A Plant-based Recombinant PlpE Vaccine for Fowl Cholera

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Amendments

Chapter 1

- Insert "LppC Cytoplasmic Membrane Lipoprotein C" in the List of Abbreviations in p xiii.
- Change *Pleuropneumoniae* to *pleuropneomoniae* (small p) in the beginning of line 10, para 4, p 5.
- Change "Figure 1" to "Figure 1.1" in p 26.
- Change "A powerful Shiga-like toxin, known as F18 fimbriae are produced by these *E. coli* strains, which have been reported to be an important virulence factor related to edema" to "F18 fimbriae produced by these *E. coli* strains have been reported to be an important virulence factor related to edema" in line 3, para 2, p 24.
- Insert "Figure 1.1" after signal peptide in line 9 and after vacuole in line 12 in para 3, p 25.
- Insert (b) after Wu et al., 2007 in lines 1, 5 & 7 in para 3, p 10 and (a) after Wu et al., 2007 in line 8, para 3, p 13. Insert (a) after 2007 in reference 243 & (b) after 2007 in reference 244 in p 58.

Chapter 2

- Insert "Figure 2.1" at the end of line 14, para 2, p 65.
- Replace "Figure 2.3 A" with "Figure 2.7 A" in line 4 and "Figure 2.3 E & F" with "Figure 2.7 E & F" in line 10, para 1, p 71.
- Replace the numbering of the subheading "2.2.12.1 and 2.2.12.2" with "2.2.11.1 and 2.2.11.2" in para 2 & 3, p 74.
- Replace the numbering of the subheading "2.2.13.1 and 2.2.13.2" with "2.2.12.1 and 2.2.12.2" in para 2 & 3, p 75.
- Replace the numbering of the subheading "2.2.14" with "2.2.13" in para 2, and "2.2.15"with "2.2.14" in para 3, p 76.

Chapter 3

- Insert "Rigano et al., 2006" after immunization at the end of line 5, para 1, p 106.
- Insert "3.1 & 3.2" after "Figures 2.6 A," in line 1, para 1, p110.
- Replace "Figure 3.13" with "Figure 3.14" after plant-PlpE in line 2, para 1, p 130.
- Delete "Figure 3.16" from p 133 and insert in p 136 after the last line "*P. multocida* infection might require inducing both humoral and cell-mediated immunity" of p 135.

Chapter 4

- Replace "Figure 4.2" with "Figures 4.4 & 4.5" para 2, p 156.
- Replace *Multocida* with *multocida* in line 9, para 1, p 166.
  Replace *Nicotiana* with *N.* in line 1, para 2, p 166.
- Delete "It was calculated that 45% of the plant-PlpE vaccine diet on average was consumed by the mice; therefore, they consumed around 45 □t was calculated that 45% of the plant-PlpE vaccine diet on average was consumed by the mice; therefore, theas fed with the plant negative control material carrying the empty vector. It was calculated that 45% of the plant-PlpE ding was observed over all 4 immunizations" and insert "It was calculated that on average 45% of the plant-PlpE vaccine diet was consumed by the mice and the same pattern of feeding was observed over all 4 immunizations" in line 3, para 2, p 157.
- Insert "Sera collected from three out of ten mice fed with plant-PlpE reacted with the insoluble *E. coli* PlpE in Western blot analysis (Figure 4.5) while rest of them did not." in line 1, para 2, p 158.
- Delete "Figure 4.8 B" and change "Figure 4.8 C" to "Figure 4.8 B" in p 165. Change "Figure 4.8 C" to "Figure 4.8 B" after subcutaneously in line 4 and "Figure 4.8 A, B & C" to "Figures 4.8 A & B" after trial in line 5, para 3, p 158.

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- Insert "Appendix 2.1" after proteins in line 4, para 2, p198.

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Abstract

Fowl cholera, caused by infection with *Pasteurella multocida* is an economically significant infectious disease of domestic birds and a major cause of economic loss in poultry industries worldwide. Commercial vaccines consist of either whole cell bacterins or live attenuated bacteria, which have disadvantages of lack of cross-protection against different strains or reversion to virulence, respectively. Moreover, those vaccines are delivered systemically. Since *P. multocida* infects via the respiratory route, a subunit mucosal vaccine eliciting systemic and mucosal immunity as well as cross-protection might offer a better solution. PlpE is an outer-membrane protein of *P. multocida*, which protects mice and chickens against fowl cholera, both in soluble and insoluble forms when produced in *E. coli* and administered systemically. Subunit vaccine antigens produced in transgenic plants can elicit protective immunity in model and target animals when administered either systemically or orally. In this thesis, the author reports the production of plant-based recombinant PlpE vaccines in *Nicotiana benthamiana* leaves and *N. tabacum* cell lines and the efficacy of those vaccines in mice and chickens against fowl cholera.

Crude plant extracts were prepared from pooled vaccine batches prepared from the homogenized freeze-dried leaf powder of *N. benthamiana* and cell suspension powder of *N. tabacum* cell line containing transient and stable transgenic plant-PlpE, respectively. An 8-10 µg dose of transient plant-PlpE vaccine adjuvanted with Alhydrogel® protected 64% of the mice against a lethal challenge infection with *P. multocida* X-73. During the second mouse vaccination trial, 100 µg transient plant-PlpE adjuvanted with Alhydrogel® protected 70% of the animals against lethal challenge with *P. multocida* X-73. Mice immunized subcutaneously with 100 µg of stable transgenic plant-PlpE with adjuvant were not protected, whereas only 10% of the mice immunized with stable transgenic plant-PlpE without adjuvant were protected.

In a chicken trial, transient plant-PlpE was tested along with soluble and insoluble *E. coli* PlpE. Chickens were immunized with 100 µg of the plant extract in Alhydrogel® and challenged with *P. multocida* strain VP161. Sera from the birds in all 3 vaccinated groups reacted with recombinant *E. coli* PlpE in Western blot analysis. Eighty and fifty percent of birds were protected in groups of chickens immunized with of soluble and insoluble *E. coli*

PlpE, respectively. No birds in the plant-PlpE immunized group were protected; however, the mean time to death was significantly delayed in birds immunized with transient plant-PlpE.

Lastly, a feeding trial was performed in mice to test the efficacy of the transient plant-PlpE as an oral vaccine. Mice fed 100 µg of transient plant-PlpE powder adjuvanted with saponin produced circulating PlpE-specific antibodies. Though no mice survived the challenge with *P. multocida* X-73, the mean time to death was significantly delayed in mice fed on transient plant-PlpE vaccine.

This is the first report of expression of any *P. multocida* protein in plants, and the first report of a plant-based recombinant fowl cholera vaccine, which protected mice against infection. Additionally, this is the first report of any bacterial antigen produced in plants that elicited a strong antibody response in chickens.

Statement

I, Sadia Sultana Deen, declare that this thesis contains no material that has been accepted for the award of any degree or diploma in any other University and, to the best of my knowledge and belief, contains no material which has been previously published or written by another person, except where due reference is made in the text of the thesis.

