock, 1983).

LPS generally consists of three distinct regions covalently attached to one another: the lipid A, the core oligosaccharide and the O-polysaccharide or O-antigenic side chain (Darveau and Hancock, 1983; Poxton, 1995; Yi and Hackett, 2000; Figure 3). Inserted directly into the outer surface of the outer membrane is the hydrophobic lipid A containing diglucosamine phosphate with five or six fatty acyl chains. A significant portion of the fatty acyl chains are unique to LPS in gram-negative bacteria. The oligosaccharide and O-antigenic side chain consists of 10 to 12 sugars and
repeating saccharide units, respectively, with the O-antigenic component determining serotype specificity (Darveau and Hancock, 1983).

**Figure 3.** Main body of *Pcc* consists of a rigid peptidoglycan layer overlaid by an outer membrane containing LPS and its three distinct regions: the O-antigen, core oligosaccharide and the hydrophobic lipid A. The O-antigen side chain is responsible for determining serotype specificity (Adapted from Tritz, 2000).

### 2.2.2 Occurrence and Disease Symptoms

*Pcc* is a typical plant pathogen of temperate regions around the world, including Ontario during early spring to late summer months. As well, the frequent use of greenhouse systems to grow various crops has meant that infection with *Pcc* can occur year-round provided that the bacterium is present. Its pathogenicity has been reported in many flowering species and vegetables including: calla lily (*Zantedeschia* ssp.),
poinsettia (*Euphorbia pulcherrima*), tomato (*Lycopersicon esculentum*), pepper (*Capsicum annuum*), potato tubers (*Solanum tuberosum*), carrot (*Daucus carota*), onion (*Allium cepa*) and lettuce (*Lactuca sativa*). Infection by *Pcc* causes head rot, stem rot and soft rot diseases in these vegetables and flowers (Howard *et al.*, 1994; Wright, 1998).

Soft rot development associated with *Pcc* in potato tubers, carrot, onion and lettuce generally begins as water-soaked lesions and later bacterial coalesce on the outer plant surfaces. The affected areas become soft, with the lesions darkening (or browning) when exposed to air, and finally becoming opaque and slimy (Toth *et al.*, 2003; **Figure 4**). Tissues macerated by lytic enzymes secreted by *Pcc* can ooze from cracks formed on affected tissues. Odor associated with bacterial soft rot is normally not apparent until the tissues collapse and are invaded by secondary organisms (other bacteria and insects; Pérombelon and Kelman, 1980).

In addition to symptoms mentioned previously, *Pcc* soft rot disease specifically affects the stems and fruit of tomato and pepper plants. In pepper, the stem discolours and several small dark lesions develop, thereafter becoming slimy. Tomato fruit also fills with a watery, soft mass kept intact by the thin outer skin. When the skin breaks, the fruit collapses and dries into a wrinkled mass of flesh and skin (**Figure 5**). Tomatoes can also be affected by stem rot which causes dark lesions, wilting and plant death, while hollowing out the base of the stems (Howard *et al.*, 1994).
Figure 4. Calla lily tuber (a) uninfected; healthy tissue, and (b) infected with *Pcc*, causing bacterial soft rot. Symptoms of *Pcc* infection are macerated and mushy tissue (b); when infected tissue is exposed to the air a strong ‘garbage-like’ odor is present (Photos by M. Bassoriello, 2007).

Figure 5. Bacterial soft rot of tomato fruit (a) and stem rot (b) caused by *Pcc* (see arrows). Areas infected with *Pcc* become water-soaked and soft, with tissue and lesion browning when exposed to air (From Howard *et al.*, 1994).
2.2.3 Pathogenicity, Mode and Mechanism of Action

2.2.3.1 Pathogenicity – LPS, enzymes & fimbriae

Plants continuously defend themselves against attack by bacteria and other pathogens (Hammond-Kosack and Jones, 2000; Dickinson, 2003). Bacteria cause rots, spots, wilts, cankers and blights by colonizing the plant apoplast. Plant tissue is extremely vulnerable to extracellular polysaccharides and cell wall-degrading enzymes secreted by Pcc. Extracellular polysaccharides (EPSs) such as LPS may aid bacterial virulence, perhaps by saturating intracellular spaces with water or by inhibiting xylem function to produce wilting (Pirhonen et al., 1988). However, a study by Perombelon (2002) and supporting literature by Hammond-Kosack and Jones (2000) suggest that EPSs are not absolutely required to play a specific role in pathogenesis.

Roughly 3.5 million LPS molecules form the outer surface of individual gram-negative bacterial cells, providing a penetration barrier to molecules larger than 700 – 1000 Daltons. Kotra et al. (1999) visualized the surface of E. coli JM109 by atomic force microscopy, revealing that LPS molecules are assembled in bundles of 600 – 3500 molecules. The chemical structure of LPS is composed of lipid A, an inner and an outer core, and the repeating units of O-Antigen (O-specific oligosaccharide) (Figure 6). The number of O-antigen repeats is the characteristic of any given strain of bacterium and could possibly range from 0 to 40 in some gram-negative bacteria (e.g. Escherichia coli). Individual LPS molecules on the surface of the bacterium are held together by electrostatic interactions, hydrophobic interactions, and hydrogen bonds. Divalent metal ions such as Mg$^{2+}$ and Ca$^{2+}$ ions bridge the anionic functionalities of the inner and outer core region of LPS (Raetz, 1990; Kotra et al., 1999; Raetz, 2002). Lugtenberg and Van
Figure 6. The chemical structure of LPS. Ions such as Mg$^{2+}$ and Ca$^{2+}$ bridge the inner and outer core oligosaccharides.

Alphen (1983) and Vaara (1992) reported that removal of these metal ions from the surface of the bacterium by chelating agents such as ethylenediaminetetraacetate (EDTA) increased the permeability of the membrane.

Increased virulence can be caused by the production of exoenzymes by Pcc. These hydrolytic enzymes initiate degradation of the plant cell wall by cleaving cell wall polymers by hydrolysis or β-elimination of ester bonds (Rosanas et al., 1995; Hammond-Kosack and Jones, 2000; Aguilar et al., 2002; Toth and Birch, 2005). In particular, enzymes such as pectate lyase (Pel), polygalacturonase (Peh), cellulase (Cel), pectin lyase (Pnl) and proteases (Prt) are major determinants that contribute to the pathogenicity and virulence of Pcc (Park et al., 1997; Vincent-Seally et al., 1999; Matsumoto et al., 2003; Hossain et al., 2005; Manatunga et al., 2005). Pectinases, considered to be the most important enzymes in pathogenesis, cause tissue maceration and, indirectly, cell death (Pérombelon, 2002). Decreased pectinase activity, as well as Pcc mutants deficient in functional Peh, Pnl and Pel genes, exhibit reduced bacterial virulence (Palva et al., 1993; Flego et al., 1997; Hammond-Kosack and Jones, 2000). Palva et al. (1993) indicated that
exogeneous application of pectinases to plant material reproduced soft rot symptoms characteristic of *Pcc*.

Considered to be an important factor for gram-negative animal pathogens, fimbriae or pili (thin, hair-like appendages made of protein subunits) allow for the adhesion of the pathogen to the host tissue (Perombelon, 2002; Boyle and Finlay, 2003; Figure 1). Seven types of fimbriae (I to VII) have been distinguished based on their length and width. In the Enterobacteriaceae family, Type I fimbriae bind bacteria to roots of grass (Boyle and Finlay, 2003). Boyle and Finlay (2003) suggested that fimbriae are pathogenicity factors on pathogenic strains of *E. coli*. Specific pathogenicity is yet to be confirmed for *Pcc*.

2.2.3.2 Mode and mechanism of Action

*Pectobacterium carotovorum* ssp. *carotovorum* causes soft rot by invading tubers and stems through wounds and cracks in the periderm caused by mechanical, freezing, insect or further mechanical injury (Pérombelon and Kelman, 1980; Howard *et al.*, 1994). Bacteria can also enter through natural openings such as lenticels in the surface of tubers. Once within the tuber, *Pcc* can survive for several months in a dormant state (Pérombelon and Kelman, 1980). While the exact cause of bacterial dormancy in the tuber is unknown, latent bacterial infection ceases once inhibiting factors are overcome (Pérombelon, 2002; Ravensdale, 2004; Snijder *et al*. 2004). Primarily, the presence of water on the tuber surface triggers the switch from latent to active infection. This has been attributed to the creation of anaerobic conditions within the tuber, thus, impairing oxygen-dependent host resistance and the synthesis of lignified materials and increased
membrane permeability resulting in nutrient leakage (Pérombelon, 2002; Ravensdale, 2004).

Active *Pcc* multiplies rapidly at temperatures ranging from 18°C to 27°C in plants and 24°C to 32°C in culture. *Pcc* moves readily in soil and splashing rain, and can be dispersed by irrigation water or hydroponic solutions (Howard *et al.*, 1994; Gracia-Garza *et al.*, 2002, 2003). In addition to spread through hydroponic solutions and soil, *Pcc* can spread from infected to healthy tubers under poor ventilation or high humidity situations during storage and transit (Howard *et al.*, 1994).

2.2.3.3 Associated disease

Research dating back to the early 1900s suggests that infection by *Sclerotinia sclerotiorum*, *Botrytis* sp., and *Rhizoctonia* sp. can predispose various plants to infection by soft rot bacteria. In potatoes, wounds arising from mechanical damage, frost injury or the presence of ring rot, late blight, leak or blackleg disease can allow invasion by *Pcc* (Howard *et al.*, 1994; Figure 7). Insects such as fruit flies and ants have also been associated with *Pcc* (Pérombelon and Kelman, 1980). After insects have laid eggs over seed tubers already infected by *Pcc*, contaminated larvae carry the bacteria into the tuber. Once the bacteria have infected the tuber, the bacteria may spread into the stems and roots (Howard *et al.*, 1994; Agrios, 1997).
Figure 7. Blackleg disease of potato tubers. Plants previously infected with bacteria and other pathogens causing blackleg, ring rot and late blight can predispose plant tissues to infection by \textit{Pcc} (Taken from Howard et al., 1994).

2.2.4 Disease Control

2.2.4.1 Cultural practices

The primary means of preventing bacterial soft rot of hydroponic greenhouse crops and specific field crops are stringent sanitation and environmental control. Prior to planting, moisture and water sources should be regulated, preventing \textit{Pcc} from thriving and creating an outbreak. Specifically, plants should be grown in well-drained soil; moisture should not be allowed to accumulate on roots; and excessive irrigation and prolonged soaking should be avoided (Howard et al., 1994). Crop rotation is also essential for vegetables in field. Rotating plants prone to \textit{Pcc} with small grains, grasses and beans may help reduce the population of soft rot bacteria in soil. Harvesting and storage procedures are also important, limiting the spread of bacteria through water, nutrient solutions or mechanical injury. Careful handling of crops during harvest and storage reduce the frequency of cuts and bruises and limit opportunities for the pathogen
to infect tissues through wounds. Growers have the means to limit soft rot bacteria during storage. For example, vegetables should be graded before storage to remove damaged or diseased roots. An infected root, bulb or tuber can lead to the infection of surrounding geophytes. Ventilation with outside air during transport and temperature and humidity controls during storage are additional practices that may limit infection or spread of bacteria. Specifically, producers should store crops at temperatures around 0°C and 90 to 95% relative humidity. Furthermore, ventilation equipment should be set to allow for regular exchange with outside air. Lastly, when soft rot becomes a recurring problem, washing and handling equipment as well as greenhouse growth rooms and storage rooms should be thoroughly disinfected before re-use (Howard et al., 1994).

2.2.4.2 Chemical control

Chemical control methods have been developed to prevent or slow the spread of soft rot pathogens. Washing tubers with sodium hypochlorite and clean water may suppress bacteria and inhibit rot. Washing tubers and tomatoes before packaging with chlorinated water reduces residual populations of soft rot bacteria. However, chlorination does not arrest soft rot development in infected fruit. Seed treatments with azoxystrobin, carboxin or thiabendazole are effective in reducing fungi that may primarily or secondarily attack plant tissues, thus reducing the possibility of secondary microorganisms prone to invade macerated tissue. Studies have shown that calcium, in the form of calcium chloride, calcium nitrate or calcium hypochlorite, added to germination water may prevent bacterial soft rot in bean sprouts (Howard et al., 1994).
2.2.4.3 Biological control

The classical approach to biological control of soft rot bacteria involves a one time introduction of biological control agents (BCAs) causing antagonistic effects to the pathogen. BCAs, such as microbes, are selected because of their ability to exploit and protect metabolic niches as well as their persistence in the environment (Knudsen et al., 1997). BCAs act upon host plants as inducers of plant resistance and can hyperparasitize pathogens or produce substances that harm the pathogen (i.e. antibiotics) (Handelsman et al., 1996; Paulitz and Matta, 1999). Biological control strategies showing success include attempts to control soft rot with a number of other bacterial species such as *Pseudomonas fluorescens* and *Bacillus subtilus*, and the use of *Erwinia* mutant isolates (Sharga and Lyon, 1998). These mutant isolates are pathogenic strains but exhibit reduced virulence (Stein, 2005; Sharga and Lyon, 1998).

2.3 Antibodies

2.3.1 Mammalian Immune System

The immune system is a remarkably adaptive defense system protecting vertebrates from disease and death caused by pathogenic infection (Alberts et al., 1994; Goldsby et al., 2000). Destruction and elimination of invasive organisms and toxic molecules are initiated by the many responses of the immune system. Because these immune reactions are destructive, it is essential that they only respond to foreign molecules and not to those of the host itself. This ability to distinguish between foreign molecules and those of the host is an essential aspect of the immune system. Almost any foreign macromolecule can induce an immune response. Immune responses are divided
into two categories: innate responses and acquired responses, which function as a highly interactive and cooperative system. Consequently, a substance capable of eliciting an immune response is referred to as an antibody generator or antigen (Ag) (Goldsby *et al*., 2000). Antibodies (Abs), also called immunoglobulins, are proteins produced by the immune system to fight specific bacteria, viruses, or other antigens.

2.3.1.1 *Innate (nonspecific) response*

The innate immune response is an array of disease-resistance mechanisms that are not specific to a particular pathogen. The innate immune system is separated into two categories. First, the innate response provides early host defense, protecting the host during lymphocyte activation. Second, effector mechanisms utilized during this response are similar to those activated during the acquired immune response to eliminate foreign pathogens (Janeway and Travers, 1994). The innate immune system is composed of four types of mechanical and cell-mediated defensive barriers (Goldsby *et al*., 2000):

- anatomic barriers (e.g., skin) provide mechanical obstruction preventing entry of microbes; entrapment of foreign microorganisms by membranes (mucous membranes);
- physiologic barriers (e.g., temperature, low pH and chemical mediators) inhibit the growth of some pathogens;
- phagocytic/endocytic barriers cause breakdown of foreign macromolecules, killing and digesting whole microorganisms;
- inflammatory barriers provide localized tissue response to injury, infection or trauma.
An important component of the innate response is phagocytic cells, such as macrophages, which are capable of ingesting and digesting antigens including bacteria (Goldsby et al., 2000). Macrophages, therefore, provide a nonspecific defense against infection. This innate method of defense is not dependent upon prior exposure to a particular pathogen (Janeway and Travers, 1994).

2.3.1.2 Acquired (specific) response

Acquired immunity is responsible for recognizing and selectively eliminating foreign molecules and microorganisms by the production of antibodies (Janeway and Travers, 1994; Kuby, 1994). The four characteristics of acquired immune response that protect the body from pathogenic invasion are antigenic specificity, diversity, immunologic memory and self/nonself recognition. In particular, the antigenic specificity of the immune system allows it to differentiate among different antigens. For example, antibodies are capable of distinguishing between two protein molecules that merely differ by a single amino acid. The diversity of the immune system permits the recognition of numerous uniquely different structures on foreign antigens. Once the immune system has recognized and responded to an antigen, it exhibits immunologic memory, providing the opportunity for a future heightened state of immune reactivity. Self/nonself recognition provides the ability of distinguishing between foreign molecules and those of the immune system (Goldsby et al., 2000). Mediating self/nonself recognition, lymphocytes, the white blood cells produced in bone marrow, leave the bone marrow and circulate in the blood and lymphatic systems, producing and displaying antigen-binding cell-surface receptors.
An acquired immune response is affected by two major groups of cells: Ag-presenting cells (APCs) and lymphocytes (Goldsby et al., 2000). Ag-presenting cells first internalize, by phagocytosis or endocytosis, process and re-express a section of the Ag on their membrane; lymphocytes with surface receptors bind to the processed Ag (Yau et al., 1998). There are two major types of lymphocytes: B lymphocytes (B cells) and T lymphocytes (T cells). When bound to APCs, T cells secrete cytokines that activate B cells and other effector cells known as cytotoxic T (T_C) cells and T helper (T_H) cells. By eliminating the Ag, cytotoxic T cells exhibit cytotoxic activity against virus-infected cells, tumor cells, and cells from foreign tissue grafts. T-cell receptors can only recognize Ag that is bound to cell-membrane proteins called major histocompatibility complex (MHC) molecules. B cells, produced within bone marrow, express a unique antigen-binding receptor on its membrane. The B-cell receptors are known as antibodies, which are the major effector molecules of acquired immunity (Kuby, 1994; Goldsby et al., 2000).

Once antibodies are produced, they circulate in the bloodstream and permeate tissues and organs where they bind to the foreign antigen that initially induced antibody production. Antibody binding inactivates bacterial pathogens and viruses by specifically blocking the toxin’s ability to bind to receptors on host cells. Antibody binding also marks the invading microorganisms for destruction, either by allowing phagocytic cells to ingest them or by activating blood proteins that kill the invaders (Goldsby et al., 2000).
2.3.2 Immunoglobulins

2.3.2.1 Basic structure of immunoglobulins

Antibodies, also known as conventional immunoglobulins (Ig), are glycoproteins present on the B-cell membrane and are secreted by plasma cells. Igs are Y-shaped homodimers consisting of two identical polypeptide chains or heavy (H) chains of 450 amino acids and two identical light (L) chains of 250 amino acids. The H and L chains are divided into variable (V) and constant (C) domains (Maynard and Georgiou, 2000; Figure 8). Together, the variable domain of the heavy and light chain ($V_H$ and $V_L$) form the unique antigen-recognition (binding) site. These variable domains, variable in amino acid sequence, are located at the N-terminal region of the antibody. This binding domain is collectively known as a paratope, as a result of its ability to bind to the epitope of the antigen (Goldsby et al., 2000). Enzymatic digestion using papain allows Ig molecules to be broken into smaller fragments known as antigen binding fragments (Fab) and crystallisable fragments (Fc). The IgG molecule produces two identical Fabs and one Fc. Instead of enzymatic digestion by papain, the enzyme pepsin was used to create a single fragment composed of two Fab-like fragments F(ab)$_2$ and a truncated Fc (Goldsby et al., 2000; Joosten et al., 2003). Azzazy and Highsmith (2002) report the use of pepsin digestion to produce Fv (fragment variable) fragments consisting of only variable regions of heavy and light chains. The Fv fragment, composed of the $V_H$ and $V_L$, contains the antigen binding site of a whole IgG antibody. At low protein concentrations, as a result of non-covalent links, native Fv fragments and their $V_H$ and $V_L$ domains tend to dissociate (Joosten et al., 2003).
Figure 8. Structure of immunoglobulin G (IgG). Monomeric antibody fragments: Fv (variable fragment), Fc (crystallisable fragment), Fab (antibody fragment), F(ab’)2 (antibody fragment with two antigen binding sites) and scFv (single chain variable fragment) are displayed above. Variable domains of heavy (V_H) and light (V_L) chains are represented by black and white ovals, respectively, while constant regions of heavy (C_H1-3) and light (C_L) chains are represented by grey and shaded ovals, respectively (Adapted from Azzazy and Highsmith, 2002.)
The constant heavy (C\textsubscript{H}) region consists of three homologous domains designated $C_{\text{H}}1$, $C_{\text{H}}2$, and $C_{\text{H}}3$ (Garrett and Grisham, 1999) (Figure 8). The non-antigen binding region or constant domain Fc fragment mediates receptor binding and complement fixation. Each L chain has two intrachain disulfide bonds, one in each of the $V_L$ and $C_L$ regions. The C-terminal amino acid in L chains forms an interchain disulfide bond with the neighbouring H chain (Garrett and Grisham, 1999). In other words, Fab fragments contain $V_H + C_{\text{H}}1$ and $C_L + V_L$ anchored by an interchain disulfide bond between $C_L$ and $C_{\text{H}}1$ (Joosten et al., 2003).