Sadia Sultana Deen

Publications

Penney CA, Thomas DR, Deen SS, Walmsley AM. Plant-made vaccines in support of the Millennium Development Goals. Plant Cell Rep. 2011 May; 30(5):789-98. Epub 2011 Jan 18. (A contribution to the special issue: Plant Biotechnology in Support of the Millennium Development Goals)

Differential fluorescent chromosome banding of four *Sida spp*. (Malvaceae). Noor SS, Deen SS, Ahmed L et al. Cytologia 68(1):25-30, 2003. (The Japan Mendel Society)

Karyotype and isozyme analysis in three forms of *Colocasia esculenta* (Araceae). Alam SS, Deen SS. Bangladesh J. Bot. 31(2): 95-98, 2002 (December)

Comparative study in two forms of *Xanthosoma violaceum* (Araceae) through karyotype and isozyme analysis. Deen SS, Alam SS. Bangladesh J. Bot. 31(1): 45-47, 2002 (June)

Conferences/workshops

"Protection of mice against *Pasteurella multocida* using a plant-made vaccine": **Sadia Deen**, Tamas Hatfaludi, Amanda Walmsley and Ben Adler: presented by the author orally at the Second workshop on Molecular Farming in Australia, held in Monash University, Clayton campus, Victoria (12-13 April, 2010)

"A plant-based vaccine protects mice against fowl cholera pathogen *Pasteurella multocida*": **Sadia Deen**, Tamas Hatfaludi, Amanda Walmsley and Ben Adler: presented by the author orally at the VIIIN (Victorian Infection and Immunity network) student Symposium, held in Walter and Eliza Hall of Institute of medical Research, Melbourne University, Victoria (3 June, 2010)

"The first report of a recombinant vaccine produced in plants inducing protective immunity in mice against the fowl cholera pathogen *Pasteurella multocida*": **Sadia Deen**, Amanda Walmsley and Ben Adler: presented by the author orally at PBVA (Plant-based Vaccine and Antibody Conference) 2011, Porto, Portugal (8-10 June, 2011)

"*Pasteurella multocida* vaccine discovery; reverse vaccinology and live attenuated strains" Keith Al-Hasani, Tamás Hatfaludi, Marina Harper, **Sadia Deen**, Amanda Walmsley, Torsten Seemann, Mark Ford, Rob Moore, Ian W. Wilkie, Noelene Quinsey, Marietta John, Ben Adler, and John D. Boyce: presented by **John D. Boyce** at International Pasteurellaceae Conference (IPC), held in LO-Skolen near Helsingør (Elsingore), Denmark (24-27 August, 2011)

"Optimization of *Agrobacterium* mediated genetic transformation system for Asian varieties of chickpea (*Cicer arietinum L.*)": **Sadia S. Deen** and Hans Joerg Jacobsen: presented by the author orally at the 4th International Symposium on Biotechnology and 1st Pakistan-China–Iran International Conference on Biotechnology, Bioengineering and Biophysical Chemistry (ICBBB'07) in University of Sindh, Pakistan (November 4-8, 2007)

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LIST OF ABBREVIATIONS

µg microgram

µm micrometre

Ab antibody

APC antigen presenting cell

bp base pair

C Celsius

CPMV cowpea mosaic virus

DC dendritic cell

DNA deoxyribonucleic acid

ELISA enzyme-linked immunosorbent assay

ER endoplasmic reticulum

GMT geometric mean titre

H hour

HRP horseradish peroxidase

IgG immunoglobulin G

Ip intraperitoneal

Kb kilobase

kDa kilo dalton

kg kilogram

LppC Cytoplasmic Membrane Lipoprotein C

mg milligram

MHC major histocompatibility complex

Min minute

ml millilitre

MP movement protein

mRNA messenger ribonucleic acid

ng nanogram

PBS phosphate-buffered saline

PCR polymerase chain reaction

RNA ribonucleic acid

rpm rotation per minute

RT room temperature

SC subcutaneous

SD standard deviation

T-DNA transferred DNA

TH1/2 T helper cell type 1/2

TMV tobacco mosaic virus

VLP virus-like particle

Chapter 1

Introduction & literature review

1.1 Introduction

Fowl cholera is caused by the gram-negative bacterium *Pasteurella multocida* (generally serotypes A:1, A:3, or A:4). It is a severe systemic disease causing significant economic loss to the poultry industry worldwide. Current vaccines against fowl cholera include bacterins, which provide only limited protection against homologous serotypes and live attenuated strains, which have reverted to virulent strains and could cause mortality in the flocks vaccinated. There is a need for more effective vaccines to control this disease caused by *P. multocida*. Extensive efforts have therefore, been made to identify the cross-protective antigens of *P. multocida* (Adler et al., 1999; Al-Hasani et al., 2007) to enable the development of subunit vaccines.

Wu et al. (2007b) published the first report on a recombinant *P. multocida* antigen PlpE conferring cross-protection to animals (mice and chickens) against heterologous strains of *P. multocida.* PlpE is a surface exposed lipoprotein of *P. multocida*, which was cloned from strain X-73 (serotype A:1) and expressed in *Escherichia coli*. The same antigen has been reported by Hatfaludi et al. (2012) to provide homologous protection to mice and chickens in urea-solubilized form. However, the mechanism by which this antigen confers protection remains unknown.

While all the commercial vaccines available against fowl cholera are injectable, edible and other mucosal vaccines may provide a more robust protective immune response (Ogra et al., 2001). Like most bacterial infections, *P. multocida* infections are believed to be initiated at mucosal surfaces, specifically the lungs and upper respiratory tracts of the hosts (Wilkie et al., 2000). Active immunity against this pathogen has been reported to be mainly humoral (Harper et al., 2006). Both humoral and cell mediated immune responses have been elicited by mucosal immunization (Rigano et al., 2006). Therefore, a recombinant, subunit vaccine consisting of one or more antigenic epitopes or proteins, especially those that target the

mucosal immune system could be an alternative approach to develop more efficient and effective vaccines against fowl cholera.

In most studies of the past 20 years, subunit vaccines have been produced in mammalian, yeast, *E. coli* and insect cell cultures (Yelverton et al., 1983). However, expensive media and the purification steps needed for recovering recombinant proteins from these expression systems increase the cost of producing vaccines. Moreover, most subunit vaccines produced in these systems are heat sensitive and require parenteral delivery. The production of subunit vaccines in transgenic plants can circumvent these problems and can be an ideal alternative means to produce mucosal vaccines (Rigano et al., 2003). It can overcome the risk of contamination with mammalian pathogens and can enable oral delivery (Streatfield & Howard, 2003a). The rigid, cellulose walls of plant cells are indicted to protect antigenic proteins from the acidic environment of the stomach (Pelosi et al., 2011), enabling intact antigen to reach the gut-associated lymphoid tissue. These characteristics simplify vaccine delivery and decrease the cost of an immunization program (Rigano & Walmsley, 2005). Studies have proven plants capable of expressing many different antigens including viral, bacterial, enteric and non-enteric as well as autoimmune antigens (reviewed by Rybicki, 2010). These antigens have demonstrated immunogenicity when tested and some have been shown to provide protection in model and target animals (Reviewed by Tacket, 2009; Tiwari et al., 2009). Plant-based vaccines have also proven to be safe in human clinical trials (reviewed by Walmsley & Arntzen, 2003).

GS60, an outer-membrane lipoprotein of *Mannheimia haemolytica* was produced in transgenic alfalfa (Ziauddin et al., 2004). This plant-based antigen was shown to be immunogenic in rabbits after administration by both systemic and oral routs (Lee et al., 2001 & 2008). ApxII is an outer-membrane lipoprotein of *Actinobacillus pleuropneumoniae*, which was produced in transgenic tobacco plants and corn seeds. When fed to mice transgenic tobacco-derived ApxII antigen was shown to be protective against a pathogen trial with the respective bacterium (Lee et al., 2006). The same antigen produced in transgenic corn seed was reported to be immunogenic when injected into mice (Min-Kyoung Shin et al., 2011). *M. haemolytica* and *A. pleuropneumoniae* belong to the same bacterial family of Pasteurellaceae as *P. multocida* and both GS60 and ApxII antigens belong to the LppC family of bacterial outer membrane lipoproteins like PlpE of *P. multocida* outer membrane (Wu et al., 2007b; Lee et al., 2008, Lee et al., 2006).

In fact, the first plant-based vaccine to be licensed by USDA (United States Department of Agriculture) was a vaccine against Newcastle disease of poultry, which was produced by Dow AgriScience in transgenic tobacco cell suspension (Cardinau et al., 2004). The ability of plants and plant cells to produce numerous antigens including two outer membrane antigens from two bacterial species belonging to Pasteurellaceae family and the first licensed plant-based vaccine being a poultry one inspired the possibility of expressing PlpE in transgenic plants with the aim of developing a plant-based PlpE vaccine against fowl cholera preferably mucosally delivered. The following pages explore this possibility in more detail.

1.2 Literature review

1.2.1 *P. multocida* and Fowl Cholera

P. multocida was first shown to be the causative agent of fowl cholera by Louis Pasteur in 1881 (Pasteur, 1880 & 1881). This gram-negative bacterium has been identified as the causative agent of many other economically important diseases in a wide range of hosts including avian fowl cholera, bovine hemorrhagic septicemia, enzootic pneumonia and swine atrophic rhinitis (Harper et al., 2006). Human infections with *P. multocida* largely arise from the bite of an infected animal, but other types of infections are occasionally reported (Weber et al., 1984). Of all the diseases caused by *P. multocida*, fowl cholera is probably well understood.

Fowl cholera is a disease of many avian species and all domestic poultry are susceptible to this highly infectious disease (Janmaat & Morton, 2010). Chickens, turkeys, ducks, and quail are the most important domestic avian species involved and the disease is very significant economically. Symptoms are variable and include respiratory problems and diarrhoea. It is spread through the flock by contaminated water, in droppings and in nasal discharges. It is a septicaemic disease that can occur in acute, sub-acute or chronic forms. The acute and sub-acute forms are associated with high mortality while chronic fowl cholera is usually associated with localized infections. Such chronic infections result in the persistence of bacteria in the flocks. *P. multocida* is capable of multiplication in the bloodstream of a bird. As a result of this bacteremia, the organism may quickly colonize many organs, contributing to the typical purulent exudative lesions of fowl cholera seen in the joints, wattles, ovaries, brain, liver, spleen, and lungs (Glisson, 1998). Shortly before a bird succumbs to the disease, the pathogen will typically multiply to very high levels in the bloodstream and tissues (Rimler & Glisson, 1997).

1.2.2 Pathogenesis of *P. multocida* and virulence factors

The mechanisms by which these bacteria can evade the innate immune system and cause systemic disease are slowly being elucidated. Disease pathogenesis is versatile and depends on the bacterial strain, the animal model used and the changing response of both the host and bacteria to the interaction (Boyce et al., 2010). The surface of Gram-negative bacteria is critical for interaction of the bacterium with the host cell environment as it mediates nutrient uptake, secretion of toxins and other products and is involved in avoidance of the host immune system. Furthermore, it is the bacterial surface molecules that are the targets for the host immunity. Key virulence factors identified to date include capsule and lipopolysaccharide (LPS). *P. multocida* strains are classified in five serogroups (A, B, D, E and F) based on capsule antigens and further divided into 16 serotypes based primarily on lipopolysaccharide antigens using the Heddleston scheme (Carter, 1955; Heddleston & Rebers, 1972). Fowl cholera strains generally belong to serogroup A, serotypes 1, 3, or 4 (Heddleston, 1962).

1.2.2.1 Capsules

Capsules are highly hydrated polysaccharides located external and adherent to the bacterial cell wall. The location of extracellular polysaccharides at the outermost surface of the bacterial cell is important because they are the first portal of entry and the last barrier to excretion of substances in and out of the bacterium. Various hypotheses have been postulated about the function of the bacterial capsule. These include protection against desiccation in the environment (Ophir & Gutnik, 1994), and evasion of phagocytosis (Smith et al., 1999) and the bactericidal activity of serum complement (Kahler et al., 1998; Vogel et al., 1997). The capsule is clearly involved in bacterial avoidance of phagocytosis and resistance to complement (Chung et al., 2001; Boyce & Adler, 2000).

1.2.1.2 Cell wall

The bacterial cell wall is the next barrier of entry into the bacterium after the capsule. The cell walls of gram-negative bacteria consist of one or a very few layers of peptidoglycan and an outer membrane. This outer membrane consists of lipopolysaccharide molecules (LPS), lipoproteins and phospholipids (Tortora, Funke & Case, 2004). Lipopolysaccharide (LPS) is a critical virulence determinant in *P. multocida* and a major antigen responsible for host protective immunity (Harper et al., 2004, 2006). *P. multocida* possesses a conserved oligosaccharide extension attached to two different, simultaneously expressed inner core structures, one containing a single phosphorylated 3-deoxy-D-manno-octulosonic acid (Kdo)

residue (glycoform A) and the other containing two Kdo residues (glycoform B). Harper et al. (2004) reported that *P. multocida* required a complete LPS structure in order to replicate *in vivo* and cause disease. However, in a recent publication, they have demonstrated for the first time that expression of a single LPS structure (glycoform B) is sufficient for *P. multocida* survival *in vivo* (Harper et al., 2007). It could be that the expression and regulation of two different glycoforms allow for *P. multocida* survival in different environments within the host.

1.2.1.3 Outer-membrane proteins

Other components of the bacterial outer membrane, such as trans-membrane proteins and lipoproteins are involved in pathogenesis and immunity. Outer membrane proteins also promote adherence to host cell surfaces. These proteins are likely to be involved in *P. multocida* virulence (Boyle & Finlay, 2003) and have been the focus of several studies investigating possible cross-protective antigens. Few of these proteins have been shown to be important for conferring protective immunity in a range of infection models (Brown et al., 2001). Hatfaludi et al. (2010) generated a common grouping for the outer-membrane lipoproteins and integral membrane proteins. They categorized the proteins solely by functional characteristics; the main functional categories are structural proteins, transport proteins, binding proteins, adhesins, protein assembly machines, and membrane-associated enzymes.

A number of other virulence factors have been identified by both directed and random mutagenesis, including *P. multocida* putative surface adhesins and iron acquisition proteins (Cox et al., 2003).