2.3.2.2 Immunoglobulin classes and subclasses

Five classes of immunoglobulins exist within vertebrates: IgG, IgD, IgE, IgA and IgM (Figure 9). IgG is the most abundant Ig in blood and has a considerable history in diagnostic applications. The number of Y-shaped units used to form an Ig as well as its heavy chain isotope is the basis for distinguishing the various Ig classes (Alberts et al., 1994). There are five $C_{\text{H}}$ region isotypes: $\mu$, $\delta$, $\gamma$, $\epsilon$, and $\alpha$ (Goldsby et al., 2000). The length of the constant regions for $\delta$, $\gamma$, and $\alpha$; and $\mu$ and $\epsilon$; is approximately 330 and 440 amino acids, respectively. The heavy chains of an antibody determine its specific Ig class: $\text{IgG}(\gamma)$, $\text{IgD}(\delta)$, $\text{IgE}(\epsilon)$, $\text{IgA}(\alpha)$ and $\text{IgM}(\mu)$, with each class containing either $\kappa$ or $\lambda$ light chains. Major variations in the amino acid sequences of the $\gamma$ and $\alpha$ heavy chains lead to further classification of heavy chains into $\gamma$ and $\alpha$ subclasses. Two subclasses of $\alpha$ heavy chains ($\alpha1$ and $\alpha2$) and four subclasses of $\gamma$ heavy chains ($\gamma_1$, $\gamma_2$, $\gamma_3$, $\gamma_4$) exist within humans (Goldsby et al., 2000).
Figure 9. Schematic representation of the five major classes of immunoglobulins. Light chains are shown in black while heavy chains are shown in white and grey. Light chains, disulfide-linked to one another, are represented by thick black lines (Taken from Barbas et al., 2001).
2.3.3 Antigen Binding Site

A comparison of the amino acid sequences of $V_L$ and $V_H$ domains suggests that sequence variability is concentrated in several hypervariable (HV) regions. These numerous HV regions, form the antigen-binding site of the antibody molecule. HV regions, also known as complementary-determining regions (CDRs), are present in every human heavy and light chain, constituting 15%-20% of the variable domain. Furthermore, the name CDR is given to HV regions because the antigen-binding site is complementary to epitope structure. The remainder of $V_L$ and $V_H$ domains is referred to as framework regions (FRs), exhibiting far less variation than HV regions. In both light and heavy chain V regions there are three CDRs (CDR1-CDR3) and four FRs (FR1-FR4) (Goldsby et al., 2000).

2.3.4 Recombinant Antibodies

The development and application of recombinant DNA technology, a series of procedures used to combine DNA sequences from various organisms, has led to the design of novel antibodies and antibody fragments. Recombinant antibodies provide numerous advantages over whole antibodies in specific applications by providing improved function and considerably lower molecular weight (Joosten et al., 2003).

2.3.4.1 Single chain variable fragment (scFv)

The recombinant version of the Fv is termed single chain variable fragment (scFv). In the scFv, the C terminus of the $V_H$ or $V_L$ chain is linked to the N terminus of
the other chain with a hydrophilic and flexible peptide linker with about 15 amino acids, improving expression and folding efficiency (Merk et al., 1999; Joosten et al., 2003; Brichta et al., 2005). The most frequently used peptide linker is the (Gly₄Ser)₃ (Joosten et al., 2003; Figure 8). Some scFv molecules have a reduced affinity compared to the parental antibody or Fab molecule. Additionally, depending on the linker used, scFv molecules can easily degrade. This dissociation of scFvs results in monomeric scFvs, which form into dimers, trimers or larger aggregates (Maynard and Georgiou, 2000; Joosten et al., 2003). In some cases, the variable regions can be connected as V₃H-linker-V₃L (V₃HV₃L) or as V₃L-linker-V₃H (V₃LV₃H) arrangements of single chain antibodies (scFv), affecting expression efficiency, stability, and the tendency to form dimers in solution (Merk et al., 1999; Maynard and Georgiou, 2000).

2.3.4.2 Polyclonal antibodies

Polyclonal antibodies make up the total population of antibodies present in animal serum (Goldsby et al., 2000). This complex population contains different Ig subclasses, with each Ab representing the secretory product from a single stimulated lymphocyte and its clonal progeny. A complex Ag such as a protein may contain distinct antigenic determinants or epitopes, each of which is specifically recognized by antibodies from a single lymphocyte clone (Dunbar and Schwoebel, 1990; Goldsby et al., 2000).

The first step for preparation of polyclonal antibodies is to obtain sufficient quantities of a highly purified immunogen that will be used to immunize the selected animal. Research has indicated that in a protein that contains as little as 1% contamination, the majority of Abs may recognize that contaminant if it is highly
immunogenic. Rabbits, the most commonly used animal for the production of polyclonal antibodies, are immunized with a highly purified antigen, adjuvant and PBS emulsification through, for example, intradermal injections. Periodically, over a number of weeks to months, rabbits are bled for antisera and re-immunized at 2 week intervals. Before the fractionation of Ig from the serum can occur, the blood must clot for several hours, followed by centrifugation to remove blood cells. IgGs are obtained following various fractionation and chromatography methods that remove unwanted Ig classes. Final fractions can be tested for the presence of IgG by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE; Dunbar and Schwoebel, 1990).

As with any current technology, the major advantages and disadvantages of polyclonal antisera must be weighed. A major advantage of polyclonal antisera is the presence of multiple subclass and high affinity Abs in the antibody population. As well, the multiple specificities of antibodies present in antisera are likely to recognize sequential as well as conformational antigenic determinants. Furthermore, polyclonal antibodies should recognize multiple determinants specific for a protein. Lastly, highly specific polyclonal antisera may easily be developed by immunization with purified proteins. In contrast, the immunogen must be highly purified to obtain the desired specificity. Individual domains of complex antigens are difficult to study because multiple antigenic determinants are recognized by the polyclonal antisera. Also, different bleedings have to be characterized individually due to changes in antibody affinity, specificity, and subclass composition (Dunbar and Schwoebel, 1990).
2.3.4.3 Monoclonal antibodies & hybridoma production

The oldest and most common procedure for producing monoclonal antibodies, identical Abs (produced by recombinant technology) targeting the same epitope of an antigen, is by hybridoma technology (Garrett and Grisham, 1999). A hybridoma is a clone of hybrid cells formed by fusion with myeloma cells. Hybridomas retain the properties of the normal cell to produce antibodies or T-cell receptors but exhibit the immortal growth characteristic of myeloma cells (Goldsby et al., 2000). More specifically, hybridoma antibody-producing cells are made by fusing mouse myeloma cells with mouse lymphocytes (Dunbar and Schwoebel, 1990). The resulting clones of hybridoma cells, which secrete large quantities of monoclonal Ab, can be cultured indefinitely (Goldsby et al., 2000). By screening the resulting hybridomas, one may select those producing useful antibodies (Dunbar and Schwoebel, 1990).

Intradermal immunization procedures used for polyclonal antibody production in rabbits are difficult to use in mice. As a result, subcutaneous injections with adjuvants, nitrocellulose implants (containing the antigen), or intrasplenic injections are used. Generally, subcutaneous immunizations using antigen suspended in adjuvant is injected into the hind foot pad of the mouse, causing eventual swelling to the popliteal lymph node after 10 to 12 days. Twenty-four to seventy-two hours after boosting, spleens are removed, dissected, minced, and their cells dispersed. Centrifugation and washing of the cell suspension is done prior to fusion with myeloma cells. Fused cells are dispensed onto a Petri dish for growth. Specially formulated agar, such as Dulbecco’s modified Eagle’s medium (DMEM), is buffered with sodium bicarbonate or 4-(2-hydroxyethyl)-1-
piperazine-ethanesulfonic acid (HEPES) and used to meet the nutritional needs of growing cells (Dunbar and Schwoebel, 1990; Goldsby et al., 2000).

Along with traditional cell culture using Petri dishes, stationary, suspension, or perfusion cultures can also be used. In a stationary culture, cells and media are placed in flasks without agitation. In contrast, in suspension culture, cells and media are agitated continuously to maintain cell suspension and gas exchange. Perfusion culture systems separate cells from media by a selective membrane, continuously bringing cells into contact with media, supplying nutrients and removing waste (Jackson et al., 1999).

After another round of centrifugation and chemical treatment using HAT medium stock (hypoxanthine, aminopterin, and thymidine) in Dulbecco’s medium (glutamine, penicillin, and streptomycin), cells are pipetted into cell culture trays and incubated for 2 weeks. Selective media such as Dulbecco’s medium are used to eliminate non-fused cells; non-fused cells will not grow in tissue culture. Hybridomas can be subcloned by microscopically selecting and pipetting out individual colonies. Dunbar and Schwoebel (1990) suggested that multiple subclonings should be completed to guarantee the monoclonality of a cell line. Lastly, the subclasses of monoclonal antibodies should be characterized. It is necessary to determine the subclass of the antibody and eliminate all other subclasses from the cultures, in turn reducing the numbers of cells that are screened. Screening of the subclass is easily performed using specialized immunoassays (Dunbar and Schwoebel, 1990; Goldsby et al., 2000).

As with polyclonal antisera, monoclonal antibodies have major advantages and disadvantages. One advantage of monoclonal antibodies is that a single homogeneous antibody is linked to a defined antigenic determinant. Large quantities of monoclonal
antibodies can be obtained since theoretically immortal cell lines can be developed. Antibodies with high affinity can be selected during screening procedures. Major disadvantages of monoclonal antibodies include expensive and time consuming procedures, epitope recognition by an antibody clone can be shared among unrelated antigens, and instability of hybridoma cell lines due to chromosome loss or tissue culture contamination (Dunbar and Schwoebel, 1990).

2.3.4.4 Camelid ‘heavy chain’ antibodies and single-domain antigen-binding fragments (V_{H}H)

The humoral immune response of Camelidae (e.g., camels, dromedaries and llamas) is unique since it contains conventional antibodies (65-75%) and a significant proportion of antibodies composed of heavy chains and devoid of light chains, referred to as heavy chain IgG (HcIgG; 25-35%) (Hamers-Casterman et al., 1993; Muyldermans et al., 2001; Figure 10). These HcIgG are abundant in the sera of all Camelidae species and total up to 75 per cent of the molecules binding to protein A (Hamers-Casterman et al., 1993). The very small (11-15 kDa) antigen-binding fragments of these antibodies (V_{H}H; Figure 10) are extremely stable (e.g., at temperatures as high as 90°C) and can be expressed at significantly higher levels (in E. coli and P. pastoris) than those of classical Fab or Fvs (Arbabi-Gharoudi et al., 1997; Frenken et al., 2000; Van der Linden et al., 2000; Jobling et al., 2003). V_{H}Hs have been raised against different bacterial or protein antigens (Arbabi-Gharoudi et al., 1997; Frenken et al., 2000). Interestingly, most V_{H}H domains contain a long CDR3 that contains a cysteine in addition to a cysteine residue located either in CDR1 or position 50 or 55 in framework region 2 (FR2), suggesting that these Abs contain a second disulphide bridge (Hamers-Casterman et al., 1993). V_{H}H
molecules can be isolated through phage display and subsequent expression in heterologous systems (Frenken et al., 2000; Van der Linden et al., 2000).

**Figure 10.** Schematic representation of the conventional IgG containing both heavy and light chains and llama (Camel) Ig containing heavy chains. Variable heavy ($V_H$) and light ($V_L$) domains of the conventional IgG are shown in dark green and light green. The $V_{H}H$ domain is shown in red. (Taken from Holliger and Hudson, 2005).

### 2.4 Phage Display Technology

First introduced by G. Smith in 1985, phage display is an effective method for producing large numbers (up to $10^{10}$) of peptides and proteins, and isolating molecules that perform specific functions (Azzazy and Highsmith, 2002; Brichta et al., 2005). Phage display involves the expression of proteins, including antibodies (such as $V_H$), or peptides on the surface of a filamentous phage. The surface of a phage is used as a
“display capsule” in which DNA sequences of interest are inserted into a location in the genome of filamentous bacteriophages expressed as a fusion product to one of the phage coat proteins (Azzazy and Highsmith, 2002). In other words, a foreign gene sequence is spliced with a gene for a specific phage coat protein, so that the foreign amino acid sequence is genetically fused to the amino acids of the coat protein, making a “hybrid fusion” protein that becomes incorporated into phage particles as they are released from the cell and displayed on the outer surface (Smith and Petrenko, 1997). The direct link between genotype and phenotype of the Ab being displayed is the common feature among various display technologies (Brichta et al., 2005). The selection of Abs and peptides from phage-displayed libraries is routinely used for the isolation of Abs for diagnostic and therapeutic applications (Azzazy and Highsmith, 2002).

2.4.1 Bacteriophages (Phages)

Bacteriophages or phages are viruses containing pili used as receptors, that infect gram-negative bacteria (Raven et al., 1999). More specifically, phages are genetic parasites that utilize their host’s cellular and molecular components to replicate themselves. The genome of a phage consists of either double stranded (ds) or single stranded (ss) DNA encapsulated by a protein sheath (Ackerman, 2001). Phages exist in families, 13 of which are delineated on the basis of host range restrictions, morphology, and genetic composition (Azzazy and Highsmith, 2002; Ackermann, 2001). According to Ackermann (2001), 5100 bacterial viruses have been morphologically characterized, with 96% of these phages exhibiting tails and 4% exhibiting cubic or polyhedral shape, filamentous (containing long filaments) or pleomorphic (variety of shapes and forms,
lemon shaped) morphologies, which lack the presence of tails (Figure 11). The genome of tailed phages, constituting the order Caudovirales, consists of a single molecule of double-stranded DNA and is divided into three large families: the Myoviridae (contractile tails), Siphoviridae (long, noncontractile tails) and Podoviridae (short tails) (Ackermann, 2001; Figure 11). Filamentous phages will be the focus of the next section since they are used for phage display.

2.4.1.1 Filamentous phages

To date, phage display work has used filamentous bacteriophages. Filamentous phages are viruses that infect strains of E. coli that contain F conjugative plasmids. The Ff filamentous phage particles (strains M13, f1 and fd), that infect E. coli via F pili, consist of ssDNA that is enclosed in a protein coat and is translocated into the bacterial cytoplasm (Figure 12). Viable filamentous phages express approximately 2700 copies of the major coat protein, gene 8 protein (g8p or pVIII), and 3 to 5 copies of the minor coat protein, gene 3 protein (g3p or PIII) which is important in phage display (Azzazy and Highsmith, 2002). g3p and g8p are involved in the cloning and detection of recombinant phage Abs and peptides. Recombinant Abs, folded proteins and peptides are typically expressed as g3p fusion proteins and are displayed at the tip of the M13 phage (Azzazy and Highsmith, 2002; Figure 12).

Upon entry into the bacterium, the ssDNA is converted by host cell machinery into double-stranded (ds) plasmid-like replicative form (RF), which undergoes rolling circle replication to make the template for expression of the phage proteins g3p and g8p,
Figure 11. Morphotypes of bacteriophages. A, B, and C, tailed phages belonging to the order of Caudovirales, represent Myoviridae, Siphoviridae and Podoviridae families, respectively. D and E represent cubic or polyhedral structures, whereas F and G represent filamentous and pleomorphic morphologies, respectively (Taken from Ackermann, 2001).
Figure 12. Schematic representation of bacteriophage M13. Widely used as vehicles for phage display, the pIII and pVIII coat proteins can be used as fusion partners for a number of proteins (Adapted from Brichta et al., 2005).

dsDNA. Phage progeny are assembled by packaging of ssDNA into protein coats which are extruded through the bacterial membrane into the medium (Smith and Petrenko, 1997; Hoogenboom et al., 1998; Azzazy and Highsmith, 2002).

2.4.1.2 Phage vectors

Viral vectors that accept and display fusions with genes III and VIII have been termed types 3 and 8, depending on the location of gene expression (Smith, 1993; Smith and Petrenko, 1997). Viable inserts of foreign peptides or proteins have been displayed at the amino-terminus of mature g3p. Remarkably, each site appears to have intrinsic limitations. For example, g8p tolerates short inserts of less than six amino acid residues possibly a result of the close packed nature of the viral surface (Kischenko et al., 1994).

In an attempt to compensate for biological intolerance with surface display sequences on M13 phages, two further vector systems were designed. The first system, the phage vector system, carries two copies of gene III or VIII. When bacterial cells are
infected, the phage incorporates both wild type and fusion copies of pIII or pVIII into the same viral particles. These vectors that are able to incorporate both wild type and recombinant copies of genes III and VIII are named “33” and “88” respectively (Smith, 1993; Figure 13). A second vector system also displaying both wild type and fusion proteins can be created using phagemid vectors (Bricha et al., 2005).

2.4.1.3 Phagemid cloning vectors

Phagemid cloning vectors (hybrids of phage and plasmid vectors) carry sequences encoding fusion proteins (Azzazy and Highsmith, 2002; Bricha et al., 2005). These vectors, carrying a copy of gene III or VIII protein fusion are termed type 3+3 or 8+8 vectors, respectively (Smith, 1993; Smith and Petrenko, 1997). Helper phages (such as VCSM13 or M13K07) and phagemids are used to co-infect the bacteria; they carry the majority of genes required for the formation of phage particles. This phenomenon, with the use of helper phages, occurs because of the lack of structural and nonstructural gene components in phagemids. Helper phages contain the necessary structural proteins for generating complete phages, incorporating wild type pIII from the helper phage and PIII fusion protein encoded by the phagemid (Azzazy and Highsmith, 2002; Figure 13). The phagemid vector system has few limitations and has been successfully used to isolate Ab fragments against a wide variety of proteins, DNA, viruses and parasites. Phagemid vectors also allow the conditional display of Ab on phages or the secretion of the Ab in the periplasmic space of E. coli in a form that can be easily detected by immunoassay technology such as ELISA (Azzazy and Highsmith, 2002).
Figure 13. Various phage display systems based on pIII coat protein. Gene pIII, foreign DNA insert and fusion products are represented as a black box, grey box and grey circle, respectively. In a type 3 vector, there is a single phage chromosome (genome) bearing a single gene III which accepts foreign DNA inserts and encodes a single type of pIII molecule. The type 33 vector bears two genes III, one of which is recombinant (Smith and Petrenko, 1997; Taken from Brichta et al., 2005).
2.4.2 Phage Display Libraries

The novel discovery that functional Ab binding sites can be displayed on the surface of bacteriophages has allowed selection of Abs to the Ag of choice without the need to immortalize B cell lines used in hybridoma technology (Hoogenboom et al., 1998; Vaughan et al., 1996). Smith and Petrenko (1997) describe a phage display “library” as a heterogeneous mixture of phage clones, each displaying a different Ab or protein on its surface. Replication of each Ab in the library can occur when the phage to which it is attached infects a bacterial host. These phages multiply to produce a large number of identical progeny phages displaying the same Ab.

Phage display libraries commonly consist of heavy and light chain variable region domains (V_H and V_L) fused to the phage minor coat protein gene III (gpIII) and displayed as a scFv (Vaughan et al., 1996; Strachan et al., 2002). These Ab fragments can be expressed in large quantities in a bacterial host, such as E. coli, more rapidly and at lower cost than generating whole antibodies. In addition, these libraries exhibit sufficient diversity (\(>10^8\)) to enable high affinity Abs to be selected against many Ags (Strachan et al., 2002). Phage display libraries employing Abs can be prepared from natural, synthetic or mixed DNA sources for antigenic specificity. Currently, the main types of phage display libraries consist of (a) immunized, (b) naïve and (c) synthetic, or (d) semi-synthetic Ab compositions.

2.4.2.1 Immunized libraries

Immune Ab libraries have two basic characteristics: (i) they can be enriched in antigen-specific Abs, and (ii) some of these Abs undergo affinity maturation by the
immune system (Clackson et al., 1991; Azzazy and Highsmith, 2002; Brichta et al., 2005). Immunized libraries are created using IgG genes of B cells from animals immunized with Ag from immune donors (Hoogenboom et al., 1998). On occasion, the recombinant Ab selected from the immune Ab library can be superior to those monoclonal Abs obtained from hybridoma technology (Brichta et al., 2005). Several disadvantages have been accounted for with immune libraries. Primarily, the length of time required for immunization, and the unpredictability of the immune response of the animal to an Ag of interest, are causes for concern. Lack of immune response and the requirement of a new library for each Ag are also significant disadvantages (Azzazy and Highsmith, 2002).

2.4.2.2 Naïve libraries

Naïve libraries can be created from collections of variable (V)-genes from IgM and/or IgG mRNA of B cells of non-immunized human donors (Brichta et al., 2005). IgM and/or IgG variable regions are amplified using oligonucleotide primer sets and PCR and are cloned into vectors for screening by phage display. IgM repertoires are preferred to IgG because IgG chains can be biased by host immune responses and will not react to self Ags. The affinity and specificity of antibodies isolated through the screening of naïve libraries is linked to library size (Maynard and Georgiou, 2000). A library consisting of $10^{10}$ clones yields antibodies with affinities in the low nanomolar range, whereas a similar library of $3 \times 10^7$ clones results in Abs with micromolar affinities. Therefore, the affinity of Abs selected from a naïve library is proportional to the size of the library, ranging from $10^6$ to $10^7$ M$^{-1}$ for a small library with $10^7$ clones, to $10^8$ to $10^{10}$
M\textsuperscript{1} for a large repertoire (Vaughan et al., 2005). Thus, it is important for naïve libraries to be as large as possible, since large libraries usually contain greater Ab sequence diversity and multiple Abs against a desired target (Maynard and Georgiou, 2000).

2.4.2.3 Synthetic and semi synthetic libraries

Fully synthetic libraries are created by cloning the germline DNA Ab gene segments. These segments are normally arranged by the immune system to generate natural immunological diversity (Maynard and Georgiou, 2000). Essentially, Abs are built artificially by \textit{in vitro} assembly of V-genes and D/J segments. V-genes may be assembled by introducing a predetermined level of randomization of CDR regions into germline V-gene segments. Synthetic libraries are often subdivided further into semi-synthetic libraries where the repertoire is constructed in a single framework (Strachan et al., 2002). This entire synthetic repertoire is subsequently cloned into an expression vector, generating a library containing $10^7$ to $10^{10}$ clones. Synthetic libraries have been generated by selecting one or more sequences within the CDR loops. The CDR3 of the heavy chain, which contains the most structural and sequence diversity in the Ag-binding site has been the target for introduction of diversity in synthetic libraries (Maynard and Georgiou, 2000). Tomlinson I and J and Griffin.1 libraries are common examples of synthetic and semi-synthetic libraries, respectively.

2.4.2.3.1 Griffin.1

The Griffin library (MRC Laboratories, Cambridge, UK) is a semi-synthetic scFv phagemid library, containing highly diverse CDR3s in both the VH and VL domains.
This library, with a $1.2 \times 10^9$ clone repertoire, was derived by recloning $V_H$ and $V_L$ from human synthetic Fab lox library vectors into the phagemid vector pHEN2 and PIT2. scFvs can be displayed on the surface of the bacteriophage when expressed in suppressor *E.coli* strains (e.g. TG1) or as soluble fragments that also contain the c-myc tag and carboxy-terminus His-tag in nonsuppressor *E. coli* strains (Watkins *et al.*, 2003). The Griffin.1 library is a more stable version of the largest synthetic Fab library made to date by Griffiths *et al.* (1994). This library was created with 49 human $V_H$ segments that were combined with a collection of 26 $V_k$ and 21 $V_\lambda$ light chain segments with partially randomized CDR3 regions (Griffiths *et al.*, 1994).

### 2.4.2.3.2 Tomlinson I and J libraries

Tomlinson I and J libraries, both synthetic, each comprise over 100 million scFv fragments cloned in an ampicillin-resistant phagemid vector and transformed into TG1 *E. coli* cells (Bricha *et al.*, 2005). The Tomlinson I and J libraries, $1.47 \times 10^8$ and $1.37 \times 10^8$ in size, respectively, are constructed in pIT2, previously constructed as the Griffin.1 library, but are based on a simple human framework for $V_H$ and $V_k$, which encodes the most common human canonical structure (Goletz *et al.*, 2002). The CDR3 of the heavy chain was designed to be as short as possible, at 7 amino acids, and still form an antigen-binding surface. Diversified (DVT) side chains were incorporated at 18 amino acid positions in the CDR3 and CDR2 to make contact with the Ag (Goletz *et al.*, 2002).
2.4.3 Selection Techniques

Antibody libraries are screened for Ag-specific clones and enriched by a technique known as “panning”. Panning allows phage displaying V_{H}Hs or scFv to be incubated with the Ag of interest (Merk et al., 1999). The selection process is achieved by repetitive rounds of phage binding to the target, washing to remove non-binding phages, elution steps to obtain the binding phage and reamplification (in *E. coli*) of the phage pool enriched for a specific binding phage (Brichta et al., 2005). Ideally, one selection cycle should be required, however, the binding of nonspecific phage limits the enrichment that can be achieved per cycle. An additional 2 to 4 cycles of selection are necessary to reduce non-specific binding (Azzazy and Highsmith, 2002).

Typically, methods with the ability to separate specific binding clones from no-binding clones can be utilized for selection. The most commonly used *in vitro* selection method is biopanning on immobilized Ag coated onto plastic tubes or chips of BIAcore sensors (Brichta et al., 2005). Phage libraries can be selected when the target Ag is immobilized onto an affinity column, plastic surfaces such as immunotubes (Maxisorb tubes; Nalge Nunc Intl., Naperville, IL), or on enzyme-linked immunosorbent assay (ELISA) plates (Merk et al., 1999; Azzazy and Highsmith, 2002). Following extensive washing of the column, immunotubes or wells of an ELISA plate, specific binders are eluted by changing the binding conditions. Elution of specific phage-displayed Abs from their specific Ag can be achieved with acidic solutions (such as HCl or glycine buffer), basic solutions (such as triethylamine), by enzymatic cleavage of a protease site constructed between the Ab and g3p, or by competition with excess Ag (Azzazy and Highsmith, 2002; Goletz et al., 2002).
2.5 Immunoassays

Immunoassays use the binding specificity of an Ab for its specific Ag to measure either the Ag or Ab. To quantitate the reaction, either the Ag or the Ab is labeled with a radioisotope or enzyme such as horseradish peroxidase (HRP) or alkaline phosphatase. Immunoassays provide a sensitive, reproducible, convenient, and generally applicable approach to the measurement of molecules of biological interest. Monoclonal Abs are extremely useful for developing highly specific immunoassays. Presently, the most widely used immunoassays are enzyme-linked immunosorbent assays or ELISAs (Parker, 1990). Membrane-based immunoassay tests (e.g. lateral flow assays or immunochromatography) can be developed using Abs conjugated to colloidal gold.

2.5.1 ELISA

ELISA, first described in 1917, combines the specificity of the antibody/antigen interaction with a detection system involving an enzyme conjugated to an antibody (Dickinson, 2003). Moreover, the enzyme conjugated Ab reacts with a colourless chromogenic substrate to generate a coloured reaction product which can be observed by eye, and the intensity of which can be measured with a spectrophotometer (Goldsby et al., 2000). The intensity of the reaction is directly proportional to the amount of bound Ab. Free Ag competes with immobilized Ags to bind to Ab. As the concentration of free Ag increases, the concentration of Ab bound to the Ag decreases; the colour intensity is inversely proportional to the concentration of Ag (Kaufman and Clower, 1995).

ELISAs can exist in homogenous (1 phase) or heterogeneous (2 phases) formats. Homogenous ELISAs are commonly used to measure quantities of low molecular weight
molecules. In homogeneous assays, an enzyme-labeled Ag is added to a sample containing an unknown concentration of the same Ag. When the Ab specific to the Ag is added, the unlabeled and the labeled Ag compete for the Ab. The unbound enzyme-labeled Ag becomes available to react with the substrate while the response of the bound labeled Ag is modified. Upon addition of the substrate, a coloured product is formed. Heterogeneous ELISAs (the focus of this section) possess many useful advantages over its homogeneous counterpart. Two such advantages include the potential of handling high numbers of samples rapidly and since one of the reactants in ELISA is attached to a solid-phase, the separation of bound substrates can be done by simple washing procedures (Crowther, 1995). A number of variations of ELISA have been developed, allowing qualitative detection or quantitative measurement of either Ag or Ab. Direct, indirect and competition ELISAs will be discussed.

2.5.1.1 Direct ELISA

2.5.1.1.1 Labeled antibody

In the first of two types of direct ELISA, the labeled Ab ELISA begins with the Ag being immobilized on a solid phase. After washing, the bound Ag is reacted directly with enzyme-labeled Abs. Following incubation and further washing, a colourless substrate is added and the colour allowed to develop (Figure 14). Monoclonal Abs, directly labeled with enzyme, are primarily used in this type of ELISA (Crowther, 1995).
2.5.1.1.2 *Labeled antigen*

This type of ELISA is primarily used for the detection and/or titration of specific Abs from serum samples. Essentially, Abs are adsorbed to the solid phase after obtaining IgG. After washing, the bound Abs react directly with enzyme-labeled Ags and an immediate reaction takes place. Following incubation and further washing, a colourless substrate is added and the colour allowed to develop (*Figure 15*). Repeated use has shown that direct-labeled Ag ELISA has poor applicability to diagnostic problems (Crowther, 1995).

*Figure 14.* In the direct antibody-labeled ELISA, Ag is attached to the solid-phase (A) and enzyme-labeled Abs are added (B). After washings and incubation (C), a substrate is added, reacts with the enzyme causing colour to develop (Taken from Crowther, 1995).
Figure 15. In the direct antigen-labeled ELISA, Abs are attached to the solid-phase (A) and can capture Ag labeled with an enzyme (B). After washings and incubation (C), a substrate is added and colour is allowed to develop. This system can form the basis for other assays such as competitive ELISA (Taken from Crowther, 1995).

2.5.1.2 Indirect ELISA

Abs can be detected or quantitatively determined with an indirect ELISA (Goldsby et al., 2000). Serum containing a primary antibody (Ab₁) is added to a microtiter plate to which an Ag has been immobilized and allowed to react. After free Ab₁ is washed away, an enzyme-conjugated secondary anti-isotype antibody (Ab₂), which binds to Ab₁, detects the presence of Ab bound to Ag (Goldsby et al., 2000). Free Ab₂ is washed away and a substrate for the enzyme is added, with the amount of specific Ab binding to Ag quantified after colour development (Crowther, 1995; Figure 16).

2.5.1.3 Competitive ELISA

Competitive ELISA is an assay format for measuring the quantity of an Ag (Goldsby et al., 1995). Competition assays, as the name implies, incorporate two
Figure 16. Indirect ELISA where Abs react with an Ag bound to a solid-phase (A). Bound Abs are detected by the addition of an antispecies antiserum labeled with an enzyme (B). After washings and incubation (C), a substrate is added and colour is allowed to develop (Taken from Crowther, 1995).

reactants that compete for binding to a third reactant. Proper competition assays involve the simultaneous addition of the two competitors (Crowther, 1995).

2.5.1.3.1 Direct antibody competition

In this technique, Abs are added simultaneously with the unbound Ag and exposed to an Ag previously absorbed to the solid phase. After washing, specific enzyme-labeled Ab (anti- to Ab₁) is added following the washing of unadsorbed Ag. Abs are usually pretitrated so that the Ag no longer contains antigenic sites for further Ab combination (Crowther, 1995).
2.5.1.3.2  *Direct antigen competition*

Direct Ag competition is the equivalent to direct Ab competition, except that the competing substance for the pretitrated conjugated Ab is Ag (Crowther, 1995). If the labeled Ab reacts with the dilution range of the competitor Ag in the liquid phase, it is washed away after the incubation step, resulting in a reduction in expected colour observed, because the labeled Ab cannot react with the solid-phase Ag. This ELISA can be predominantly used to quantify Ags or used as a comparison for the relative affinity of binding of two Ags in the same serum.

2.5.1.3.3  *Indirect antibody competition*

Indirect Ab competition ELISA is essentially the same as indirect ELISA, however, a competing Ab is added to the solid-phase Ag either before or simultaneously with specific Abs that have been pretitrated. If the competing Ab is able to bind to the Ag, then it prevents the pretitrated Ab from reacting. This is observed as a decrease in the expected colour as compared to controls without competition (Crowther, 1995).

2.5.1.3.4  *Indirect antigen competition*

Also an indirect ELISA, indirect Ag competition has a pretitrated Ab against the solid-phase bound Ag by the use of antispecies conjugates. It is the addition of the antispecies enzyme conjugate that quantifies the bound Abs. If the Ag shares antigenic determinants with the solid-phase Ag, it binds to the pretitrated Abs preventing their reaction with the solid phase Ag. In contrast, if there is no sharing of antigenic
determinants, the Abs are not bound and can freely react with the solid-phase Ag (Crowther, 1995).

2.5.2 Lateral Flow Immunoassay

The use of membrane-based assay tests for rapid on-site screening provides a simple and low-cost alternative to expensive and time-consuming instrumental methods (Zhao et al., 2008). Methods using membranes as solid supports offer certain advantages, such as multianalyte tests. The one-step lateral flow assay, also known as a ‘dipstick test’, is the most advanced and user-friendly format providing results within 5-10 minutes. The lateral flow method uses colloidal gold-labeled Abs, in which a reactant (Ag or Ab) is transported to its binding partner (Ag or Ab) immobilized on the membrane surface (Kolosova et al., 2007; Figure 17). Colloidal gold is a suspension (or colloid) of sub-micrometre-sized particles of gold in solution. The solution is usually either an intense red colour (for particles less than 100 nm), or a dirty yellowish colour (for larger particles). The nanoparticles themselves can come in a variety of shapes (spheres, rods and cubes) (Kolosova et al., 2007; Zhao et al., 2008). The use of gold nanoparticles associated with the lateral flow technology allows for short assay time, visual interpretation of results, long-term stability and cost-effectiveness.
Figure 17. Membrane-based assay test (lateral flow assay) provides results in minutes. Abs conjugated to colloidal gold are applied to the conjugate pad. Monoclonal or polyclonal Abs or specific Ags are applied to the test line, while a secondary (un-conjugated) Ab is applied to the control line.
3. Detection and Quantification of Pcc in Hydroponic Solutions Using ELISA and Dipstick Assay Formats

3.1 Introduction

The gram-negative bacterium Pectobacterium carotovorum ssp. carotovorum (Pcc), previously called Erwinia carotovora ssp. carotovora, is the principal cause of soft rot disease of calla lily (Zantedeschia ssp.) in greenhouses (Pérombelon and Kelman, 1980; Wright, 1998; Snijder et al., 2004; Ravensdale, 2007). Lipopolysaccharide (LPS), a major surface component of Pcc and other gram-negative bacteria, is specifically implicated in plant pathogenesis (Fukuoka et al., 1997; Nakhamchik et al., 2007). Approximately 3.5 million LPS molecules, in bundles of 600 to 3500, cover the surface of gram-negative bacteria (Kotra et al., 1999). LPS consists of three distinct structural regions: lipid-A (responsible for endotoxicity), the core oligosaccharide and the O-polysaccharide (responsible for specificity) (Liu, 1997; Yi and Hackett, 2000).

In 2009, the Ontario greenhouse industry comprised approximately 480 ha and was worth $1.9 billion. In Ontario, estimated losses attributed to bacterial soft rots were 12% to 20% in each of several crops (OMAFRA, 2009). Currently, a significant part of the greenhouse industry routinely recirculates hydroponic nutrient solutions. This recirculation increases the incidence and severity of disease due to Pcc and other pathogens such as Pythium and Fusarium spp. in the irrigation solution (Schuerger, 1998; Blom, 2003; Sutton et al., 2006). Approximately 100 colony forming units per milliliter (CFU/ml) of Pcc caused about 10% loss of calla lily (Zantedeschia sp.) plants (Gracia-Garza et al., 2002; Gracia-Garza et al., 2004). Consequently, there is a need to find fast,
user-friendly, disease specific and quantitative tools for detecting Pcc in greenhouse hydroponic systems (Blom, 2001).

The common method for detecting Pectobacterium spp. is through polymerase chain reaction (PCR), which can detect 2-4 CFU/ml (Kang et al., 2003). Immunoblot and enzyme-linked immunosorbent assay (ELISA)-based detection systems, using pathogen specific antibodies, have been commercially developed for P. atroseptica and P. wasabiae with detection limits of ca. 10³ CFU/ml. ELISA is typically one of the most sensitive and frequently used methods for estimation of pathogens (i.e. bacteria) and LPS molecules (Hill and Matsen, 1983; Freudenberg et al., 1989). To this end, antibodies can be used to specifically capture and quantify pathogens and their toxins in hydroponic systems.

PCR- and ELISA-based tests are not easy to apply on-site (i.e., in the greenhouse). A colorimetric assay could provide a more rapid, simple, and inexpensive detection tool for use in hydroponic greenhouses (Jin et al., 2005). A simple colorimetric assay, currently being used in the medical and environmental fields, is a lateral flow assay more commonly known as a ‘dipstick’ assay utilizing the concepts of ELISA. Pathogen specific antibodies are linked to colloidal gold and attached to a paper medium, ready to be used for detection and quantification of pathogens in samples.

A rapid, user-friendly dipstick assay for detection of Pcc, based on a specific Pcc ELISA, would be of considerable benefit to the greenhouse industry. The present report describes a competitive inhibition ELISA (CI-ELISA) for the detection of Pcc using anti-HK Pcc and anti-Pcc LPS. A user-friendly dipstick assay for detection of Pcc was also developed.
3.2 Materials and Methods

3.2.1 Bacterial Strain Preparation

The one *Erwinia* sp. and five *Pectobacteria* ssp. used in this study, *E. amylovora* Burrill, *P. carotovorum* ssp. *atroscopicum* van Hall, *P. carotovorum* ssp. *betavasculorum* Thomson et al., *P. carotovorum* ssp. *carotovorum* Jones (*Pcc*), *P. carotovorum* ssp. *wasabiae* Goto and Matsumoto and *P. chrysanthemi* Burkholder, are listed in Table 1. All *Erwinia* and *Pectobacterium* spp./ssp. were purchased from the American Type Culture Collection (ATCC) (Manassas, VA). Freeze-dried bacterial cultures were revived according to the ATCC protocol supplied with the cultures. Subsequently, a portion of the cultures were placed in 15% (v/v) glycerol solution (≥99% glycerol G5516, Sigma-Aldrich, Oakville, ON), aliquoted and stored at -80°C, while another portion of the culture was grown on nutrient broth (NB) agar plates (8 g NB and 15 g granulated agar in 1 L dH₂O, autoclaved at 121°C for 15 min; NB and agar; Difco, Detroit, MI) at approximately 26°C for 24 to 48 h or until cell growth was apparent. Plates were stored at 4°C and new plates were prepared every two months.