1.2.1.4 Iron acquisition proteins

Gram-negative organisms adapt to grow in the absence of free iron by producing a number of novel proteins on their surface which are able to sequester iron from host iron-binding proteins such as transferrin, lactoferrin, heme compounds and others (Bullen et al., 1972, 1978; Griffiths et al., 1980, 1985). Members of the Pasteurellaceae are excellent examples of this phenomenon (Deneer & Potter, 1989, a & b; Ogunnariwo & Schryvers, 1990; Ogunnariwo et al., 1991, 1997) and a number of groups have demonstrated that vaccines enriched in these novel proteins were capable of inducing enhanced protection following immunization (Gilmour et al., 1991; Gerlach et al., 1992; Potter et al., 1999). Recombinant subunit vaccines based on the transferring-binding protein TfbB are available for *A. pleuropneumoniae* and conventional vaccines have been developed for both bovine and

ovine isolates of *M. haemolytica*. However, no iron-binding protein has been shown to be immunogenic and protective in chickens against *P. multocida* (Bosch et al., 2004; Cox et al., 2003). A prominent 84 kDa outer-membrane protein was expressed by Ikeda & Hirsh (1988) from *P. multocida* A:3 grown under iron-depleted conditions. This protein was immunogenic in turkeys and present in 15 different serotypes of *P. multocida* with varying molecular masses (84–92 kDa) grown under iron-depleted conditions.

1.2.2 Vaccination against Fowl Cholera

Vaccination to prevent fowl cholera is an important aspect of controlling the disease, particularly in broiler breeders and turkeys (Glisson, 1998).

1.2.2.1 Commercially available vaccines

There are two broad categories of vaccines commercially available, live and inactivated or killed vaccines. The killed vaccines available commercially contain primarily Serotypes 1, 3, and 4 as those are the most common serotypes found in commercial poultry. However, the killed vaccines are adequate only for homologous protection in the birds (Glisson, 1998). There are three live *P. multocida* vaccines available. These vaccines vary in virulence. The M-9 vaccine is the least virulent, the CU vaccine is the most virulent, and the *P. multocida*-1 vaccine is intermediate in virulence. All three vaccines are serotype 3 and 4 (Glisson, 1998). All *P. multocida* live vaccines readily infect turkeys orally; therefore, they can be administered in the drinking water. However, live *P. multocida* vaccines do not readily infect chickens orally and must be applied by wing-web stab (Derieux, 1984). Unlike inactivated vaccines, live *P. multocida* vaccines induce an immune response that is not serotype-specific. Birds immunized with a live *P. multocida* vaccine are protected against all *P. multocida* serotypes. There are several advantages and disadvantages to the use of killed or live *P. multocida* vaccines. The live *P. multocida* vaccines are low virulent organisms and have the potential to induce chronic fowl cholera in both chickens and turkeys (Rimler & Glisson, 1997). Live vaccines provided orally to turkeys induce only a transient immune response and require frequent vaccination in order to maintain adequate protection. The inactivated *P. multocida* bacterins do not induce disease but have the disadvantage of providing protection limited to the serotypes in the bacterin (Rimler & Glisson, 1997). With either a live vaccine or an inactivated bacterin, a bird must be vaccinated at least twice to obtain an adequate immune response. As both types of vaccines have some limitations, a wide variety of vaccination programs have been developed to accommodate different management situations (Janmmat & Morton, 2010). Broiler breeder pullets should be vaccinated twice at

least 4 weeks apart with either two inactivated vaccines, two live vaccines, or one live vaccine and one killed vaccine. Young meat turkeys are typically vaccinated with a live vaccine at about 6, 10, and 14 weeks of age.

1.2.2.2 Use of antibiotics

Several different antibiotics such as tetracyclines and sulfonamides have been used by the poultry farmers to treat fowl cholera though antibiotic susceptibility patterns vary among field isolates of *P. multocida* (Glisson, 1998). However, the recent and forthcoming bans on non-therapeutic use of antibiotics will increase the demand for new treatment options to promote animal's health (Anonymous 2005 & 2007b). The currently available vaccines against *P. multocida* are therefore less than ideal and the search and development of an effective subunit vaccine continues.

1.2.3 Subunit vaccines

There are powerful tools available now, such as phage display expression libraries (Wang & Yu 2004) and reverse vaccinology approach (Rappuoli & Covacci 2003), which can be used to identify novel putative immunogenic proteins. These proteins can be isolated from the infectious agent and directly administered as subunit vaccines. It is also possible to isolate these immunogenic proteins and express in heterologous systems such as bacterial, mammal, fungal, insect or plant cell cultures as recombinant vaccines. These recombinant vaccines offer the benefit of avoiding the purification of the proteins of interest from the virulent agent. Expression levels can be maximized and antigenic proteins with new desired features can be expressed by using genetic engineering technique (Chargelegue et al., 2005).

Subunit vaccines have advantages over live attenuated and inactivated vaccines including the excellent safety profile and possibility of co-delivery of multiple subunit antigens. These vaccines have the ability to induce strong humoral and cell-mediated immune response. Subunit vaccines against a variety of respiratory and enteric viruses, including BVDV, BRSV, PI3, rotavirus, and coronavirus have been successfully tested, although none of these is used on a commercial basis (Van Drunen Littel-van den Hurk et al., 1993; Romero et al., 1994). Bacterial subunits have proven more successful than their viral counterparts (Jones et al., 1996; Lee et al., 2003). Recombinant vaccines are commercially available for respiratory pathogens *Mannheimia haemolytica* and *Actinobacillus pleuropneumoniae* based upon the leukotoxins produced by these organisms, as well as transferrin-binding proteins.

1.2.3.1 Subunit vaccine candidates against *P. multocida*

To date, many outer-membrane proteins of *P. multocida* have been tested in immunization experiments as subunit vaccines for fowl cholera. Only three have provided protection against *P. multocida* challenge. Soluble and insoluble PlpE both in mice and chickens protected against heterologous challenges (Wu et al., 2007b; Hatfaludi et al., 2012). OmpH protected mice against homologous, and chickens against heterologous challenges (Lee et al., 2007; Luo et al., 1999). Lastly, fragments of recombinant filamentous haemagglutinin protein FhaB2, elicited protection in turkeys (Tatum et al., 2009). However, no subunit vaccines based on individual protein antigens have been developed commercially.

OmpH

OmpH is a 37 kDa channel-forming, surface protein of *P. multocida* (Lugtenberg et al., 1986). Purified native OmpH from a *P. multocida* A:3 strain was shown to elicit homologous protection in chickens, however, the recombinant protein was shown to stimulate little protection (Luo et al., 1997). Lee et al. (2007) expressed full length recombinant OmpH with an N-terminal thioredoxin as well as three sub-fragments of the same recombinant OmpH protein. It was shown that full-length OmpH was able to elicit 70% protection in mice against homologous challenge, whereas three sub-fragments of the protein elicited lower protection in mice (30–50%) without any preference for any of the sub-fragments.