3.2.2 Preparation of Heat Killed Bacteria

All *Pectobacterium* and *Erwinia* spp. were initially started using glycerol stocks and grown overnight (26°C for 24 h, 150 rpm) as small-scale (10 ml) NB cultures and subsequently used to inoculate larger (1 L) cultures. Small- and large-scale cultures were grown to an optical density (λ = 600 nm) of 0.1, which is equivalent to ca. 1 x 10⁸ CFU *Pcc* per ml. Growth curves were prepared for each *Pectobacteria* ssp. For heat killing,
Table 1. Strains of *Pectobacterium* and *Erwinia* used 

<table>
<thead>
<tr>
<th>Strain</th>
<th>Host</th>
<th>Geographical origin</th>
<th>ATCC(^b) number</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Erwinia amylovora</em></td>
<td>Pear (<em>Pyrus communis</em>)</td>
<td>United Kingdom</td>
<td>15580</td>
</tr>
<tr>
<td><em>P. carotovorum</em> ssp. atrosepticum</td>
<td>Potato (<em>Solanum tuberosum</em>)</td>
<td>United Kingdom</td>
<td>33260</td>
</tr>
<tr>
<td><em>P. carotovorum</em> ssp. betavasculorum</td>
<td>Sugar beet (<em>Beta vulgaris</em>)</td>
<td>California</td>
<td>43762</td>
</tr>
<tr>
<td><em>P. carotovorum</em> ssp. carotovorum</td>
<td>Potato (<em>Solanum tuberosum</em>)</td>
<td>Denmark</td>
<td>15713</td>
</tr>
<tr>
<td><em>P. carotovorum</em> ssp. wasabiae</td>
<td>Japanese horseradish (<em>Eutrema wasabi</em>)</td>
<td>Japan</td>
<td>43316</td>
</tr>
<tr>
<td><em>P. chrysanthemi</em></td>
<td>Chrysanthemum (<em>Chrysanthemum morifolium</em>)</td>
<td>United States</td>
<td>11663</td>
</tr>
</tbody>
</table>

\(^a\) Details provided by ATCC. \(^b\) American Type Culture Collection.
10-ml cultures were prepared and placed into a steamer (~100°C) for 1 h. To ensure that cells were dead, 100 µl of culture was added to a NB agar plate and incubated at 26°C for 24 h.¹ Heat killed (HK) cells were centrifuged at 12,000 g (Sorval, DuPont Instruments, Mississauga, ON; Model RC-5B) for 15 min, and supernatant removed. Cells were washed three times with 10 ml phosphate buffered saline (PBS) (1 x PBS; 8 g NaCl, 1.44 g Na₂HPO₄, 0.24 g KH₂PO₄, 0.2 g KCl in 1 L dH₂O; pH 7.4) and centrifuged at 12,000 g for 5 min between washes. After the final wash, cells were resuspended in 1 ml PBS and stored at 4°C for no more than 48 h.

3.2.3 Infection of Calla Tubers with *Pcc*

To ensure the *Pcc* culture was pathogenic, a healthy tuber of calla lily (*Zantedeschia elliottiana*) was infected with *Pcc*. The tuber was washed with tepid water, the thin outer membrane peeled, and other surface blemishes were removed to ensure infection sites would appear more noticeable. Thin slices (~5 mm) of the inner tuber tissue were cut and placed in Petri dishes. Three slices of tuber were placed into separate Petri dishes, with one dish containing tubers that were not inoculated with *Pcc* (negative control) while the other dish contained tuber slices wounded and inoculated with approximately (20 µl of cell suspension) 10⁴, 10⁶, and 10⁸ CFU per inoculation site. Each site was inoculated by means of a syringe with a sterile 18 gauge needle. The needle punctured the tuber tissue and the cell suspension was released into the tissue. Injected cell suspension that escaped the puncture sites was drained so it would not pool

¹ Note: when cells were not present on the plate after overnight incubation, cells were designated as dead or ‘heat killed’.
around the tuber slice. Petri dishes were placed at 37°C, and viewed after 24, 36 and 72 h and visually compared to the negative control.

3.2.4 LPS Isolation

Large-scale 1-L cultures of all Pectobacterium ssp. were grown at room temperature (RT; ~24-26°C) in NB for 24 to 48 h with shaking at 150 rpm. Cultures were centrifuged at 12,000 g for 15 min at 4°C in 250-ml centrifuge tubes. Once the supernatant was removed, centrifuge bottles containing cell pellets were placed at -20°C for 1.5 h in preparation for lyophilization. To lyophilize the bacterial cells, the bottles with the cell pellets were placed in a freeze-drier (Labconco, Kansas City, MO, Model 4.5, Vacuum/Temperature) for approximately 24 h, after which the lyophilized cells were stored at 4°C. LPS from all of the Pectobacterium ssp. were isolated using the method of Yi and Hackett (2000). One to 10 mg of lyophilized bacterial cells were suspended in 200 µl of Tri-Reagent (Molecular Research Center Inc., Cincinnati, OH) and incubated at RT for 15 min to separate the LPS from the cell wall. After incubation and complete cell homogenization, 20 µl of chloroform per mg of cells were added, the mixture vigorously vortexed, incubated at RT for 10 min and centrifuged at 12,000 g for 10 min, to create an aqueous/organic phase separation. The aqueous (upper) phase was transferred to a new 1.5-ml centrifuge tube. To ensure complete removal of LPS from the chloroform phase, 100 µl of distilled water (dH2O) was added to the organic phase, the

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2 Note: Tri-Reagent is used for the isolation of total RNA or the simultaneous isolation of RNA, DNA and proteins from bacterial samples. A biological sample was homogenized in Tri-Reagent and the homogenate was separated into aqueous and organic phases by the addition of chloroform followed by centrifugation.
mixture vortexed, incubated at RT for 10 min and centrifuged for 10 min at 12,000 g; this extraction step was repeated twice more. All aqueous phases were combined and dried for approximately 16 h in a freeze-drier. The crude LPS was dissolved in 500 µl of cold 0.375 M magnesium chloride (MgCl₂) in 95% ethanol and centrifuged for 15 min at 12,000 g. The pellet was suspended in 200 µl dH₂O and lyophilized to yield a fluffy white solid LPS totaling 15-20% of the starting material dry weight. Lyophilized LPS was stored at 4°C; aliquots were weighed and resuspended in PBS when needed.

3.2.5 Quantitative Estimation of Purified LPS

The carbocyanine colorimetric assay was performed for quantitative estimation of the purified LPS (Janda and Work, 1971; Yi and Hackett, 2000; Kroll et al., 2005; D’Silva, 2006). The amount of purified LPS was estimated from a standard curve (correlation coefficient, 0.995) generated by using 1-20 µg LPS (Janda and Work, 1971; D’Silva, 2006). Approximately 0.5 µg of LPS was dissolved in 0.5 ml of H₂O, followed by the addition of 0.3 ml of carbocyanine dye reagent and 0.2 ml ascorbic acid. The mixture was incubated at 4°C in the dark for 10 min and the optical density was read at a wavelength of 472 nm.

3.2.6 LPS Analysis

Purified LPS was electrophoresed on a 12% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) and stained using the PlusOne™ Silver Staining Kit (GE Healthcare Bio-Sciences Inc., Baie d’Urfé, Québec, Canada) as described by Tsai and Frasch (1982) and Kroll et al. (2005).
3.2.7 Preparation of Polyclonal Antibodies

Two New Zealand white rabbits (*Oryctolagus cuniculus*; NZW; female, 2.8-3.0 kg, 11-13 weeks old) were used for the immunizations. One rabbit was injected subcutaneously with 10 μg *Pcc* LPS in a 1:1 PBS to Titermax® emulsion (Sigma-Aldrich, Oakville, ON), while the other rabbit was injected with 10⁵ CFU/ml of heat killed *Pcc* in a 1:1 PBS:Titermax® emulsion. All injections were performed with Titermax® adjuvant (Sigma-Aldrich, Oakville, ON). Rabbits were injected four times (~250 μl per injection site; two injection sites) at the first time of injection and, thereafter, once at a different injection site every two weeks. After four months, three more immunizations (~250 μl per injection site; two injection sites) per rabbit were performed. Rabbits were bled (~5 ml blood collected) during the weeks between injections. A terminal heart bleed (~200 ml) of the rabbits was collected one week following the last injection. Serum was prepared by centrifugation at 2,700 g for 10 min at 4°C following clotting at RT for 1-4 h. All serum samples were aliquoted and stored at -80°C.

3.2.8 Serum Titer Analysis

To observe increases in antibody titer over the course of the rabbit immunizations, serum samples were analyzed for antibodies raised against *Pcc* LPS and HK *Pcc* using an indirect assay described by Desphande (1996). Microtiter plates (96-well flat bottom polystyrene high binding microplates; Costar; Corning, NY) were coated with *Pcc* LPS (10 μg / 100 μl sodium phosphate, 2% formalin (37% (w/v) formaldehyde in H₂O; Sigma-Aldrich, Oakville, ON) and HK *Pcc* (10⁸ CFU) per ml in 0.1 M bicarbonate buffer, pH 9.6-9.8) overnight (o/n) at RT and 4 °C, respectively. After incubation, the
wells were decanted and blocked with 2% w/v skim milk powder in PBS (MPBS; 200 μl/well) for 2 h at RT, while being shaken at ca. 30 rpm. The wells were washed three times with PBS-Tween (PBST; 0.05% Tween 20). Diluted sera (pre-immune to fourth bleed; 1:100 serum dilution in 100 μl MPBS) were added to designated wells and kept for 1.5 h at RT. Following three washes with PBST (200 μl/wash), secondary antibody (1:6,000 dilution in 100 μl MPBS) was added to each well for 1 h at RT. The secondary antibody used was goat anti-rabbit IgG (GAR-HRP; Jackson Labs, West Grove, PA) conjugated to horseradish peroxidase (HRP). After decanting and three washes with PBST (200 μl), TMB substrate (100 μl; 1-Step Turbo TMB; Thermo Fisher Scientific, Nepean, ON) was added and left to develop for 1 h at RT in the dark. The optical density (OD) was determined spectrophotometrically at a wavelength of 450 nm after neutralizing with 0.2 M H₂SO₄ (100 μl). All dilutions were run in triplicate, absorbance readings were averaged, and background subtracted. Negative and positive controls consisted of PBS, secondary antibody only, substrate only, uncoated wells incubated with serum dilutions, and coated wells incubated with only secondary antibody only or only substrate.

3.2.9 Checkerboard ELISAs

To determine the appropriate dilutions of rabbit serum and coating antigen required to optimize the final diagnostic assay, both were titrated using a checkerboard assay (Desphande, 1996). Pcc LPS or HK Pcc was used to coat 96-well polystyrene microtiter plates. The Pcc LPS coating antigen was titrated (100 μl/well) down the plate in 1:2 serial dilutions in formalin-sodium phosphate buffer from an initial stock solution.
The HK Pcc coating antigen was titrated (100 µl/well) down the plate in 1:2 serial dilutions in bicarbonate buffer from a stock preparation. The plates were incubated o/n at 4°C (RT for formalin). The plate was washed three times with PBST buffer and then blocked with 2% MPBS (200 µl/well) for 2 h at RT. The rabbit anti-Pcc LPS and anti-HK Pcc sera were diluted 1:4,000 in MPBS, added in 1:2 serial dilutions (100 µl/well) across the appropriately coated plates and allowed to incubate for 1 h at RT. After incubation, the wells were washed three times with PBST. GAR-HRP was added (100 µl/well) at a dilution of 1:6,000 in MPBS and allowed to incubate for 1 h at RT. Unbound conjugate was removed by washing three times with PBST. TMB substrate was added (100 µl/well) and allowed to react for 1 h at RT in the dark. The reaction was neutralized using sulfuric acid (100 µl, 0.2 M), and the optical density was observed at a wavelength of 450 nm.

3.2.10 Competitive Inhibition ELISAs

Indirect CI-ELISAs were developed for both Pcc LPS and HK Pcc to assess the specificity and reproducibility of the assay. A standard curve showing competition between the coating conjugate and the free Pcc in solution was prepared through the use of a competitive inhibition assay (Furzer et al., 2006). Polystyrene microtiter plates (96 wells) were coated (100 µl/well) with Pcc LPS in formalin-sodium phosphate buffer (1 µg/ml) or HK Pcc in 0.1 M bicarbonate (10⁸ CFU/ml) and incubated overnight at RT and 4°C, respectively. Plates were blocked with 2% MPBS (200 µl/well) for 2 hours at RT. The rabbit anti-Pcc LPS and anti-HK Pcc sera were diluted to 1:8,000, as determined by the checkerboard assay, in 2% MPBS and mixed with various concentrations of Pcc LPS
or HK Pcc to create standard curves. Serum solution (without competitor or “free” antigen) as well as solutions of serum and “free” Pcc LPS or HK Pcc were applied (100 μl/well) to the coated and blocked wells and were incubated for 1 h at RT.

Free pathogen concentrations varied from $3 \times 10^{-2} \mu g/ml$ to 50 μg/ml, and approximately 1 CFU/ml to $10^8$ CFU/ml for Pcc LPS and HK Pcc, respectively. After three washes with PBST, 100 μl/well GAR-HRP (1:6,000 dilution in 2% MPBS) was added and allowed to react for 1 h at RT. Thereafter, the wells were washed three times with PBST buffer. TMB substrate was added (100 μl/well) and left to develop for 1 h at RT in the dark. The reaction was stopped with 0.2 M H$_2$SO$_4$ (100 μL) and read spectrophotometrically at wavelength 450 nm (OD$_{450}$). The CI-ELISAs were developed to determine the specificity of the anti-Pcc LPS and anti-HK Pcc sera to both Pcc LPS and HK Pcc cells. Inhibition was calculated by plotting the ratio of absorbance and maximum absorbance observed ($A/A_o$) against the concentration of the antigen (Furzer et al., 2006).

3.2.11 Cross Reactivity of Polyclonal Serum

The CI-ELISAs were also tested for the ability to recognize and differentiate between HK Pcc and Pcc LPS, and other environmental pathogens (i.e. cross-reactivity; Vega-Warner et al., 2000; Table 2). The collected serum was analyzed for cross-reactivity with various Pectobacterium spp., E. amylovora and other common pathogens (i.e. Pythium aphanidermatum Edson, PaNie (Pythium aphanidermatum Necrosis inducing elicitor), Escherichia coli (Sigma-Aldrich, Oakville, ON), Listeria.
Table 2. *Pectobacterium* spp. and other common pathogens tested for cross-reactivity against *Pcc*.

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Antigen concentration (µg/ml, CFU/ml)(^a)</th>
<th>Type of antigen used in assay</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. amylovora</em></td>
<td>50; 10(^8)</td>
<td>L(^b), W(^c)</td>
</tr>
<tr>
<td><em>P. carotovorum</em> ssp. <em>atrosepticum</em></td>
<td>50; 10(^8)</td>
<td>L(^b), W(^c)</td>
</tr>
<tr>
<td><em>P. carotovorum</em> ssp. <em>betavasculorum</em></td>
<td>50; 10(^8)</td>
<td>L(^b), W(^c)</td>
</tr>
<tr>
<td><em>P. carotovorum</em> ssp. <em>carotovrum</em></td>
<td>50; 10(^8)</td>
<td>L(^b), W(^c)</td>
</tr>
<tr>
<td><em>P. carotovorum</em> ssp. <em>wasabiae</em></td>
<td>50; 10(^8)</td>
<td>L(^b), W(^c)</td>
</tr>
<tr>
<td><em>P. chrysanthemi</em></td>
<td>50; 10(^8)</td>
<td>L(^b), W(^c)</td>
</tr>
<tr>
<td><em>Pythium aphanidermatum</em></td>
<td>2.38 mg/ml</td>
<td>E(^d)</td>
</tr>
<tr>
<td>PaNie</td>
<td>1 µg/ml</td>
<td>E(^d)</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>50 µg/ml</td>
<td>L(^b)</td>
</tr>
<tr>
<td><em>Listeria monocytogenes</em></td>
<td>10(^8)</td>
<td>W(^c)</td>
</tr>
<tr>
<td><em>Fusarium graminearum</em></td>
<td>2.2 mg/ml</td>
<td>E(^d)</td>
</tr>
</tbody>
</table>

\(^a\)Initial competition concentrations. \(^b\)LPS (L). \(^c\)Heat killed whole cell (W). \(^d\)Cell extract (E).
monocytogenes Murray et al., and Fusarium graminearum Schwein, through the use of a competitive inhibition ELISA (CI-ELISA).

Both anti-HK Pcc serum and anti-Pcc LPS serum were analyzed for cross-reactivity. Separate 96-well polystyrene microtiter plates were coated with Pcc LPS (1 μg/ml) in sodium phosphate formalin buffer or HK Pcc (10^8 CFU/ml) in 0.1 M bicarbonate buffer, pH 9.6-9.8 (100 μl/well) and incubated overnight at RT and 4°C, respectively. Following incubation, wells were blocked with 2% MPBS (200 μl/well) for 1 hour at RT. The rabbit anti-Pcc LPS and anti-HK Pcc sera were diluted appropriately, as determined by the checkerboard assay, in 2% MPBS and mixed with various concentrations of Pcc LPS or HK Pcc to derive a standard curve. Serum was tested for binding to five other Pectobacterium spp. LPS as well as various unrelated pathogens. Solutions of serum alone (without competitor), and solutions of serum and “free” pathogen were applied (100 μl/well) to the coated and blocked wells and left to incubate for 1 h at RT.

Concentrations of free antigen varied from 3.1 x 10^{-3} to 50 μg LPS /ml and approximately 1 CFU to 10^8 CFU HK Pcc/ml. Following three washes with PBST, GAR-HRP was added (100 μl/well) at a dilution of 1:6,000 in 2% MPBS and allowed to react for 1 h at RT. Unbound conjugate was removed by washing three times with PBST buffer. TMB substrate was added (100 μl/well) and left to develop for 1 h at RT in the dark. The antibody binding was determined spectrophotometrically at 450 nm after neutralizing with 0.2 M H_2SO_4 (100 μL). Cross-reactivity was expressed as a percentage of inhibition observed as compared to the inhibition by Pcc observed at the IC_{50} of the standard curve (Vega-Warner, 2000; Furzer et al., 2006).
3.2.12 Preparation of Standard Curves in Nutrient Solution and PBS

For *Pcc* LPS and HK *Pcc* primary standard curves were prepared. A series of diluted standards, in 2% MPBS, ranging from $3 \times 10^{-2}$ to 50 µg/ml (*Pcc* LPS) and approximately $10^0$ to $10^8$ CFU/ml (HK *Pcc*) were prepared from the primary standard by serial dilution (Miller *et al*., 1991). ELISAs were also performed using MPBS and hydroponic nutrient solutions spiked with *Pcc* LPS or HK *Pcc* at concentrations of 0.1, 0.2, 0.5, 1.5, and 4.0 µg/ml and $10^1$, $10^2$, $10^3$, $10^4$, $10^5$ and $10^6$ CFU/ml, respectively.

Two hydroponic solutions were tested: (1) greenhouse hydroponic solution (pH 6.0; EC 1.2; Boekestyn Greenhouses, Jordan Station, ON), and (2) greenhouse hydroponic solution (pH 6.2; EC 1.3; Westbrook Greenhouses, Beamsville, ON). Both solutions were filtered through 0.22 µm filters, followed by plating on media to test for the presence of potential microorganism contamination. Any nonspecific matrix effects were accounted for by determining the absorbance with MPBS and hydroponic nutrient solution alone (without ‘free’ *Pcc* and without ‘free’ *Pcc* and rabbit serum added).

Standard curves showing the competition between the free *Pcc* and the coating antigen were prepared using CI-ELISAs. The standard curves were constructed from dose-response data points using a GraphPad Prism, Version 5.01 (GraphPad Software, Inc., CA). The linear range of the CI-ELISA was determined by plotting the ratio of sample absorbance to maximum absorbance observed for the control ($A/A_o$) against the concentration of *Pcc*. The IC$_{50}$ or concentration of *Pcc* required for 50% inhibition of the mean blank was inferred. The limit of quantitation (LOQ), defined as the concentration of *Pcc* that gives a signal ten times the standard deviation of the mean blank for zero competition ($A_o$), and the limit of detection (LOD), defined as the concentration of *Pcc*
that gives a signal three times the standard deviation of the mean blank for zero competition ($A_o$), were also calculated (Armbruster et al., 1994; Clegg et al., 2001; Furzer et al., 2006).

3.2.13 Purification of Polyclonal Antibodies For Lateral Flow Assay

Five millilitres of polyclonal rabbit anti-HK $Pcc$ serum (from the terminal bleed) was dialyzed against 20 mM PBS buffer (pH 7.0) o/n at 4°C with 10,000 MW cut-off dialysis tubing (Spectrum Laboratories, Inc, Rancho Dominguez, CA). The dialyzed serum was centrifuged (1 min; 10,000 g), filtered through a 0.22 μm syringe filter, loaded onto a 5-ml Protein G HiTrap column (GE Healthcare, Piscataway, NJ) and purified using the ÄKTA FPLC system (GE Healthcare Bio-sciences AB, Uppsala, Sweden). A purified IgG fraction was eluted with 100 mM glycine buffer (pH 2.7) and neutralized with 1 M Tris/HCl (pH 8.8) to pH 7.0. The fraction was dialyzed (10,000 MW dialysis tubing) overnight against pre-chilled PBS (pH 9.0) at 4°C. An OD reading at 280 nm was taken and the protein concentration was determined using the BCA-protein assay (Pierce, Rockford, IL). SDS-PAGE (12% gel) was used to confirm the purity of the antibody fraction (IgG) under non-reducing condition, followed by staining with Coomassie dye.

3.2.14 Lateral Flow (Dipstick) Assay Development

A ‘Lateral Flow (LF) Development Kit’ with detailed instructions was purchased from Millenia Diagnostics, Inc (MDx; San Diego, CA). The colloidal gold particles (MDx; San Diego, CA) and the purified polyclonal antibodies (adjusted to 1 mg/ml, pH 9.0) used to prepare the LF assay indicator conjugate (i.e. anti-HK $Pcc$ IgG-gold
conjugate) were prepared according to the manufacturer’s instructions. Briefly, the anti-HK Pcc IgG was titrated prior to the mixing of each IgG concentration with colloidal gold to determine the appropriate concentration needed to stabilize the colloidal gold prior to conjugation. The optimum gold-IgG mixture (approximately 2.3 μg protein (IgG) per 1 ml gold) was incubated for 30 min at RT, followed by the addition of 10 μl conjugate block solution (MDx; San Diego, CA) per 1 ml gold. The conjugate was centrifuged at 7,000 g for 45 min at 4°C. After centrifugation, the pellet was re-suspended to 1/10 of the starting volume (100 μl) in the conjugate stabilizing solution (MDx; San Diego, CA). To prepare the conjugate for dispensing on the glass fiber/conjugate pad, 20% (w/v) sucrose (Sigma-Aldrich, Oakville, Ontario, Canada) and 5% (w/v) trehalose (Sigma-Aldrich, Oakville, Ontario, Canada) were added and then vortexed until dissolved. The purified anti-HK Pcc-colloidal gold (40 nm particles, \( \text{OD}_{540} \) of 1.0) conjugate, and the positive control, i.e. goat anti-rabbit IgG (GAR, Fc fragment specific; Thermo Scientific, Rockford, IL), were evaluated in a series of titration experiments to determine optimum concentrations for the assay (Al-Yousif et al., 2002). The antibody-gold conjugate was used within one week of its preparation.

The glass fiber/conjugate pads (3.0 cm x 2.5 cm) were saturated with 3 ml lateral flow block solution (MDx; San Diego, CA) and incubated for 5 min at RT. Glass fiber pads were dried on a paper towel in a 37°C incubator until completely dry (~1 hour) and stored in a desiccator container. The conjugate was dispensed with a pipette (0.5 μl x 16 spots) in the middle of the pad at 8 μl/cm followed by drying on aluminum foil at 37°C for 45 min. To prevent sticking during the drying process, the conjugate pads were
periodically lifted from the foil. The conjugate pads were stored in a desiccator container.

3.2.14.1 Membrane striping/immobilization of proteins on nitrocellulose

Whole \textit{Pcc} cells, $10^8$ CFU HK \textit{Pcc} were prepared in 100 µl striping buffer (MDx; San Diego, CA) and vortexed for 10 sec. The control-line protein (i.e. GAR; 2.3 mg/ml) was also diluted in 100 µl striping buffer and vortexed for 10 seconds. After testing various Hi-Flow nitrocellulose membranes (HF90, HF120, HF135, HF180 and HF240) supplied with the LF kit, HF240 (slowest wicking time) was chosen for use in the LF assay since a high sensitivity was required. Goat anti-rabbit (GAR; control) and HK \textit{Pcc} (test) were dispensed, in two respective lines, with a pipette (0.5 µl x 16 spots) on the membrane at 8 µl/cm. The control line and test line were applied along the width of the membrane and were approximately 1 cm apart. The membrane was dried at 37°C until the lines were no longer visible (10 min) after which the membrane was blocked with 3 ml of lateral flow block solution. The block was added to the bottom of the strip so that it wicked to the top of the membrane. Strips were left to block for 2 min at RT, followed by drying at 37°C for 1 h before being stored in a desiccator container.

3.2.14.2 Assembly of lateral flow test

All solid components were mounted onto a backing card. The nitrocellulose membrane was the first to be placed on the backing card, followed by slight overlap with the fiber conjugate pad and sample pad (sample application). The absorbent pad
(wicking pad) was aligned with the top edge of the backing card. Once assembled, the card was cut into approximately 5 mm strips with scissors (Figure 18).

**Figure 18.** Overview of a typical (a) lateral flow (dipstick) test strip, and the format used for this thesis (b). A typical dipstick assay uses a ‘sandwich’-based assay format, where the conjugate pad contains IgG-gold labelled conjugate, and the test line contains a primary IgG; whereas the dipstick format (competition) used for this thesis contains an antigen-based (HK Pcc) test line.
3.2.14.3 Analysis of Pcc in hydroponic solutions

Greenhouse hydroponic solutions from Boekestyn Greenhouses and Westbrook Greenhouses were used for the analyses. Nine 3-ml glass tubes were filled with 1 ml nutrient solution, tubes 2 to 8 were spiked with $10^1$, $10^2$, $10^3$, $10^4$, $10^5$, $10^6$ and $10^7$ CFU/ml of live Pcc, respectively. Tube 1 did not contain Pcc. LF test strips were placed one by one into separate tubes, with the bottom half of the sample pad submersed in the test solution. Each sample was allowed to wick until the sample reached the bottom of the absorbent pad (~2-5 min). Test strips were removed from each solution and placed at RT until dry (~10 min). Following drying, the membrane was evaluated visually. The presence of a line at the positive control position on the membrane (i.e. GAR antibody line) indicated that the assay was functioning properly.

3.3 Results

3.3.1 Infection of Calla Tubers with Pcc

Decay (soft rot) developed in the inoculated tuber slices but not in the controls (Figure 19). Tissue slices inoculated with approximately $10^4$, $10^6$, and $10^8$ CFU/ml darkened near injection sites within 24 h, and softened by 48 h (Figure 19). After 72 h the tissues darkened further and began to disintegrate (Figure 19). During 24 h to 72 h an unpleasant odour characteristic of Pcc infection (reminiscent of garbage) was noted. At 24 h, slices injected with $10^8$ CFU/ml appeared to have decayed more rapidly than those injected with $10^6$ or $10^4$ CFU/ml (Figure 19).
Figure 19. Sliced calla tubers infected with *Pcc* at three concentrations (10⁴, 10⁶ and 10⁸ CFU/ml) after 0h, 24 h, 48 h, and 72 h. Infected tissue was compared to uninfected tissue after 24 h, 48 h and 72 h. Red circles indicate where the tissue was injected with *Pcc*. 

<table>
<thead>
<tr>
<th></th>
<th>10⁴</th>
<th>10⁶</th>
<th>10⁸</th>
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<tbody>
<tr>
<td>Uninfected</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
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<tr>
<td>72 h</td>
<td>![Image]</td>
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</tbody>
</table>
3.3.2 SDS-PAGE and Silver Staining of LPS

When the LPS of the Pcc spp. were compared to LPS standards of P. aeruginosa and E. coli (Darveau and Hancock, 1983; Kroll et al., 2005), as determined by SDS-PAGE and silver staining, all three has similar bands ranging from 18 to 25 kDa, with the most prevalent band for Pcc appearing at 20 kDa (Figure 20). Silver staining was used to confirm glycosylation since the aldehyde groups of the sugars react with the silver (Kroll et al., 2005). According to Brade et al. (1987), Holst et al. (1993) and Poxton (1995), the silver stain identifies the Pectobacterium spp. that contains smooth (lipid-A, core oligosaccharide and O-polysaccharide units) and rough (void of O-polysaccharide) LPS. Smooth LPS exhibits a regular distribution of bands or ‘ladder pattern’ of spaced bands while rough LPS was shown as a smeared band (Figure 20). The smooth LPS bands demonstrate the repeating oligosaccharide units of O-polysaccharide that increase in molecular mass (Poxton, 1995).

3.3.3 Polyclonal Serum Titers and Checkerboard Assays

The anti-Pcc LPS and anti-HK Pcc in the rabbit sera steadily increased from the first to the fourth injection (Figure 21 and Figure 22). The fourth bleed was used for all further ELISAs in this study since the serum was the most sensitive, with a signal to noise ratio of approximately ten. The noise is the average background signal (OD$_{450}$) of an uncoated well. Since the terminal bleed exhibited similar titer sensitivity to the fourth bleed, and provided a greater total volume of serum, it was used for developing the dipstick assay.
Figure 20. Silver staining of *Pectobacterium* spp. LPS separated on 12% polyacrylamide gels. (a) First lane (left), benchmark pre-stained protein marker (Invitrogen); second lane, *Pseudomonas aeruginosa* LPS standard (Sigma-Aldrich); third and fourth lane, *Pcc* LPS; (b) first lane (left), benchmark pre-stained protein marker (Invitrogen); second lane, *P. chrysanthemi* LPS; third lane, *E. coli* LPS standard (Sigma-Aldrich); fourth lane, blank; fifth lane, *Pcc* LPS; sixth lane, *E. amylovora* LPS; seventh lane, *P. c. atroseptica* LPS; eighth lane, *P. c. betavasculorum* LPS; ninth lane, blank; and tenth lane, *P. c. wasabiae* LPS.
**Figure 21.** Analysis of rabbit serum for anti-\textit{Pcc} LPS antibodies (i.e. rabbits immunized with \textit{Pcc} LPS) when microtitre plate was coated with \textit{Pcc} LPS.
Figure 22. Analysis of rabbit serum for anti-HK Pcc antibodies (i.e. rabbits immunized with HK Pcc) when microtitre plate was coated with Pcc LPS.
The results of the checkerboard ELISAs suggested that a 1:8,000 dilution of rabbit anti-Pcc LPS and rabbit anti-HK Pcc serum and a dilution of 1 μg/ml Pcc LPS and 10^8 CFU/ml HK Pcc, respectively, were the appropriate concentrations to use for the development of the CI-ELISA (Figure 23).

3.3.4 Antiserum Cross-Reactivity

To determine the specificity of the assay, it was important to analyze both the anti-Pcc LPS and anti-HK Pcc sera with various Pectobacterium ssp. and other common pathogens, some of which may contaminate greenhouse hydroponic solutions, for the potential to interfere with anti-serum binding. The cross-reactivity data are summarized in Table 3. Little cross-reactivity was observed with the pathogens tested in the ELISA format, which means that the assay is specific for Pcc with virtually no cross-reactivity (<1%) against other Pectobacterium sp. and the other gram negative pathogens tested (Table 3). Cross-reactivity was expressed as a percentage of inhibition observed versus the inhibition by Pcc observed at the IC_{50} of the standard curve (Furzer et al., 2006). Non-linear regression curves of the competitive inhibition ELISAs conducted with anti-Pcc LPS serum and Pcc LPS coated microtiter plates also showed that there was little cross-reactivity with LPS from various other gram negative pathogens (Figure 24).

3.3.5 Standard Curves and Detection of Pcc

After plotting the A/A_o against the concentration of free Pcc, the linear working range of the Pcc LPS CI-ELISA was determined to be 23 to 780 ng/ml. The IC_{50} or concentration of Pcc required for 50% inhibition of the mean blank, A_o, was calculated to
Figure 23. Checkerboard ELISA assay to determine the best antigen and serum concentrations to use for development of CI-ELISAs. (a) *Pcc* LPS: an initial coating concentration of 1 µg/ml was serially diluted 1:2; (b) HK *Pcc*: an initial coating concentration of $10^8$ CFU/ml was serially diluted 1:10.
**Table 3.** Cross-reactivity of anti-*Pcc* LPS serum from rabbit with LPS from various *Pectobacterium* spp. and other gram negative pathogens.

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>% Activity</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. carotovorum subsp. carotovorum</em></td>
<td>100.00</td>
<td>0.43</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>0.90</td>
<td>0.037</td>
</tr>
<tr>
<td><em>Pectobacterium chrysanthemi</em></td>
<td>0.66</td>
<td>0.045</td>
</tr>
<tr>
<td><em>P. carotovorum subsp. atrosepticum</em></td>
<td>0.50</td>
<td>0.035</td>
</tr>
<tr>
<td><em>P. carotovorum subsp. betavascularorum</em></td>
<td>0.47</td>
<td>0.024</td>
</tr>
<tr>
<td><em>E. amylovora</em></td>
<td>0.46</td>
<td>0.025</td>
</tr>
<tr>
<td><em>P. carotovorum subsp. wasabiae</em></td>
<td>0.44</td>
<td>0.046</td>
</tr>
<tr>
<td><em>Fusarium graminearum</em></td>
<td>0.17</td>
<td>0.0067</td>
</tr>
<tr>
<td><em>Pythium aphanidermatum</em></td>
<td>0.14</td>
<td>0.045</td>
</tr>
<tr>
<td><em>PaNie</em></td>
<td>0.075</td>
<td>0.021</td>
</tr>
<tr>
<td><em>Listeria monocytogenes</em></td>
<td>0.083</td>
<td>0.012</td>
</tr>
</tbody>
</table>

* LPS of *Pectobacterium* spp. used.  
  b Measure of cross-reactivity expressed as a percentage of inhibition observed with LPS of other species compared to inhibition by *Pcc* LPS observed at the IC$_{50}$ of the CI-ELISA standard curve.  
  c Standard deviation (n=3).
**Figure 24.** Line of best fit driven by *Pcc*. CI-ELISAs were conducted with anti-*Pcc* LPS serum and *Pcc* LPS coated microtiter plates; see previous page as well. Antigens used for competition included *Pcc* LPS (■) and the LPS from various other gram negative pathogens (●).
be 131 ng/ml (Figure 25). The limit of detection (LOD), defined as the concentration of *Pcc* that gives a signal three times the standard deviation of the mean blank for zero competition (A₀), was 23 ng/ml. The limit of quantitation (LOQ), defined as the concentration of *Pcc* that gives a signal ten times the standard deviation of the mean blank for zero competition (A₀; Armbruster *et al.*, 1994; Clegg *et al.*, 2001; Furzer *et al.*, 2006) was 76 ng/ml. The linear working range of the HK *Pcc* CI-ELISA was 10¹ to 10⁶ CFU/ml, while the IC₅₀ was 1.3 x 10³ CFU/ml (Figure 26). The LOD and LOQ were 81 CFU/ml and 216 CFU/ml, respectively.

### 3.3.6 Analysis of HK *Pcc* and *Pcc* LPS in Hydroponic Solutions

Hydroponic nutrient solution was spiked with *Pcc* LPS or HK *Pcc* at concentrations of 0.1, 0.2, 0.5, 1.5, and 4.0 µg/ml and 10¹, 10², 10³, 10⁴, and 10⁵ CFU/ml, respectively (Table 4). The concentration of bacteria and LPS in nutrient solution was determined using a standard curve (prepared in MPBS buffer). The statistical comparison of the concentration of either antigen in hydroponic solution and MPBS spiked with *Pcc* LPS or HK *Pcc* indicated that there were no significant differences between the estimated and true values as determined using the Tukey test. For example, the lowest recoveries of *Pcc* LPS and HK *Pcc* were 86% and 88%, respectively. Non-specific matrix effects were also determined by comparing the OD₄₅₀ values in MPBS and hydroponic nutrient solution for binding of serum to the antigen coated plates (i.e., without ‘free’ HK *Pcc* or without ‘free’ *Pcc* LPS).
Figure 25. Standard curve for the \textit{Pcc} LPS ELISA (y = -0.4540 \log(x) + 1.537; r^2=0.9963). Data are presented as the means ± standard errors, n=6; IC\textsubscript{50} = 131 ng/ml. Rabbit anti-Pcc LPS serum was diluted 1:8,000, and the coating concentration of \textit{Pcc} LPS was 1 µg/ml.
Figure 26. Standard curve for the HK Pcc ELISA (y = -0.09264 log(x) + 0.8016; \( r^2 = 0.9721 \)). Data are presented as the means ± standard errors, n=6; IC\(_{50} = 1.3 \times 10^3 \) CFU/ml. Rabbit anti-HK Pcc serum was diluted 1:8,000, and the coating concentration of HK Pcc was 10^8 CFU/ml.
Table 4. Estimation of *Pcc* LPS and HK *Pcc* concentrations in spiked nutrient solutions using ELISA<sup>a</sup>. Estimates were made using a standard curve prepared in MPBS.

<table>
<thead>
<tr>
<th>LPS</th>
<th>mean recovered (µg/ml)</th>
<th>% recovered</th>
<th>SD&lt;sup&gt;b&lt;/sup&gt;</th>
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<tr>
<td>spiked (µg/ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.0</td>
<td>4.30</td>
<td>108</td>
<td>3</td>
</tr>
<tr>
<td>1.5</td>
<td>1.30</td>
<td>86</td>
<td>3</td>
</tr>
<tr>
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<td>0.54</td>
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</tr>
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</tr>
<tr>
<td>0.1</td>
<td>0.11</td>
<td>110</td>
<td>6</td>
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<thead>
<tr>
<th>HK</th>
<th>mean recovered (CFU/ml)</th>
<th>% recovered</th>
<th>SD&lt;sup&gt;b&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>spiked (CFU/ml)</td>
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<td>7.2 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>117</td>
<td>2</td>
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<tr>
<td>1.6 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>1.4 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>90</td>
<td>1</td>
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<td>1.0 x 10&lt;sup&gt;3&lt;/sup&gt;</td>
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<td>4</td>
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<td>3</td>
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<td>7.1 x 10&lt;sup&gt;1&lt;/sup&gt;</td>
<td>103</td>
<td>2</td>
</tr>
</tbody>
</table>

<sup>a</sup>For each concentration, n = 3. <sup>b</sup>Standard deviation.
3.3.7 Lateral Flow Assay and Analysis of *Pcc* in Hydroponic Solutions

The polyclonal rabbit anti-HK *Pcc* serum (terminal bleed) purified using protein G affinity chromatography was analyzed by SDS-PAGE to confirm purity. The gel shows the purified polyclonal antibodies (IgG), with heavy chain and light chain fragments present at approximately 50 kDa and 25 kDa, respectively (Figure 27). The antibody-gold conjugate was prepared using this serum and used within one week.