FHAB2 peptides

It was demonstrated that a filamentous hemagglutinin (fhaB2) mutant of *P. multocida* was highly attenuated in turkeys (Tatum et al., 2005). The reduced pathogenicity of the FHA mutant was hypothesized to be related to decreased capacity to colonize the respiratory epithelium or to an impaired ability to invade tissues. Three gene fragments encoding approximately the one-third of fhaB2, derived from *Pasteurella multocida* strain P-1059 (serotype A:3) were over-expressed individually in *Escherichia coli*. The recombinant peptides were purified, pooled, and administered to turkeys. Turkeys immunized twice with the recombinant peptides were significantly protected against intranasal challenge with *P. multocida* strain P-1059.

PlpE

Rimler (1987) reported a cross-protective antigen of *P. multocida* from infected turkey tissues. Rimler later purified a protein of approximately 39 kDa that was believed to be one of the cross-protection antigens previously found (Rimler, 2001). In addition to Rimler's work, Ali et al. (2004) purified a 39 kDa protein from *P. multocida* that was believed to be a

cross-protective antigen, but it remains unknown whether the 39 kDa proteins purified by these two groups are the same protein. The 39 kDa protein prepared by Rimler (2001) was identified to be *Pasteurella* lipoprotein B (PlpB) using a peptide mass fingerprinting assay. This report however did not verify whether PlpB could confer cross protection. Tabatabai & Zehr, (2004) also found a 39 kDa cross-protective factor protein in addition to four other outer membrane proteins of the membrane proteome of *P. multocida.* It was falsely determined that the 39 kDa cross-protective protein was *Pasteurella* lipoprotein B, or PlpB (Wu et al., 2007b).

Lipoprotein E (PlpE) from *Mannheimia haemolytica* is a lipid-modified, surface-exposed outer membrane protein that is important in complement-mediated killing of *M. haemolytica* and this was found to be highly immunogenic in cattle (Pandher et al., 1998). Addition of recombinant PlpE to the commercial *M. haemolytica* vaccine markedly enhanced the vaccine-induced resistance against experimental challenge with serotypes 1 and 6 (Confer et al., 2006). PlpE of *M. haemolytica* has sequence homology of 18% with the OmlA of *Actinobacillus pleuropneumoniae*. OmlA is a lipid-modified outer membrane protein that confers protection against experimental challenge with *A. pleuropneumoniae* to pigs. A bioinformatics-based sequence search showed that a gene annotated *plpE* is present in the published genome sequence of *P. multocida* strain pm-70, serotype A:3 (May et al., 2001). The 1.0 kb expected PCR product of *plpE* was obtained from 15 reference strains of *P. multocida* and the complete nucleotide sequencing of the gene from 6 reference strains showed 90.8-100% identity. The *plpE* is an open reading frame of 1008-1019 nt encoding a PlpE protein of 37.4-37.7 kDa. This gene has the potential to encode a lipoprotein of 335 amino acids that has 24.3% sequence identity with PlpE of *M. haemolytica* and 19.1% identity with OmlA of *A. pleuropneumoniae*. PlpE amino acid sequences from different strains of *P. multocida* showed that they shared 90–100% identity. The amino acid sequence alignment showed that the PlpE from *M. haemolytica* and *P. multocida* and OmlA from *A. pleuropneumoniae* had a putative signal peptide of 19-20 residues, followed by a consensus lipoprotein processing site (LVAC). Additionally, the second residue of the mature PlpE and OmlA is a serine or glycine, which allows the targeting of PlpE and OmlA for the outer membrane (Seydel et al., 1999). All PlpE and OmlA proteins investigated have a stretch of glycine or serine residues after the signal peptidase cleavage site. Sequence alignment shows that PlpEs and OmlA have one unique feature in common: all of them contain a stretch of serine and glycine residues at their amino termini (Pandher et al., 1998; Gerlach et al.,

1993). Site-directed mutagenesis or nested deletion at the amino termini of these proteins is required to investigate whether these residues are important for the function of PlpE and OmlA. Sequence comparison showed that *P. multocida* PlpE lacks tandem repeats, which is a characteristic of *M. haemolytica* PlpE (Confer et al., 2003).

It is most likely that this protein was initially incorrectly named as *Pasteurella* lipoprotein B (PlpB) by two different groups (Tabatabai & Zehr, 2004). Indeed, proteomics analysis indicated that native PlpB has a molecular mass of 30.1 kDa (Boyce et al., 2006; Wu et al., 2007b). Although PlpB was initially identified as a cross-protective antigen, recombinant PlpB failed to protect either mice or chickens against *P. multocida* challenge, despite stimulating an antibody response (Wu et al., 2007b; Hatfaludi et al., 2012).

The report published by Wu et al. (2007b) indicates for the first time that the 39 kDa *Pastuerella* lipoprotein E (PlpE) is the cross-protective antigen, since immunization with a single dose of purified soluble recombinant PlpE produced in *E. coli* cells conferred 70% protection to mice against challenge with strains X-73 (A:1), P-1059 (A:3) and P-1662 (A:4) (Wu et al., 2007b). In addition to mice, they have also reported that recombinant PlpE conferred 100% protection to chickens against challenge with strain X-73, and gave some protection against P-1662 (Wu et al., 2007b). The same antigen was reported by Hatfaludi et al. (2012) to be produced and purified from *E. coli* cells but in urea-soluble form. This urea-solubilized recombinant antigen protected chickens completely (100%) against *P. multocida* infection with X-73 after two sub-cutaneous immunizations and provided 70% protection in mice (Hatfaludi et al., 2012). Therefore, PlpE is the only recombinant subunit vaccine that showed complete protection in animals against *P. multocida* infections.

1.2.4 Novel strategy for vaccine formulation and delivery

The success of a vaccine depends on its formulation and delivery. Traditionally, most vaccines are administered systemically, either intramuscularly or subcutaneously. Since over 90% of pathogens enter and initiate infection at mucosal surfaces, the best target for an effective vaccine is the mucosal surface in order to reduce the ability of the pathogen to become established (Mestecky et al., 1997). Thus, the induction of immune responses should ideally occur at the mucosal site (Mestecky et al., 1997). Rational vaccine development includes novel adjuvants, immunomodulators and delivery devices for mucosal administration of vaccines. These enhance and modulate the immune response and can be used to deliver the vaccine to either specific compartments or to facilitate uptake via the

mucosal surfaces (Gentschev et al., 1996, 2002; Autenrieth & Schmidt, 2000, Sizemore et al., 1996; Darji et al., 2000; Dietrich et al., 2003; Loessner & Weiss, 2004). The development of oral vaccines for animals has been a goal of many groups, with most work focused on the use of live attenuated or live vectored vaccines though these live vaccines bear the potential risk of infection for the vaccine administering personnel (Streatfield, 2005). Recombinant subunit vaccines are predominantly expressed in suspension cultures of *Escherichia coli* or mammalian cells with a few exceptions of yeast or insect cells (Yin et al. 2007). Their production relies on fermentation technology of suspension cells in bioreactors, which requires an enormous upfront capital investment, it has intrinsic severe constraints in scalability and, thereby a considerable impact on manufacturing costs and ultimately on market price (Yin et al. 2007). Plant expression platforms are safe alternatives to the conventional systems in the production of novel recombinant subunit vaccines and could offer less expensive options depending on the specific expression system use (Streatfield & Howard, 2003b).