When testing the 5-mm wide strips with the two nutrient solutions, it was apparent that the nutrient solution only (negative control) strips developed quicker than those placed in nutrient solution spiked with HK *Pcc*. This may be attributed to the presence of the spiked *Pcc* cells and cell debris. The test and control lines became darker (after >5 min), the longer each strip was in contact with the test solution. A single line indicated that the test was working, i.e., the presence of a dark control line and light or no visible spots indicated the presence of high concentrations of *Pcc* (10⁵, 10⁶ and 10⁷ CFU/ml). Conversely, the presence of a darker colored test line (spots) and a dark control line indicated the presence of *Pcc* in lower concentrations (10¹, 10², 10³, and 10⁴ CFU/ml). The darker the test line, the lower the *Pcc* concentration (Figure 28). Once the test strips were developed, the reaction lines, which formed within 5 min of sample application, were permanent.
Figure 27. Protein G affinity chromatography purified polyclonal HK *Pcc* antibody (IgG) fraction. First lane (left), benchmark prestained protein marker (Invitrogen); second lane, column flow through; third lane, non-purified (before purification) polyclonal HK *Pcc* serum; fourth lane (right), elution of heavy chain and light chain fragments of the antibodies (after purification); bands are present at approximately 50 kDa and 25 kDa, respectively.
Figure 28. Lateral flow test strips detecting no HK Pcc lane (1) and HK Pcc at various concentrations (lanes 2-8). If a test solution contains no Pcc, both the control and test lines appear; however, if Pcc is present above a certain concentration, only the test line will appear since the Pcc in the sample binds to anti-HK Pcc IgG-gold conjugate preventing its binding to the immobilized HK Pcc at the test line. The absence of a control line indicates the test is not functioning properly.
3.4 Discussion

Since immunoassays are used for detection and for quantitative applications (Lipton et al., 2000), therefore, optimization of an immunoassay is essential. The optimal conditions used were sera dilution of 1:8,000 and coating antigen concentration of 1μg/ml (Pcc LPS) and 10⁸ CFU/ml (HK Pcc). The overall specificity of an immunoassay, with optimized conditions, depends on the specificity of the antibodies used (Hennion et al., 1998; Furzer et al., 2006). The rabbit anti-Pcc LPS serum and anti-HK Pcc serum were analyzed for cross-reactivity with various Pectobacterium ssp. and other common pathogens. Little or no cross-reactivity was observed, with the highest values being less than 1% for E. coli and P. chrysanthemi. Because the serum antibodies appear to be directed against LPS for both the HK Pcc and Pcc LPS sera, the lack of cross-reactivity is likely due to differences between the other pathogens and Pcc (i.e. different genera and hence different types of the O-polysaccharides composing the LPS). Since the antisera did not cross-react with the pathogens tested, the test was considered specific for Pcc only. A specific assay is essential for detecting the target in question; however, specific tests eliminate the detection of other potentially harmful pathogens which one may also want to detect.

After achieving specificity, the immunoassays showed limits of quantitation and detection of 76 ng/ml (7.6 x 10⁻² μg/ml) and 23 ng/ml (2.3 x 10⁻² μg/ml) for Pcc LPS and 216 CFU/ml and 81 CFU/ml for HK Pcc. The IC₅₀ for Pcc LPS and HK Pcc assays were 1.1 x 10⁻¹ μg/ml and 1.3 x 10³ CFU/ml. Generally, current Pectobacterium ssp. detection occurring through the use of PCR-based detection, is able to detect as low as 2-4 CFU/ml (Kang et al., 2003). According to studies by Blom (2001), Gracia-Garza et al. (2002) and
Gracia-Garza et al. (2004), a mean of 100 CFU/ml of Pcc bacteria in a recirculating system using subirrigation would result in approximately 10% loss of plants (Zanthedeschia sp.). The CI-ELISA developed here for Pcc was not as sensitive (~10^1 CFU/ml) as previously described PCR-based methods; however, the ELISA-based method for detection takes less time to conduct and is more user-friendly. Since gram-negative bacteria contain approximately 3.5 million LPS molecules (Raetz, 1990; Raetz, 2002), some of which dissociate from the surface, the Pcc LPS assay could be used as a primary or secondary means of detection when a small number of bacteria (~1 to 100 CFU/ml) are present in solution.

The accepted range for mean recovery of spiked samples should be between 70 and 110% (Christensen and Granby, 2001; Furzer et al., 2006). After spiking hydroponic solutions with varying concentrations of Pcc LPS and HK Pcc, samples were quantified using the ELISA; approximately 86-110% and 88-117% of the samples were recovered from solution, respectively. Furthermore, no significant differences were determined between quantities spiked into the two hydroponic solutions (Westbrook and Boekestyn) and MPBS (P<0.05), thus, matrix effects did not hamper the ELISA.

The preliminary user-friendly lateral flow (dipstick) assay developed using polyclonal rabbit anti-HK Pcc serum conjugated to colloidal gold was formatted in reverse order to those of commercial lateral flow assays (i.e., decreasing intensity of test line versus increasing intensity). However, the lateral flow test detected Pcc at densities in the range of 10^5 CFU/ml, within 5 min. The 5-mm test strips were used to detect Pcc in nutrient solutions. Dark coloured test lines represented low concentrations of Pcc in solution. Specifically, the presence of darker red colored lines (spots) and a dark control
line indicated the presence of $Pcc$ in lower concentrations ($10^1$, $10^2$, $10^3$ and $10^4$ CFU/ml). The presence of a red line (dark control line) and very light or no visible spots indicated the presence of high concentrations of $Pcc$ ($10^5$, $10^6$ and $10^7$ CFU/ml). The data presented are only preliminary and further optimization of the dipstick assay is required. Replacing the HK $Pcc$ on the nitrocellulose strips with HK $Pcc$ antibodies, in a typical dipstick assay format, may provide a more specific assay with a lower level of detection then currently achieved.

In conclusion, two sensitive polyclonal immunoassays for the specific detection of $Pcc$ were developed along with a rapid, simple and inexpensive dipstick assay. The dipstick assay is ideal for use by growers because it is easier to use than previous commercial detection methods and will give results within minutes. Further optimization of both detection methods by substituting monoclonal antibodies for polyclonal antibodies may increase the sensitivity of the assay for growers.
4. PRODUCTION AND SELECTION OF V\textsubscript{H}H ANTIBODY FRAGMENTS AGAINST

PECTOBACTERIUM CAROTOVORUM SUBSP. CAROTOVORUM LPS

4.1 Introduction

Antibodies have historically proven to be excellent diagnostic and therapeutic tools. Animal immunization has provided a wealth of valuable antisera and monoclonal antibodies as research agents. Since their discovery nearly two decades ago in Camelidae species (e.g., camels, dromedaries and llamas), heavy chain immunoglobulin Gs (HcIgGs), which naturally lack a light chain and constant region 1 have been shown to bind to antigens with high affinity (Arbabi-Gharoudi \textit{et al.}, 1997; \textbf{Figure 8} and 10). The humoral immune response of Camelidae species is unique since it results in the production of both conventional antibodies (ConvIgG; 65-75%) and heavy chain antibodies (HcIgG; 25-35%) (Hamers-Casterman \textit{et al.}, 1993; Muyldermans \textit{et al.}, 2001). These HcIgGs are abundant in the sera of all Camelidae species with ca. 75 per cent of the molecules binding to protein A (Hamers-Casterman \textit{et al.}, 1993). The antigen-binding fragments of these antibodies (V\textsubscript{H}H) are very small (11-15 kDa; \textbf{Figure 10}), extremely stable at high temperatures (90\degree C), and can be expressed in \textit{E. coli} and \textit{Saccharomyces cerevisiae} or \textit{Piccia pastoris} at significantly higher levels than Fab or Fvs (Arbabi-Gharoudi \textit{et al.}, 1997; Frenken \textit{et al.}, 2000; Van der Linden \textit{et al.}, 2000; Jobling \textit{et al.}, 2003). Furthermore, through sequence and structural analysis, Muyldermans and Lauwereys (1999) revealed a number of unique features of HcIgG. For example, some V\textsubscript{H}H domains have a long CDR3 that contains a cysteine in addition to the one located
either in CDR1, or in position 50 or 55 of framework region 2 (FR2), suggesting that these antibodies contain a second disulphide bridge (Hamers-Casterman et al., 1993).

The most common method of isolating large numbers (up to $10^{10}$) of $V_{H}$ molecules is through phage display and subsequent expression in heterologous systems (i.e. *E. coli*; Frenken et al., 2000; Van der Linden et al., 2000; Azzazy and Highsmith, 2002; Brichta et al., 2005). Phage display involves the expression of $V_{H}$ on the surface of filamentous phages. Antibody libraries are screened for antigen-specific clones (i.e. *Pcc* LPS) and enriched by selection or panning. Panning allows antigen-specific phage displaying $V_{H}$s to be isolated and used in various diagnostic and therapeutic applications (Azzazy and Highsmith, 2002).

There has been limited success with the isolation of anti-lipopolysaccharide (LPS) single-domain antibodies. For example, recently El Khattabi et al. (2006) attempted, unsuccessfully, to select and characterize a llama $V_{H}$ against *Neisseria meningitides* LPS. It was suggested that purified LPS molecules may not have elicited a HcIgG response in llamas, thus, leading to the inability to select an anti-LPS $V_{H}$ from a phage-displayed library. In order to address this problem, the construction of an anti-*Pectobacterium carotovorum* subsp. *carotovorum* (*Pcc*) library, along with the successful selection and expression of an anti-*Pcc* LPS $V_{H}$ is described.

### 4.2 Materials and Methods

#### 4.2.1 Immunization and Antiserum Preparation

A young adult male llama (*Lama glama*; two-year-old; 125 kg; Figure 29) was injected 8 times, subcutaneously, in the lower rear back; on days 1, 14, 42, 56, 70, 84, 98,
Figure 29. Clipper, a two-year-old male llama (*Lama glama*), was immunized with *Pcc* LPS and heat killed *Pcc* to stimulate an immune response. Blood and serum collected from the llama were used for multiple applications (i.e. ELISAs, library construction, etc.).

Injections were prepared using 250 μg/ml mixture of *Pcc* LPS and HK *Pcc* (ca. 70:30 w/v) in PBS. All injections consisted of 1:1 (v/v) antigen/PBS-Titermax® emulsion (Sigma-Aldrich, Oakville, ON). On alternate weeks between injections, approximately 200 ml of blood were collected from the jugular vein. Prior to immunization (i.e. Day 0), approximately 200 ml of pre-immune blood was collected. Llama serum was prepared by placing tubes of blood at RT for 1-4 h or at 37°C for 30 min (allows for better separation of plasma and blood cells), followed by centrifugation at
2,700 g for 10 min. All serum samples were aliquoted and stored at -80°C. Part of the serum was used to complete serum titer ELISAs, while another portion (1 to 5 ml) was dialyzed against PBS (pH 7, 4°C; dialysate volume was 100 times that of the sample volume) o/n and fractionated twice by protein G chromatography (HiTrap, GE Healthcare, Uppsala, Sweden) using a gradient pH elution (Hamers-Casterman et al., 1993). Following elution, each fraction was neutralized (1M Tris/HCl (pH 8~8.8) and then dialyzed against PBS (pH 7, 4°C; dialysate volume was 100 times sample volume) o/n. Purified and dialyzed fractions were filtered through 0.22 µm syringe filters and stored at 4°C o/n. The protein concentration was determined using both A280 readings and the BCA-protein assay (bicinchoninic acid (BCA); Pierce, Rockford, IL).

4.2.2 Serum Titer Analysis

As with the serum titer analysis for the rabbit immunizations outlined in the previous chapter, serum samples from llama were analyzed for antibodies raised against Pcc LPS and HK Pcc using an indirect assay. Microtiter plates (96-well flat bottom polystyrene high binding microplates; Costar, Corning, NY) were coated with Pcc LPS (10 µg/100 µl 2% formalin (from 37% (w/v) formaldehyde in H₂O stock; Sigma-Aldrich, Oakville, ON) overnight (o/n) at RT. Following incubation, the wells were emptied and blocked with 2% w/v milk powder in PBS (MPBS; 200 µl/well) for 2 h at RT, while

3 Note: purification of the crude serum by protein G chromatography provides the separation of ConvIgG and HcIgG fractions.
4 Note: the dialysate was removed and replaced with fresh buffer at least twice during the o/n period.
5 Note: see Chapter 3 for bacterial strain and HK bacteria preparation and growth, LPS isolation, quantitative estimation of purified LPS, and LPS analysis.
shaking at approximately 30 rpm. The wells were washed five times with PBS-Tween (PBST; 0.05% Tween 20). Diluted sera (pre-immune and fourth (Day 56), seventh (Day 98), and eighth (Day 112) bleeds; 1:50 serum dilution in 100 μl MPBS) were added to each well and kept at RT for 1.5 h with gentle shaking. After five washes with PBST (200 μl/wash), goat anti-llama IgG (GAL-HRP; Bethyl Labs, Montgomery, TX) secondary antibody (1:1,000 dilution in 100 μl MPBS) was added to each well for 1 h at RT with gentle shaking. Following incubation with the secondary antibody, the wells were washed five times with PBST. A tertiary antibody, swine anti-goat-HRP conjugate (1:3,000 dilution in 100 μl MPBS; Cedarlane Labs, Burlington, ON), was added to the wells. After decanting and 5 washes with PBST (200 μl), TMB substrate (100 μl; 1-Step Turbo TMB; Thermo Fisher Scientific, Nepean, ON) was added and incubated for 1 h at RT in the dark. The antibody binding was determined spectrophotometrically (OD) at a wavelength of 450 nm after neutralizing with 0.2 M H₂SO₄ (100 μl). All dilutions were run in triplicate, absorbance readings were averaged, and background subtracted.

Negative controls (no coating antigen) included secondary Ab only, tertiary Ab only, and substrate only wells incubated with serum dilutions, and coated wells with secondary antibody only, tertiary antibody only, and substrate only.

4.2.3 Library Construction

After titer analysis of sera and the affirmation of an increased titer followed by a plateau after several immunizations, the blood (100 ml) from the fourth (Day 56), seventh (Day 98), and eighth (Day 112) llama bleeds was collected and each mixed with an EDTA-H₂O solution (ethylenediaminetetraacetic acid (EDTA) dipotassium salt
dihydrate; 1.13 mg/ml dissolved in sterile ddH$_2$O; final volume 6 mM) to prevent clotting. Leucocytes isolated from the blood, after pooling 1.5 ml of each of the bleeds from the three harvest days, were lysed and prepared according to the QIAamp RNA Blood Mini Kit (Steps 1-6: Leukocyte isolation; QIAGEN Inc., Mississauga, ON). Lysed leukocytes were stored in buffer (Qiagen buffer RLT plus β-Mercaptoethanol) at -80°C until library construction proceeded. On the first day of library construction the total RNA was isolated from the lysed leucocytes, according to the QIAamp RNA Blood Mini Kit protocol (Steps 7-13: Total RNA extraction). RNA concentration was determined using A$_{260}$/A$_{280}$ readings from the spectrophotometer.\(^6\)

First strand cDNA was prepared using the First-Strand cDNA Synthesis Kit (GE Healthcare Biosciences, Uppsala, Sweden). Total RNA was heat-denatured by incubating the RNA solution at 65°C for 10 min followed by incubation on ice. In a separate microcentrifuge tube, DTT (1 µl Dithiothreitol; provided in kit) was added to the bulk first-strand cDNA reaction mix (11 µl; provided in kit), followed by 1 µl of *Not* I-d(T)$_{18}$ cDNA primer (0.2 µg) and heat-denatured RNA (20 µl), respectively. This mixture was incubated for 1 h at 37°C; the entire procedure resulted in the transcription of total RNA (5 µg/20 µl) to cDNA. The cDNA (cDNA concentration was not quantified; however, 1, 2 and 5 µl volumes were used in PCR amplification to determine the optimal concentration for subsequent amplification) was used as a template for six 50-µl amplifications of the variable heavy (V$_{H}$H) domains of the heavy chain immunoglobulin using polymerase chain reaction (PCR). Specific forward primers (10 pmol; sense hinge-}

\(^6\) Note: A$_{260}$/A$_{280}$ readings should range between 1.6 to 1.9 when total RNA is diluted in water.
specific primers MJ1, MJ2 and MJ3) were used in all of the reactions, in conjunction with the anti-sense hinge-specific CH2 reverse primer (10 pmol) for three reactions and the anti-sense hinge-specific CH2b3 reverse primer (10 pmol), for the remaining three reactions. The CH2 and CH2b3 primers, which correspond to the CH2 domain DNA sequence, were used to amplify the VH-CH1-CH2 region of ConvIgG or VH-H-CH2 of the HcIgG (Arabbi-Gharoudi et al., 1997; Bell et al., 2009). A complete list of primer sequences used for the library construction can be found in Table 5. DNA Taq polymerase (2.5 units; Hoffmann-La Roche Ltd., Mississauga, ON) and 0.25 mM dNTPs were used for each reaction. Taq polymerase was added to the reactions immediately before the start of the PCR cycles: 94°C for 3 min; 30 cycles of: 94°C for 45 sec, 55°C for 45 sec, 72°C for 1.5 min; 72°C for 7 min; 4°C ∞. The 650 bp amplicons from the CH2b3 reactions were purified using the QIAquick PCR Purification kit (QIAGEN Inc., Mississauga, ON) while the 650 and 750 bp amplicons from the CH2 reactions were purified using the Qiagen QIAquick Gel Extraction kit (QIAGEN Inc., Mississauga, ON).7 Fifty-percent of amplified product (i.e. VH CH2; 2.6 µg/20 µl) was obtained from the CH2 amplification and 50% from the CH2b3 amplification.

All amplified VH CH1 product (1 µl/PCR reaction) was used as a template for 20 PCR reactions (50 µl). The PCR amplifications were performed using the Expand High Fidelity PCR System (Hoffmann-La Roche Ltd., Mississauga, ON) in 50 µl reactions with the MJ7 forward and MJ8 reverse primers (10 pmol of each per reaction;

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7 Note: Gel purification and extraction were performed to extract the correct bands and to ensure no VH contamination; since the conventional VH CH1 CH2 coding sequence is also amplified by these primers.
Table 5. Primer oligos used in library construction.

<table>
<thead>
<tr>
<th>Name</th>
<th>Oligonucleotide Sequence 5’ → 3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>MJ1</td>
<td>GCCCAGCAGGCCCAGCAGCTGACGGTTGAGT</td>
</tr>
<tr>
<td>MJ2</td>
<td>GCCCAGCAGGCCCAGCAGCTGACGGTTGAGT</td>
</tr>
<tr>
<td>MJ3</td>
<td>GCCCAGCAGGCCCAGCAGCTGACGGTTGAGT</td>
</tr>
<tr>
<td>C12</td>
<td>CGCCATACAGGCTACCGTTA</td>
</tr>
<tr>
<td>C12b3</td>
<td>GGGGTACCTGTATCCACGAGGACCAGCTGA</td>
</tr>
<tr>
<td>MJ7</td>
<td>CATGTGTAGACTCGCGGCCCAGCAGCTGA</td>
</tr>
<tr>
<td>MJ8</td>
<td>CATGTGTAGACTCGCGGCCCAGCAGCTGA</td>
</tr>
<tr>
<td>PN2</td>
<td>CCCTCATATGTAGGTAAGATCT</td>
</tr>
<tr>
<td>M13</td>
<td>CAGGAAACAGCTATGACC</td>
</tr>
</tbody>
</table>

Provided by Dr. Mehdi Arbabi-Ghahroudi. Note: S = C/G; M = A/C; K = G/T.

see Table 5), 0.25 mM dNTPs, 2.5 units of DNA Taq polymerase and 1.0 mM MgSO₄. DNA Taq polymerase was added to the reactions immediately before starting the following PCR cycles: 94°C for 3 min; 35 cycles of: 94°C for 30 sec, 55°C for 30 sec, 72°C for 1 min; 72°C for 7 min; 4°C ∞. The amplified product was confirmed using an agarose gel. All amplicons were purified using the QIAquick PCR purification kit to yield 20 μg/370 μl DNA, of which 15.8 μg were restriction digested using SfiI. The digestion was done 2 μg at a time in 50 μl (30 μl DNA, 5 μl H₂O, 5 μl 10x NEB buffer 2, 5 μl 10x BSA, 5 μl/100 units SfiI; New England Biolabs, Ipswich, MA) preparations. Each preparation was incubated for 24 h at 50°C after the addition of 100 μl of mineral

8 Note: The purpose of this reaction is to introduce the SfiI restriction sites at the 5’ and 3’ end of the VH/H sequence.
oil to the top of each preparation. Digested \( V_{H} \) DNA (15.8 \( \mu \)g) was purified using four spin columns from the QIAGen PCR purification kit. After ethanol precipitation, 22.5 \( \mu \)g/80 \( \mu \)l DNA was yielded.