1.2.5 Plant-based vaccines

Although vaccination programs are one of the most cost efficient health interventions, the development of a new vaccine is an enormous economic effort, mainly due to the research and manufactory costs and to the expense of complying with the legal and regulatory requirements needed for market authorization (Heldens et al. 2008). Most of the currently marketed vaccines are still based on the conventional strategies used in the last 200 years (Potter et al., 2008). However, production of antigenic proteins in plants relies on a comparatively newly developed and proven technology (Rybicki, 2009 & 2010). Plants are attractive as protein factories because they can produce large volumes of products efficiently and sustainably and, under certain conditions, can have significant advantages in decreasing manufacturing costs (Hood et al. 1999; Giddings 2001). Plant systems are far less likely than mammalian cells or whole transgenic animal systems to harbor microbes pathogenic to humans. In addition, they are able to perform post-translational modifications typical of eukaryotic organisms (Vitale & Pedrazzini 2005). These cost, scale, and safety advantages make plant-based pharmaceuticals very promising for the development of new improved animal vaccines. Several plant expression platforms are available and many different plant species have been utilized. Expression systems include stable transgenic or transplastomic plants or plant cell lines, with inducible or constitutive expression, plant virus-based and *Agrobacterium tumefaciens*-based transient expression systems as well as seed-specific

expression. Candidate vaccines have been produced for human and animal viruses and bacteria and other parasites; for allergens; and as cancer therapeutics (Dirnberger et al., 2001; reviewed by Rybicki, 2009; Tacket, 2009; Tiwari et al., 2009). Plant-based veterinary vaccines have been shown to provide protection in model animals and some of them have been reported to protect specific species of interest after relevant pathogen challenge trials (Dalsgaard et al., 1997; reviewed by Rice et al., 2005; Ling, Pelosi & Walmsley, 2010; Santi et al., 2006).

1.2.5.1 Plant expression platforms

Different plant types and systems have been used for expression of vaccine antigens (Rybicki, 2009). These include various *Nicotiana* spp., *Arabidopsis thaliana*, alfalfa, spinach, potatoes, duckweed, strawberries, carrots, tomatoes, aloe plants and single-celled algae. Proteins have been expressed in seeds of maize, rice, beans and tobacco, in potatoes, tomatoes and strawberries, in suspension cell cultures of tobacco and maize, in hairy root cultures and in transformed chloroplasts of a variety of plant species. Vaccine antigens have been successfully produced in *A. thaliana* including antigens from transmissible gastroenteritis virus of swine (Gomez et al., 1998), infectious bursal disease of chickens (Wu et al., 2004), *Mycobacterium tuberculosis* (TB) ESAT-6 antigen (Rigano et al., 2006). Tomatoes have been engineered to express a variety of antigens, including rabies virus glycoprotein G (McGarvey et al., 1995), respiratory syncytial virus F glycoprotein (Sandhu et al., 2000), a Yersinia pestis F1-V antigen (Alvarez et al., 2006). Transgenic potato tubers have been used to produce *Escherichia coli* heat-labile enterotoxin (LT-B) (Haq et al., 1995), Rabbit haemorrhagic disease virus (RHDV) protein VP60 (Castanon et al., 1999), a combination of Cholera, *E. coli* and rotavirus vaccine (Yu & Langridge, 2001) and Newcastle disease virus envelope proteins (Berinstein et al., 2005), among many others. Edible leafy crops such as alfalfa, spinach, lupins and lettuce have been used for antigen production as well. The VP1 antigen of Foot and mouth disease virus (FMDV) was produced in transgenic alfalfa (Wigdorovitz et al., 1999a; Dus Santos et al., 2002). Rabies virus was expressed in transgenic spinach (Modelska et al., 1998). Measles virus was expressed in transgenic lettuce (Webster et al., 2006).

Seeds tend to preserve proteins very well for long periods, even under ambient conditions because of ‘glassification’ and other desiccation-dependent stabilisation conditions (Hiroi & Takaiwa, 2006; Lamphear et al., 2002; Streatfield et al., 2003). A number of viral and bacterial antigens and antibodies have been successfully produced in corn seeds (Karaman et

al., 2006). Chikwamba et al. (2002) demonstrated that *E. coli* LT-B toxin subunit could be produced in transgenic corn seeds as functional antigen. A transmissible gastroenteritis virus (TGEV) subunit vaccine was produced in maize seeds for pigs (Lamphear et al., 2004). Rice has been investigated as a production and delivery vehicle, for the same reasons as maize, and probably has a significant advantage over maize in that plants are self-fertilised, which limits potential escape of vaccine genes (Hiroi & Takaiwa, 2006; Takaiwa, 2007). VP2 protein of Infectious bursal disease virus (IBDV) was produced in transgenic rice seeds (Wu et al., 2007a). Other seed systems such as legumes have been investigated (Reviewed by Tiwari et al., 2009). A Rabbit haemorrhagic disease virus (RHDV) protein VP60 was fused with CTB and this chimeric protein was produced in pea (*Pisum sativum*) (Mikschofsky et al., 2009). The use of *Nicotiana* spp. and mainly *N. tabacum* for production of a variety of recombinant proteins has been well documented (Kang et al., 2005; Kehm et al., 2001; reviewed by Rybicki, 2009; Sharma & Sharma, 2009; Tiwari et al., 2009).

1.2.5.2 Plant expression systems

There are three main methods for production of recombinant vaccines in plants: stable transformation of the nuclear genome, stable transformation of the chloroplast genome by using the gram-negative bacterium *Agrobacterium tumefaciens* (Herrera-Estrella et al., 1992) and transient transformation either by viral infection or *Agrobacterium* infiltration.

Stable expression systems

Stable transformation allows the integration of the transgene into the plant genome. As a result, the acquired character is transferred to the next generations while with transient infection the acquired trait is not genetically transmissible and a new transformation event will have to be performed on every new plant.

Nuclear transformation

Efficient transformation protocols have been developed for different plant species. Genetically engineered *Agrobacterium tumefaciens* is used to transfer the T-DNA with the gene of interest using the natural capacity of the bacterium to infect plant cells and insert the piece of foreign DNA in to the plant's nucleus. Stable nuclear transformation can also be performed by particle bombardment, also known as the biolistic method. Metal particles are coated with naked DNA and shot inside the plant tissue using a gene gun (Christou 1995). In this case the transferred DNA integrates randomly in the genome so that its expression is subject to positional effects. Transgenic plants have the advantage of permitting large scale, low-cost biomass production of selected high-expressing genes using agricultural practice

and the potential for crossing transgenic lines to obtain multiple proteins expressed in the same plant. Moreover, they are able to confer heat stability to the heterologous protein, especially when expressed in grains and, if edible plants are used, there is the intriguing possibility of direct delivery through oral administration avoiding antigen purification and needle administration.