Dr. Yan Luo from the National Research Council of Canada (NRCC; Ottawa, ON) prepared and provided the pMED1 vector (235 ng/\( \mu \)l; **Figure 30**) to be used in the ligation process. The vector was double digested using \( SfiI \) at 50°C o/n. The reaction products were purified using the QIAGen PCR purification kit. The pMED1 \( SfiI \) was further digested with \( PstI \) and \( XhoI \) at 37°C for 5 h. The vector was purified using the QIAGen PCR purification kit. Ten reactions of 140 ng of \( SfiI \) digested \( V_{H} \) each were ligated into 800 ng of \( SfiI \)-digested pMED1 each at an approximate molar ratio of 2:1 insert:vector. Twenty microliter ligation reactions were prepared with 3.4 \( \mu \)l of vector, 1.9 \( \mu \)l of \( V_{H} \), 2 \( \mu \)l (45 U) of ligase, 10 \( \mu \)l 2x buffer from the LigaFast Rapid DNA Ligation System (Promega, Madison, WI), and 2.7 \( \mu \)l ddH\(_2\)O. After 20 minutes at RT the ligation products were purified using five spin columns from the QIAGen PCR purification kit. The elutions were pooled and concentrated by ethanol precipitation.

The purified, ligated product was transformed into TG1 *E. coli* cells. Specifically, 50 reactions were prepared separately, each with 1 \( \mu \)l DNA and 50 \( \mu \)l electrocompetent TG1 *E. coli* cells (Stratagene, La Jolla, CA) and immediately electroporated using Gene Pulser cuvette with a 0.1 cm gap (Bio-Rad Laboratories, Mississauga, ON) at 1800 V with 25 \( \mu \)F and 200 \( \Omega \).

The transformed cells were immediately transferred to 1 ml of pre-warmed SOC medium (tryptone, yeast extract, NaCl, ddH\(_2\)O, MgCl\(_2\), MgSO\(_4\) and 20% (w/v) glucose). Ten milliliters of the TG1 in SOC media were added to each of 5 sterile 50-ml tubes and
Figure 30. Map of the pMED1 vector. The vector was double digested using SfiI. The multiple cloning site (MCS; shown beneath the vector map) was double digested with PstI and XhoI. The MCS displays gene segments, restriction sites and purification/detection tags (Taken from Arbabi-Ghahroudi et al., 2009).
incubated for 2 hours at 37°C with shaking at 250 rpm, prior to being centrifuged at 4,000 g for 30 min at 4°C. Pellets were resuspended in a total of 30 ml 2xYT (yeast extract tryptone with NaCl; Becton Dickinson Biosciences/Difco, Mississauga, ON) containing 2% (w/v) glucose and 100 μg/μl AMP (Ampicillin; Sigma-Aldrich, Oakville, ON). A 10-μl aliquot was taken to determine library size, while glycerol (30% w/v) was added to 5-ml aliquots (the rest of the stock, i.e. Library stock), which was stored at -80°C. To determine library size, 100 μl of *E. coli* (10⁻², 10⁻⁶, 10⁻⁸, 10⁻¹⁰, 10⁻¹²) were plated on LB-AMP plates and incubated at 30°C o/n.

To estimate the percentage of the clones containing the insert, colony PCR (94°C for 5 min; 30 cycles of: 94°C for 30 sec, 55°C for 30 sec, 72°C for 1 min; 72°C for 7 min; 4°C ∞) was performed on 20 colonies from the dilution plates using the PN2 forward primer and M13 reverse primer (see Table 5). The insert ratio was determined by agarose gel electrophoresis (1.5% gel). Digestion of the PCR product (8 μl PCR product, 1 μl 10X BSA, 1μl BstN1; incubation for 1 hour at 60°C) was performed to test the library diversity. An estimate of library diversity was revealed by agarose gel electrophoresis (1.5% gel). DNA sequencing was performed on the colony PCR amplicons (10 colonies containing an insert).

### 4.2.4 V_H Selection by Phage Display

A 5 ml aliquot of library stock was used to inoculate 300 ml 2xYT containing 100 μg/ml AMP and 1% glucose. The cells were grown at 37°C with shaking at 250 rpm until OD₆₀₀ was 0.353. A solution of M13KO7 helper phages (9 x 10¹¹ plaque forming units (pfu); New England BioLabs, Inc., Ipswich, MA) was added to the cells at a ratio of
20:1, at a temperature of 37°C, without shaking for 30 min. The culture was grown for an additional hour at 37°C with shaking at 250 rpm, followed by centrifugation at 3,300 g for 10 min. The pelleted cells were resuspended in 300 ml 2xYT-AMP-KAN (100 µg/µl AMP, 50 µg/µl kanamycin) and incubated o/n at 37°C with shaking at 250 rpm.

After 16 h, the culture (except for 500 µl, which was kept for titration of the original phage library) was transferred to a 250-ml centrifuge bottle and centrifuged at 4,400 g for 15 min at 4°C. The phages were aliquoted into 50-ml centrifuge tubes and precipitated with 1/5 volume (e.g. 10 ml PEG-NaCl to 40 ml supernatant) polyethylene glycol (PEG)-NaCl (20% w/v PEG 6000, 2.5 M NaCl). The tubes were inverted to ensure uniform mixing and placed on ice for 1-1.5 h. After incubation, the precipitated phage solution was centrifuged at 13,800 g at 4°C for 15 min. The white pellets were resuspended in 1 ml of sterile PBS (pH 7.4) by pipetting, transferred to 1.5-ml tubes and centrifuged at 10,000 g for 30 min at 4°C to remove bacterial debris (formation of a brownish pellet). The supernatant was transferred to a clean 1.5-ml tube while the bacterial pellet was discarded. The supernatant was centrifuged for a second time (10,000 g for 15 min, 4°C) to ensure the removal of any further bacterial debris. An aliquot (10 µl) of the supernatant was kept for titration, 300 µl were used for panning, and the remaining solution was stored at 4°C. To determine the input phage titer, titrations were performed by first adding 10 µl of phage solution into 990 µl PBS to obtain a 10⁻² dilution and repeating this process until 10⁻¹². Ten microliters of a dilution (10⁻⁶ to 10⁻¹²) were added to 90 µl of exponentially growing TG1 E. coli cells (grown to OD₆₀₀ = 0.4)

---

9 Note: tubes containing white pellets were flipped upside-down on paper towel to remove excess supernatant.
and incubated for 15 min at 37°C. After infection, to determine titer and helper phage packaging ratio, 100 μl of each dilution were plated on 2xYT-AMP (100 μg/μl) and 2xYT-KAN (50 μg/μl) media, respectively, and incubated o/n at 32°C. After incubation, colony forming units were counted and used to determine the phage titer.

For the first round of selection, two wells of a 96-well microtiter plate (Nunc A/S, Roskilde, Denmark) were coated with 100 μl of Pcc LPS in formalin-sodium phosphate buffer, while one well contained a negative control (formalin-sodium phosphate only). Plates were incubated o/n at RT. Following incubation, the wells were emptied and blocked with 200 μl of a blocking agent in sterile PBS (see Table 6) for approximately 2 h at 37°C or o/n at 4°C. After blocking, amplified phages (100 μl; ~10¹² pfu) were added to each well (i.e. add to a coated (positive control) and non-coated well (negative control)) and incubated for 2 h at 37°C. Non-bound phages were removed by washing the wells with PBST (250 μl; the number of washes were increased in subsequent rounds of selection; see Table 6). Bound phages were eluted from each well with 100 μl/well of 100 mM triethylamine (TEA) and after 8 min the TEA containing phage from each well were pipetted up and down 10x. After another 2 mins, the eluate containing the phages was immediately transferred to 1.5-ml tube containing 200 μl 1 M tris-HCl (pH 7.5) to neutralize the pH. A 2 μl sample of the infected TG1 was removed from each well and used for “after” panning titration, while the remaining TG1 was stored on ice until required for use during the next section of the procedure. Before and after panning titers were determined for all rounds of panning.

10 Note: see Table 6 for details of the rounds of selection (i.e. LPS coated, blocking agent and amount of washing).
**Table 6.** Phage titers (polyclonal) for three rounds of panning against *Pcc* LPS by phage display. The LPS coating concentration, number of washes and blocking agent were different for each round of panning.

<table>
<thead>
<tr>
<th>Panning round</th>
<th>µg LPS coated</th>
<th># washes</th>
<th>Blocking agent</th>
<th>Eluted phage (uncoated)</th>
<th>Eluted phage (coated)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>40</td>
<td>6</td>
<td>2% MPBS</td>
<td>7.2 x 10⁵</td>
<td>5.8 x 10⁵</td>
</tr>
<tr>
<td>2</td>
<td>30</td>
<td>7</td>
<td>2% Casein</td>
<td>5.8 x 10⁴</td>
<td>3.1 x 10⁵</td>
</tr>
<tr>
<td>3</td>
<td>20</td>
<td>8</td>
<td>4% MPBS</td>
<td>3.9 x 10⁴</td>
<td>1.7 x 10⁶</td>
</tr>
</tbody>
</table>

The eluted phages (600 µl) were added to 2 ml TG1 *E. coli* cells (OD₆₀₀ = 0.3 – 0.5) and incubated for 15 min at RT. After incubation, a 2 µl aliquot of infected TG1 cells were added to 200 µl; 10 µl and 100 µl were used for titration on 2xYT plates containing 100 µg/µl AMP. The rest of the infected TG1-cell mixture was added to prewarmed (37°C) 2xYT in a 50 ml tube to make a total of 8 ml (2.598 ml cells & 5.402 ml 2xYT). The culture was grown at 37°C for 30 min at 250 rpm after the addition of AMP (final concentration of 50 µg/µl). Following incubation, another dose of AMP (to a final concentration of 100 µg/µl) was added to the culture; the culture was grown for an additional 30 min at 37°C with shaking at 250 rpm. M13KO7 helper phages (9 × 10¹⁰ pfu) was added to the 8-ml culture and allowed to incubate for 15 min at 37°C without shaking. The infected culture was transferred into a 500 ml flask containing 92 ml 2xYT.

Note: only 150 µl of eluted phage from the negative control well was added to 1 ml TG1.
with 100 μg/ml AMP and grown for 1.5 h at 37°C with shaking at 250 rpm. After 1.5 h, KAN (50 μg/μl final concentration) was added to the culture; the culture was grown o/n at 37°C with shaking at 250 rpm to amplify phages for the next round of selection.

4.2.5 Polyclonal Phage ELISAs

The progress of V_{1H}-bearing phage selection against immobilized *Pcc* LPS was monitored by performing polyclonal phage ELISAs, specifically using polyclonal phages from each of the three rounds of panning. Microtiter plates (96-well flat bottom polystyrene high binding microplates; Costar, Corning, NY) were coated with *Pcc* LPS (50 μg/100 μl 2% formalin (from 37% (w/v) formaldehyde in H₂O stock) and incubated o/n at RT. After overnight incubation, wells were washed three times with PBS (250 μl; pH 7.4) to remove unbound antigen, followed by blocking with 4% MPBS (250 μl) o/n at 4°C. After blocking, the wells were washed three times with PBST, followed by the addition of 100 μl of amplified phage (approximately 10^5/well) eluted from each round of panning, and incubated at RT for 2 h. The phage solution was discarded, and the wells were washed four times with PBST. The bound phages were detected using 100 μl/well of anti-M13 IgG conjugated to HRP (GE Healthcare, Baie d’Urfé, QC) diluted 1:5000 in 2% MPBS. Plates were incubated for 1 h at RT with gentle shaking, followed by washing four times with PBST (250 μl). Subsequently, the reactions were developed with TMB substrate (100 μl/well; Pierce Biotechnology, Rockford, IL) for 45 min to 1 h at RT, followed by neutralization with 0.2 M H₂SO₄ (100 μl/well). The optical density was measured spectrophotometrically at 450 nm.
4.2.6 Monoclonal Phage ELISAs

In 96-well culture plates (Corning Incorporated Life Sciences, Acton, MA), individual colonies from round 2 (82 colonies) and round 3 (73 colonies) of selection were inoculated with 100 µl/well of 2xYT-AMP (100 µg/µl) with 1% glucose. The cultures were grown o/n at 37°C and 250 rpm. Approximately 2 µl of each inoculum was transferred to new 96 cell-well culture plates containing 200 µl of 2xYT-AMP (100 µg/µl) and 1% glucose per well. The cultures were grown at 37°C with shaking (250 rpm) for 2 h. After incubation, 25 µl 2xYT-AMP (100 µg/µl), 1% of glucose and 10 µl M13KO7 helper phages (1 x 10^9 pfu) were added to the transferred inoculum. The cultures were shaken at 250 rpm for 1 h at 37°C and centrifuged at 1,800 g for 10 min at 4°C. The supernatant was carefully pipetted out of each well, making sure not to disturb the pellet, and discarded. The pellets were resuspended in 2xYT-AMP-KAN (200 µl/µl per well; 100 µg/µl AMP; 50 µg/µl KAN) and grown at 30°C o/n with shaking at 250 rpm. The cultures were centrifuged at 1,800 g for 10 min at 4°C. The supernatant (50 µl) was used directly in the monoclonal phage ELISAs; the pellets were discarded.

Microtiter plates (96-well, flat bottom polystyrene high binding microplates; Costar; Corning, NY) were coated with Pcc LPS (50 µg) in formalin-sodium phosphate buffer (100 µl) o/n at 4°C. Negative control wells were coated with 100 µl formalin-sodium phosphate buffer. Wells were blocked with 4% MPBS (250 µl) o/n at 4°C, emptied and washed three times with PBST. Supernatant (50 µl) of the monoclonal phage amplifications were added to the wells, along with 50 µl of 4% MPBS, and

12 Note: glycerol stocks of the original 96-well plates were prepared by adding glycerol (15% final concentration) and then stored at -80°C.
incubated for 1.5 h at RT. After the wells were washed ten times with PBST (250 µl), anti-M13 IgG conjugated to HRP (100 µl; GE Healthcare, Baie d’Urfé, QC) was added at 1:5000 dilution in 2% MPBS and incubated for 1 h at RT. The wells were washed ten times with PBST (250 µl), developed with TMB substrate (100 µl), and stopped after the addition of 0.2 M H₂SO₄ (100 µl). The optical density was determined spectrophotometrically at 450 nm. Clones that were good binders (greater than 0.3 background at 450 nm) were sequenced using the universal M13RP primer at the Laboratory Services Division, University of Guelph.

4.2.7 Soluble Expression of Selected Clones

Monoclonal phage-display V₄₉H that bound to LPS were used to infect exponentially growing E. coli strain HB2151 (OD₆₀₀ of 0.4; 2xYT media containing 100 µg/ml AMP and 1% (w/v) glucose) and grown for 30 min at 37°C. Dilutions of the cultures (50 µl; 10⁻², 10⁻⁴ and 10⁻⁶) were plated on TYE (10 g bactotryptone and 1 g of yeast extract; Difco, Detroit, MI) AMP (100 µg/µl) containing 1% glucose and grown o/n at 37ºC. Individual colonies were used to inoculate 5 ml LB-AMP (LB; Luria-Bertani; Difco, Detroit, MI) plus 1% glucose. After o/n growth at 30ºC with shaking at 250 rpm, a sample (600 µl) was used to inoculate 60 ml LB-AMP-glucose (0.1% glucose) and grown at 37ºC (shaking at 250 rpm) until the OD₆₀₀ was approximately 0.9. Once an OD₆₀₀ of 0.9 was reached, the cultures were induced by adding isopropyl beta-D-1-

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¹³ Note: negative and positive controls consisted of no phage and 1:8,000 rabbit serum.

¹⁴ Note: the remaining culture was stored at -80°C after adding glycerol to a final concentration of 15%)

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thiogalactopyranoside (IPTG; 1 mM final concentration) and incubation was continued o/n at 37°C with shaking at 150 rpm. Cultures were centrifuged at 10,000 g for 10 min at 4°C. The supernatant and sonicated pellets were analyzed by SDS-PAGE and Western blotting. Large-scale cultures of good expressors were started in 2 x 10 ml LB-AMP-glucose (as described above) and were used to inoculate 1 L of LB-AMP-glucose. At OD$_{600}$ of 0.9, IPTG was added and the cultures were grown with shaking (250 rpm) o/n at 30°C.

Clone (P245) that showed good expression was purified using immobilized metal ion affinity chromatography (HisTrap™ HP column 5 ml; GE Healthcare, Uppsala, Sweden) and the ÄKTA FPLC system (GE Healthcare Bio-sciences Uppsala, Sweden). The column was washed with ddH$_2$O and equilibrated with IMAC A buffer (10 mM Hepes and 500 mM NaCl). The pellet containing $V_H^H$ was resuspended in IMAC A buffer, sonicated, and the supernatant was loaded onto the column and washed with buffer (IMAC A). The clone P245 was eluted with IMAC B (10 mM Hepes, 500 mM NaCl and 500 mM immidazole) buffer using a step gradient (5%, 10%, 15%, 20%, 30%, 50%, 70% and 100%).$^{15}$ Elutions containing $V_H^H$ were dialyzed (using 6,000-8,000 MW dialysis membrane and 1x PBS) o/n at 4°C and concentrated before SDS-PAGE, Western blot and ELISA analyses were performed. Protein concentration was measured at 280 nm ($A_{280}$), and $V_H^H$ concentration was estimated (http://expasy.org/tools/protparam.html) using an extinction coefficient based on the predicted amino acid sequence of each $V_H^H$ (Pace et al., 1995). Purified $V_H^H$ was stored at 4°C.

$^{15}$ Note: protein eluted between 5% and 20%.
4.2.8 Soluble ELISA

Indirect CI-ELISAs were performed to assess the binding of the V\textsubscript{H}H (clone P245). A standard curve showing competition between the coating conjugate and the free V\textsubscript{H}H in solution was prepared through the use of a competitive inhibition assay (Furzer et al., 2006). Ninety-six well polystyrene microtiter plates were coated (100 µl/well) with \textit{Pcc} LPS in formalin-sodium phosphate buffer (50 µg/well) and incubated overnight at RT. Plates were blocked with 4% MPBS (200 µl/well) for 2 h at RT. The V\textsubscript{H}H (10 µM) in 4% MPBS, as determined using a checkerboard assay, was mixed with various concentrations of \textit{Pcc} LPS to create a standard curve. V\textsubscript{H}H solution (without ‘free’ \textit{Pcc} LPS) as well as solutions of V\textsubscript{H}H and ‘free’ \textit{Pcc} LPS were applied (100 µl/well) to the coated and blocked wells and were incubated for 1 h at RT. Free pathogen concentrations varied from 0.9 µg/ml to 12.5 µg/ml for \textit{Pcc} LPS. After three washes with PBST, 100 µl/well Penta-His\textsuperscript{TM} antibody (1:500 dilution in 4% MPBS; QIAGEN Inc., Mississauga, ON) was added and allowed to react for 1 h at RT. Thereafter, the wells were washed three times with PBST buffer. TMB substrate was added (100 µl/well) and left to develop for 1 h at RT in the dark. The reaction was stopped with 0.2 M H\textsubscript{2}SO\textsubscript{4} (100 µL) and read spectrophotometrically at 450 nm (OD\textsubscript{450}). The CI-ELISAs were developed to determine the specificity of the V\textsubscript{H}H to \textit{Pcc} LPS. Inhibition was calculated by plotting the ratio of absorbance to maximum absorbance observed (A/A\textsubscript{o}) against the concentration of the antigen (Furzer et al., 2006).
4.3 Results

4.3.1 Anti-\textit{Pcc} LPS Polyclonal Serum Titer Analysis

The anti-\textit{Pcc} LPS titers increased steadily from day 56 to day 112 (Figure 31).