Chloroplast transformation

Chloroplast transformation can only be achieved by the biolistic method. Stable transformation of the chloroplast offers several distinct advantages in areas of transgene targeting, product yield, and regulatory compliance (Daniell, 2006). Since the chloroplast genome allows for homologous recombination, the gene of interest can be precisely targeted to a specific locus of the genome (Daniell, 2006). The chloroplast is inherited maternally therefore; this technology reduces the risk of potential transgene escape by pollen dissemination (Daniell, 2006). Unfortunately, chloroplasts are unable to perform typical eukaryotic post-translational modifications, such as glycosylation and only a few plant species have been transformed with this technology, primarily tobacco (Bock 2007). Singh et al. (2009) have reviewed protocols for transformation, expression and oral administration of transgenic chloroplast-derived antigens. An early demonstration of the potential of chloroplast expression was done by Daniell et al. (2001). They reported the production of viable *E. coli* LT-B and Cholera vibrio CT-B antigens in tobacco chloroplasts (Daniell et al., 2001). *Clostridium tetanii* toxin Fragment C was produced at high level (Tregoning et al., 2003) in transgenic tobacco chloroplast as well as *Bacillus anthracis* (anthrax) protective antigen (Watson et al., 2004; Aziz et al., 2005; Koya et al., 2005). Bacterial-derived antigens express well in chloroplasts, because of their origin and similar mechanisms for folding proteins and a lack of downstream processing such as glycosylation (Kang et al., 2003 & 2004). However, 2L21 peptide from virulent canine parvovirus (CPV) was expressed in tobacco chloroplasts as a C-terminal translational fusion with the cholera toxin B subunit (CTB) (Molina et al., 2004), which could elicit neutralising antibodies in mice and rabbits although not via the oral route of administration (Molina et al., 2005). The severe acute respiratory syndrome coronavirus (SARS-CoV) spike protein is a membrane-associated glycoprotein, which also apparently accumulates at high level in transformed chloroplasts (Li et al., 2006).

Transient expression systems

Transient expression systems include virus-based and *Agrobacterium*-mediated somatic expression, usually in whole plants. These systems rely either on the ectopic expression of *Agrobacterium tumefaciens* (*A. tumefaciens*) directly infiltrated into plant tissues (agroinfiltration) or on viral-based expression vectors. Both systems generally offer several advantages over the generation of transgenic plants, including production speed and high expression yields (Palmer et al., 2006; Kohl et al., 2006).

Virus-based transient expression

In virus-based transient expression system, the genes of interest are inserted into a viral genome in such a way that the heterologous protein is produced in the plant as a "by-product" of virus replication. The use of plant viral vectors offers several advantages. Recombinant protein expression can reach very high levels in a relatively short time ranging from 3 to 14 days post-infection, depending on the system used (Gleba et al., 2007; Yusibov et al., 2006). Fully functional and systemic infectious vectors are easily transmissible by mechanical inoculation, making large-scale infections feasible. Several expression vectors have been developed using different types of plant viruses; the most common are based on single stranded positive RNA viruses like the tobacco mosaic virus (Lico et al. 2009). One of the first plant-based vaccines was the recombinant TMV with malarial epitopes exposed on virion surfaces (Turpen et al., 1995). Other plant viruses that have successfully been used for either peptide display or expression of whole antigens include the potato virus X (PVX), bamboo mosaic virus (BaMV), papaya mosaic virus (PapMV), cowpea mosaic comovirus (CPMV) (reviewed by Brennan et al., 2001; Canizares et al., 2005), bean yellow dwarf mastrevirus (BeYDV), alfalfa mosaic (AlMV) and cucumber mosaic viruses (CMV) among others (Brennan et al., 1999; Langeveld et al., 2001; Yang et al., 2007; Lico et al., 2009).

Agrobacterium infiltration based expression

In this transient expression system, *Agrobacterium tumefaciens* suspension carrying the gene/s of interest is infiltrated in to the intercellular spaces in plant leaves via syringe or vacuum. This results in mobilisation of T-DNA into the nuclei of a large proportion of the cells and subsequent expression of any transgene from integrated or episomal DNA (reviewed by Fischer et al., 1999). The technique has become very useful for determining which given genes can be expressed in plants, and in which cell compartments the protein accumulates best (Lee et al., 2008). Gene of interest can be fused with different signal peptides to express the protein in different organelles of plant cell. Agro-infiltration system has been used to accumulate high levels of different heterologous proteins, ranging from

reporter genes (green fluorescent protein and β-glucuronidase) to complex multimeric molecules, such as immunoglobulins. Main advantages of this system consist in the possibility of expressing long gene sequences, flexibility and ease of expressing more than one gene simultaneously in the same cell, allowing efficient assembly of multimeric proteins. Moreover, this system could be easily applied to industrial scale for massive production of recombinant proteins (D'Aoust et al., 2008). A major drawback of this technology is the post-transcriptional gene silencing (PTGS) response that may take place in the infiltrated plant tissue (Hammond et al., 2001). A viral suppressor protein of Tomato Bushy Stunt virus (TBSV-P19) called P19, was able to prevent PTGS and enhance transient expression yields of a range of heterologous proteins up to 50 folds (Voinett et al., 2003).

Viral vectors for *Agrobacterium* infiltration

A technology termed 'Magnifection' has been developed by the new Icon Genetics (Halle, Germany) company in this field in recent times. It couples the agro-infiltration with delivery of cDNA encoding a 'deconstructed' TMV-based vector, (Gleba et al., 2005). The system has been used in a landmark vaccine model exercise to produce recombinant *Yersinia pestis* antigens F1, V and fusion protein F1-V. Purified antigens injected into guinea pigs were protective against an aerosol challenge of virulent *Y. pestis* (Santi et al., 2006). The first really acceptable production level of HBsAg in a plant system was also achieved using MagnICON viral vectors (300 mg⁄kg leaf fresh weight) (Huang et al., 2008). A novel version of an agro-infiltration-delivered TMV based vector entitled 'launch vector' (Musiychuk et al., 2007) has also been used to produce many other antigens including HPV E7 protein and H5N1 influenza virus haemagglutinin and neuraminidase domains fused to a thermostable lichenase (LickM). Both vaccines were protective in animals (Massa et al., 2007; Mett et al., 2008). The same group recently used the same vector and fusion system to produce Fraction 1 (F1) and V antigens of *Y. pestis*, and demonstrated protection of cynomolgus macaques against aerosolised bacteria (Chichester et al., 2009). Other new vector systems used for amplified protein expression in plants include those derived from the plant single-stranded DNA Gemini viruses and nano viruses.

1.2.5.3 Cell culture systems

Plants can be cultured in vitro in different systems such as hairy roots, immobilized cells, and cell suspensions for production of heterologous proteins, such as vaccine antigens. These systems circumvent the presence of contaminants associated with mammalian or other culture systems (Fischer et al., 2004). The cell suspension culture is commonly used and is generally

regarded to be the most suitable for large-scale bioreactors complying with GMP guidelines (Rybicki, 2009). Different cell lines like NT1 and BY2 have been generated using *Nicotiana tabacum* or tobacco mainly (Mayo et al., 2006). A number of microalgae systems like *Chlorella* and *Chlamydomonas* spp. have also been investigated (Fuhrmann, 2004; Siripornadulsil et al., 2007; Surzycki et al., 2009; Sun et al., 2003). Most commercial applications of plant cell suspension cultures are aimed at the production of secondary metabolites, for example, the antitumor agent Taxol. However, the use of plant cell suspensions as production systems of recombinant proteins has only been evaluated during the past decade (Shin et al., 2003; Su et al., 2004). Although the cost of biomass production is greater for plant cell suspension cultures than for whole plants, this system offers several advantages over whole plants (Becerra-Arteaga et al., 2006). For example, the time to evaluate a genetic manipulation is shorter (because a plant does not have to be regenerated), the conditions are more controlled in a reactor than in the field or in a greenhouse, and the transgenic gene is contained within the production facility. Moreover, if the protein is secreted into the culture medium, the purification is relatively easy because plant cells are grown in a simple, chemically defined medium. A very successful example of a veterinary vaccine produced in plant cell suspension culture is the poultry vaccine against Newcastle disease virus (Cardinau et al., 2004), the only licensed plant-based vaccine till now.