Once high polyclonal serum titers were obtained, the sera were fractionated into HcIgGs and ConvIgGs by protein G chromatography. The antibodies in the serum were separated and used to determine the suitability of creating a $V_{\text{H}}$ library. Purity of fraction was analyzed using SDS-PAGE and Western blotting (Figure 32). The individual IgG chains migrate as separate bands, allowing for the visualization of the separate HcIgG (46 kDa) and ConvIgG fractions (25 and 50 kDa). The HcIgG fraction was confirmed to be pure, which is of importance since contamination with ConvIgG could lead to false results (i.e.
**Figure 32.** ConvIgG and HcIgG fractions from immunized llama. The Coomassie-stained SDS-PAGE (A) and Western blot (B; probed with goat anti-llama IgG-HRP and swine anti-goat IgG-HRP) show reduced HcIgG (46 kDa) and conventional heavy (ConvIgG H) and conventional light (ConvIgG L) at 50 and 25 kDa, respectively.

...what seems to be HcIgG response but is really ConvIgG signal response). Furthermore, there was no contamination of the ConvIgG fraction with HcIgG appearing on the Western transfer of the ConvIgG fraction.

ELISAs were performed to determine the binding of HcIgG and ConvIgG to Pcc LPS (Figure 33). The HcIgG fraction was approximately 10X less concentrated than the ConvIgG fraction. Nonetheless, both the HcIgG and ConvIgG showed good binding to Pcc LPS, although the response of ConvIgG appeared to be better than that of HcIgG, which may be explained by the lower concentration of HcIgG that was used. On the basis of these results it was decided to construct the V_{H}H library from the leucocytes of
this llama; therefore, RNA from day 56, 98 and 112 was isolated from leucocytes and used to construct the phage-display library.

Figure 33. The activity of ConvIgG and HcIgG binding to Pcc LPS as assessed by ELISA. Sera from Day 56, 98 and 112 are shown in blue, red and green, respectively.

4.1.1 Phage Display Library Construction

A library (against Pcc LPS and HK Pcc) of $6.0 \times 10^5$ clones/ml with a 50% insert ratio and high sequence diversity was constructed. Analysis of 10 clones by agarose gel electrophoresis and sequencing showed 100% diversity among the clones (Table 7). When analyzing the CDRs of each clone, it was evident that both CDR2 and 3 were diverse in terms of identities and length. The CDR1 showed the least diversity.
Table 7. Predicted amino acid sequences of ten random clones (pre-selection) from the anti-Pcc LPS V_{15}H library. Sequence diversity of the clones is illustrated in the complementary determining regions (CDRs).

<table>
<thead>
<tr>
<th>Clone #</th>
<th>CDR1</th>
<th>CDR2</th>
<th>CDR3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>GGSTFDSAMS</td>
<td>GDFSGDKLTVVR</td>
<td>NFFLYAGYGPSSE</td>
</tr>
<tr>
<td>2</td>
<td>AFGSFSMIGG</td>
<td>RVYAASTIFTATRA</td>
<td>GCISKEDSTYYR</td>
</tr>
<tr>
<td>3</td>
<td>GSFFSIDTMA</td>
<td>SITRFDRTTIYAPVR</td>
<td>TFINLLGKSYNA</td>
</tr>
<tr>
<td>4</td>
<td>GRTICISAIG</td>
<td>RSKDGRDVSTYYG</td>
<td>TGIDGHGATRKVYDMD</td>
</tr>
<tr>
<td>5</td>
<td>EFFIDTAMS</td>
<td>FIDSGSGGPNDP</td>
<td>RSFKDGRRTTSAVSK</td>
</tr>
<tr>
<td>6</td>
<td>GSFIIEDGIG</td>
<td>TTTLGKSTPDS</td>
<td>NVRYTEARCDAV</td>
</tr>
<tr>
<td>7</td>
<td>ASDPGSVMG</td>
<td>DFIKVPSESDTAADP</td>
<td>FPRGRRESGSAYD</td>
</tr>
<tr>
<td>8</td>
<td>GRFSTFFYIS</td>
<td>FINLWRKSYR</td>
<td>DRGSAKYTDYDSPD</td>
</tr>
<tr>
<td>9</td>
<td>GDTIFSTDDD</td>
<td>GIDVRTNYGGG</td>
<td>RGYGFILYWSPLV</td>
</tr>
<tr>
<td>10</td>
<td>GFGYVMHG</td>
<td>STRFVICDEYA</td>
<td>DGVVKDYDTAMTSA</td>
</tr>
</tbody>
</table>

4.1.2 Panning/Selection Titer and Monoclonal Phage ELISAs

Selection of clones from the constructed library was conducted by first selecting clones that bound to LPS using polyclonal phage ELISAs to assess eluted phage after each of the three rounds of panning; however, the results were inconclusive (high background values; non-specific binding) for round 2 and 3 (Figure 34; other data not shown). Therefore, the progression from one round to the next was also monitored by phage titers from the elutions of each round of selection (Table 6).
Figure 34. Polyclonal phage ELISA to assess eluted phage after each of the three rounds of panning. The data is presented as means (absorbance 450 nm) of triplicates after background subtraction. Vertical bars indicate the standard deviation.

When panning stringency was increased, both by decreasing the concentration of the coating antigen while increasing the number of washes, and by changing the blocking agent, the phage titre of the background controls was reduced from $7.2 \times 10^5$ in round 1 to $3.9 \times 10^4$ in round 3. Conversely, the number of apparent specific binders (e.g. eluted phage-coated) to LPS remained constant, i.e. $5.8 \times 10^5$ in round 1 and $1.7 \times 10^6$ in round 3. Titration signal to noise ratio (background) provided an approximation of the possible number of colony screenings required to find specific binders amongst non-specific binders (from the library) in the subsequent monoclonal phage ELISAs.

Monoclonal phage ELISA is the best method to assess the affinity of an individual antibody clone to bind to an antigen. Note: the overall binding affinity of a clone to the
antigen is relative to the titer of the M13 phage displaying antibody. Consequently, 82 and 73 colonies from the 2nd round and 3rd round of panning were individually screened by phage ELISA, respectively. After screening the 155 clones, four binding clones to \textit{Pcc} LPS, known as P211 (OD\(_{450}\) = 0.47), P212 (OD\(_{450}\) = 0.57), P245 (OD\(_{450}\) = 0.55) and P311 (OD\(_{450}\) = 0.23) were selected due to high OD\(_{450}\) readings from the monoclonal phage ELISA, when compared with other selected clones (a typical OD reading for a positive binder was considered to be 0.2 or higher; whereas, negative binders had OD values of 0.1 or lower; data not shown). Note: absorbance values for each of the four positive binding clones represent triplicate mean values after background subtraction. After comparison of the sequences of the four clones, only P245 contained the \textit{V\textsubscript{H}} gene (Table 8).

4.1.3 Sequence Analysis of \textit{V\textsubscript{H}} Antibodies

Comparison of the sequence of P245 \textit{V\textsubscript{H}} with the \textit{V\textsubscript{H}} subfamily sequences in Harmsen et al. (2000) revealed that P245 belongs to subfamily \textit{V\textsubscript{H}H1} (Table 8). No other clones containing the \textit{V\textsubscript{H}} gene were found that bound to the LPS; this may be the result of the small size of the library (as previously described, the titer of the llama Hc\textsubscript{IgG} fractions were much lower than that of the Conv\textsubscript{IgG}, which could have attributed to the size of the library constructed). Protein characteristics for P245 were calculated using proteomics tools from the online Expert Protein Analysis System (ExPASy; Table 9). P245 is hydrophilic according to the negative GRAVY score (GRand AVerage hYdrophobicity; hydrophilicity). The calculated pI of P245 was 7.84, close to the pH
### Table 8. Amino acid alignment of selected anti-\textit{Pcc} V_H clone (P245).

<table>
<thead>
<tr>
<th>V_H</th>
<th>FR1</th>
<th>CDR1</th>
<th>FR2</th>
<th>CDR2</th>
<th>FR3</th>
<th>CDR3</th>
<th>FR4</th>
</tr>
</thead>
<tbody>
<tr>
<td>P245</td>
<td>QVQLEESGGGLVQPGGSLRLSCAAS</td>
<td>GRTSIYCMG</td>
<td>WFRQAPGRKFVA</td>
<td>INWNGNDDRTYADSVKGF</td>
<td>RFTISRDNAKNTVYLQMNSLRFEDTAVYTCAG</td>
<td>DTTWLRYGQSGDGY</td>
<td>WGGSTQTVSS</td>
</tr>
</tbody>
</table>
which panning was carried out (pH ~ 7.5). P245 has a molecular weight of 14602.5 Da (Table 9).

Table 9. Protein parameters for the anti-\textit{Pcc} LPS \textit{V}\textsubscript{H} selected. GRAVY represents GRand AVerage hYdrophobicity. Hydrophilicity is represented by the negative value.

<table>
<thead>
<tr>
<th>\textit{V}\textsubscript{H}H</th>
<th>P245</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular Weight (Da)</td>
<td>14602.5</td>
</tr>
<tr>
<td>Molar extinction coefficient</td>
<td>22512.5</td>
</tr>
<tr>
<td>Theoretical pI</td>
<td>7.84</td>
</tr>
<tr>
<td>Negatively charged amino acids</td>
<td>10</td>
</tr>
<tr>
<td>Positively charged amino acids</td>
<td>11</td>
</tr>
<tr>
<td>GRAVY</td>
<td>-0.489</td>
</tr>
</tbody>
</table>

4.1.4 Soluble Expression of P245 Anti-\textit{Pcc} LPS \textit{V}\textsubscript{H}H

According to Georgiou and Segatori (2005), \textit{V}\textsubscript{H}Hs can be harvested from the culture supernatant by simple centrifugation since \textit{V}\textsubscript{H}Hs leak into the growth medium from the periplasm. However, P245 \textit{V}\textsubscript{H}H did not express well in small culture volumes (50 ml), i.e. the \textit{V}\textsubscript{H}H signal on SDS-PAGEs and Western blots was hard to see. In an attempt to resolve the issue with low \textit{V}\textsubscript{H}H expression, large-scale (1L) cultures were grown and the supernatant purified and concentrated. This supernatant was visualized on SDS-PAGE and by Western blot (Figure 35). Large cultures (1L) yielded 0.98 mg/L of protein after purification. The SDS-PAGE and Western blot show the \textit{V}\textsubscript{H}H as a band at
~14 kDa. Further analysis of P245 V_{HH} was performed by competitive inhibition ELISA.

**Figure 35.** Reducing SDS-PAGE (left; Coomassie stained) and Western blot (right) of V_{HH} P245 purified from *E. coli* (HB5121). Note: the Western blot was probed with anti-penta-HIS IgG.

### 4.1.5 Competitive Inhibition ELISA

Checkerboard ELISAs were performed to determine the optimal concentrations of both *Pcc* LPS and V_{HH} to use for the CI-ELISA (i.e., 50 μg/well and 1/2 dilution, respectively). Free-*Pcc* LPS was used in the range of 0.9 μg/ml to 12.5 μg/ml (**Figure 36**). Maximum coating and V_{HH} working concentrations could not be determined because the ELISA signal could not be saturated at the concentrations used.
**Figure 36.** Competitive inhibition ELISA (CI-ELISA) of $V_H$ P245 versus $Pcc$ LPS. The IC$_{50}$ = 0.77 µg/ml, and refers to the concentration of free Pcc LPS required to reduce the absorbance ($A_0$) by 50%. The equation of the line is $y = -0.5421 \log(x) + 0.2543$. 
4.2 Discussion

The construction and screening of the hyperimmunized phage display library (6.0 x 10^5 clones/ml) was successful in yielding one unique anti-

\textit{Pcc} \text{LPS} \text{V}_{\text{H}} \text{H} (P245) representing the \text{V}_{\text{H}} \text{H1} subfamily. P245 \text{V}_{\text{H}} \text{H} was expressed solubly, with its binding affinity being determined by using IC_{50} value of the Cl-ELISA, i.e 52.7 \mu M.

The immune response of an animal is important for recombinant antibody development, since a strong immune response in the immunized animal provides the basis for construction of a hyperimmunized library (Arbabi-Gharoudi \textit{et al.}, 1997). All immunizations of the llama with \textit{Pcc} \text{LPS} resulted in sera with affinity for \textit{Pcc} \text{LPS}. Fractionation of the llama serum into conventional and heavy chain IgGs was used to determine the suitability of creating a \text{V}_{\text{H}} \text{H} library. The HcIgG response was lower than the ConvIgG response. HcIgGs lack light chains and compensate by having, on average, longer CDR loops than ConvIgGs. However, it was suggested by El Khattabi \textit{et al.} (2006) that finding suitable HcIgG and hence \text{V}_{\text{H}} \text{H} may be difficult because of the poor pocket formed for binding to a glycosylated structure. This fact, coupled with the poor immunogenicity of the glycosylated epitopes on \textit{Pcc}-LPS, make the selection of a high affinity \text{V}_{\text{H}} \text{H} difficult. El Khattabi \textit{et al.} (2006) showed that immunization of llamas with purified LPS did not result in detectable immune reactions, and that attempts to select anti-LPS \text{V}_{\text{H}} \text{H} from the \text{V}_{\text{H}} \text{H} phage library of an immunized llama were not successful.

The research presented in this chapter demonstrates that although the library constructed was small, an anti-Pcc LPS \text{V}_{\text{H}} \text{H} was derived from a hyperimmunized phage display library. Perhaps the existence of a small library and the selection of few \text{V}_{\text{H}} \text{Hs could be
attributed to the fact that purified LPS molecules may not be fully immunogenic in llamas.

The data pertaining to the \( V_{H} \) isolated in this research suggest that many other methods and additional studies should be performed. Specifically, the library should be panned with HK \( Pcc \) or live \( Pcc \), since the llama was immunized with HK \( Pcc \) as well as \( Pcc \) LPS. It may be interesting to pan the library against \( Pcc \) flagella as well. Furthermore, analysis of the \( V_{H} \) for cross-reactivity to \( Pectobacteria \) spp. should be completed. To ensure the binding of the \( V_{H} \) to LPS is not an artifact, surface plasmon resonance should be performed.

Phage display is a powerful tool for the detection of a particular domain of the antigen (Azzazy and Highsmith, 2002; El Khattabi et al., 2006). However, phage display in \( E. coli \) may not be the ideal method of selecting for \( V_{H} \)s against LPS, since anti-LPS \( V_{H} \)s may interact with endogenous LPS molecules (\( E. coli \)), resulting in the loss of specific anti-LPS \( V_{H} \)s. In other words, the LPS of \( E. coli \) could possibly interfere with selecting a good binder to \( Pcc \) LPS. Therefore, the use of yeast (\( P. pastoris \)) display could be an alternative to \( E. coli \).

The anti-\( Pcc \) LPS \( V_{H} \) isolated here represents a candidate for the development of diagnostics. The use of anti-\( Pcc \) LPS antibodies in a \( Pcc \) detection system is appealing because anti-\( Pcc \) LPS \( V_{H} \)s can be produced at low cost on a large scale using various microorganisms (\( E. coli \) or \( P. pastoris \)), resulting in an affordable and user-friendly diagnostic system (i.e. paper-based dipstick assay or filtration device). Ultimately, such paper-based devices could demonstrate the practicality of an instrument-independent
method for visual detection of \textit{Pcc} in different media and under various circumstances on-site.
5. **Overall Discussion and Future Directions**

The overall research objectives of this thesis were to produce high-affinity antibodies (polyclonal serum-based and/or V\textsubscript{H}Hs) against HK *Pcc* and *Pcc* LPS and then use them for the development of sensitive, rapid, and user-friendly assays for the detection (LOD\textsubscript{LPS} = 23 ng/ml and LOD\textsubscript{HK} = 81 CFU/ml) and quantification (LOQ\textsubscript{LPS} = 76 ng/ml and LOQ\textsubscript{HK} = 216 CFU/ml) of *Pcc* in greenhouse hydroponic solutions. The results of this research provide evidence that HK *Pcc* and/or *Pcc* LPS can be used to generate antigen-specific polyclonal and monoclonal (V\textsubscript{H}H) antibodies, which can also recognize *Pcc* or *Pcc* LPS with high binding affinity. Furthermore, the results also demonstrated the sensitivity and feasibility of using *Pcc*-specific (HK *Pcc* and/or *Pcc* LPS) polyclonal antibodies for the detection and quantification of *Pcc*, using ELISA and colorimetric paper–based assay technologies. These results also indicated the future possibility of using *Pcc*-specific V\textsubscript{H}Hs for the detection and quantification of *Pcc* as well.

Due to the scope of this research, it would be beneficial to continue, repeat and perform new facets of the research presented in this thesis. For example, HK *Pcc* and *Pcc* LPS rabbit polyclonal antibodies should be further tested against live *Pcc* cells to determine accurate binding specificity and affinity. As well, llama polyclonal antibodies should be tested for their efficacy in detecting and quantifying *Pcc* through ELISA and dipstick-based assays, following the procedures used for the rabbit polyclonal antibodies in chapter 3.

With regard to the hyperimmunized phage display library constructed and panned (discussed in chapter 4), many additional studies should be performed. Specifically, the library was only panned with *Pcc* LPS, but should also be panned with HK *Pcc* since the
llama was also immunized with HK Pcc along with Pcc LPS. Furthermore, it may be of value to pan the library against live Pcc cells and/or Pcc flagella as well, to distinguish if other high-affinity anti-Pcc V\textsubscript{H}Hs could be derived. Preliminary soluble ELISAs using the anti-Pcc LPS V\textsubscript{H}H against HK-Pcc and live Pcc cells were performed; however, further repeats are required for definitive conclusions. As with the cross-reactivity studies performed in chapter 3, analysis of the derived V\textsubscript{H}H for cross-reactivity to Pectobacteria spp. should be completed. Moreover, surface plasmon resonance should be performed to ensure the binding of the V\textsubscript{H}H to LPS is not an artifact. Since E. coli was the expression system used for phage display, a possible interference between the LPS of E. coli and the selection of a good binder to Pcc LPS may have occurred. Thus, the use of a yeast (P. pastoris) expression system could be a preferable alternative to E. coli.

While a user-friendly lateral flow (dipstick) assay was developed using polyclonal anti-HK Pcc serum conjugated to colloidal gold, further optimization of the assay is required. Using a typical dipstick assay format (i.e., replacing HK Pcc on the nitrocellulose strips with HK Pcc antibodies) may provide increased specificity, and detection and quantification at lower levels than observed in chapter 3. Furthermore, using llama polyclonal antibodies instead of rabbit polyclonal antibodies (chapter 3) could possibly increase assay specificity, while decreasing commercial cost for users. Although ELISA and dipstick-assays were optimized using nutrient solutions from commercial hydroponic systems, the assays should also be tested on-site, with large-scale solution volumes, since this is the ultimate application of the assay.
In conclusion, the research conducted in this thesis illustrates the ability to create sensitive, rapid, and user-friendly assays for the detection and quantification of *Pcc*. Although future research is required, producing various fully functional, sensitive, rapid, and user-friendly polyclonal and/or monoclonal-based assays for specific greenhouse industry requirements is feasible.
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