1.2.5 Plant-based veterinary vaccines

The production of subunit vaccines for animal health have been widely validated using all the different plant heterologous gene expression approaches previously described; numerous candidate vaccines have been proven to elicit humoral and mucosal immune responses against toxins, viruses, bacteria and parasitic pathogens. Regrettably there are still no examples of licensed products on the market except a tobacco plant cell derived vaccine for Newcastle disease (Cardinau et al. 2004), developed by Dow AgroSciences. Plant-based vaccines for veterinary purposes have been summarized by Streatfield and Rice et al. in 2005 and Floss et al. in 2007. The majority of the published studies of vaccine candidates expressed in plants for animal infectious disease control are against viral pathogens. The likely reason for this is not a lack of potential microbial pathogens or protective antigens but a lack of cost-effective means to treat animal virus infections (Joensuu, 2008). Microbial infections can usually be controlled by antibiotics. However, the recent emergence of antibiotic resistant strains and calls for reduced antibiotic use will increase the demand for new treatment options (Joensuu et al, 2008; Anonymous, 2005). Most of the plant-based

vaccines have only been preliminary tested in laboratory animals (mainly mice), but studies have also shown protection in the target animals like cattle, sheep, pigs and chickens (Khandelwal et al., 2003; Khandelwal et al., 2011; Loza-Rubio et al., 2012; Lamphear et al., 2001; Zhou et al., 2004; Guerrero-Andrade et al., 2006). Few examples of those will be briefly described.

1.2.5.1 Applications for poultry

There have been relatively more plant-based vaccine vaccination and challenge studies completed in poultry. Vaccination and challenge trials are reported for Newcastle disease virus (Cardineau et al., 2004), infectious bursal disease virus (Miller et al., 2004; Wu et al., 2004), infectious bronchitis disease virus (Zhou et al. 2003, 2004) and avian influenza virus (D'Aoust et al., 2008).

Infectious bursal disease

Live attenuated vaccines are currently employed for controlling infectious bursal disease virus (IBDV) in poultry, but highly attenuated strains give only moderate protection, while less attenuated vaccines can produce disease in vaccinated chickens instead. The VP2 protein of IBD virus was expressed in *A. thaliana* and showed a serum antibody response in chickens when fed with leaf extracts of the transgenic plants (Wu et al. 2004). Subsequently, the plant-based vaccine was compared to a commercial vaccine given by ocular/nasal drop (Wu et al., 2004). All chickens vaccinated with commercial or plant-based vaccine showed immune response. 90% of the birds were protected in the orally vaccinated group and 60% in the group receiving the plant material subcutaneously. The commercial vaccine alone or with the oral plant vaccine boost provided approximately 80% protection. These studies are encouraging with respect to the potential to induce protective immunity by feeding transgenic plant material.

Miller et al. (2004) expressed the VP2 protein of IBDV in tobacco cell cultures and demonstrated of efficacy by challenge. In the study, birds received three doses of vaccine (days 7, 21 and 35) either via the subcutaneous, the oral gavage (non-adjuvanted) or via the intrabursal route of administration (non-adjuvanted). All vaccines delivered via the subcutaneous route of administration were protective against challenge except for the VP2 antigen formulated with drakeoil emulsion adjuvant. Those vaccines delivered via oral gavage or intrabursal were not protective.

Newcastle disease virus

Including the first FDA-approved plant-based vaccine made in tobacco cell culture (Cardinau et al., 2004), Newcastle disease virus has been the target of many recent studies for a plant-based vaccine. Newcastle disease virus (NDV) belongs to the family *Paramyxoviridae*. NDV is highly infectious, affecting domestic poultry and wild birds. Virulent forms of NDV cause devastating disease of poultry and are, therefore, economically important avian viruses. Virus surface glycoprotein F and HN epitopes have been displayed on the surface of Cucumber mosaic virus particles (Zhao & Hammond 2005; Natilla et al. 2006). Full length glycoproteins were expressed in transgenic potato (Berinstein et al. 2005) and tobacco (Hahn et al. 2007) leaves, and maize (Guerrero-Andrade et al. 2006) and rice seeds (Yang et al. 2007). Using stable transformation, Berinstein et al (2005) demonstrated NDV F or HN protein accumulation in transgenic potato leaves. Mice subsequently fed with the transgenic potato leaves developed NDV-specific IgG and IgA antibodies. *F* gene of NDV was produced in stable transgenic maize plants. Chickens were orally administered transgenic corn and displayed antigen-specific immune responses and were protected against NDV challenge (Guerrero-Andrade et al. 2006). Stable transgenic tobacco expressing HN of NDV was also produced. Immunogenicity of the transgenic tobacco was confirmed when chickens fed with lyophilized tobacco leaves displayed low HN-specific IgG immune responses (Hahn et al. 2007).

The hemagglutinin/neuraminidase protein (HN) of NDV was expressed in tobacco cell cultures and chickens were vaccinated with plant-cell-produced HN (adjuvanted or non-adjuvanted) at 2 days of age and 16 days of age via the subcutaneous, intranasal, oral gavage or oral gavage followed by feeding routes of administration (Cardinau et al., 2004). Birds were challenged 14 days after the second vaccination. One hundred percent of the non-vaccinated control birds died of NDV and 100% of the positive control birds (receiving native NDV subcutaneously) survived. Seventy five percent and 80% of the birds immunized sub-cutaneously with the plant-cell-produced HN vaccine (non-adjuvanted and adjuvanted, respectively) were protected, but no protection was observed (100% mortality) in case of the intranasal, oral gavage or oral feeding routes of administration. In a subsequent study, these researchers reported that 100% of the birds receiving adjuvanted 250 mg of plant-based HN and 60% of the birds receiving 20 mg non-adjuvanted plant-based HN were protected while 100% of the unvaccinated controls died post challenge. They used MPL–TDM adjuvant (Corixa, Inc) for this experiment (Cardinau et al., 2004).

Avian influenza virus

Several reports of plant-based influenza vaccines for animals and humans have been published. *Agrobacterium*-mediated transient expression of HA in *Nicotiana benthamiana* was demonstrated by D'Aoust et al. (D'Aoust et al., 2008). High accumulation (50 mg/kg) of virus-like particles (VLPs) made from HA antigen was observed. Mice immunized intramuscularly with two doses of 0.5 µg of the purified H5 VLPs were protected against H5N1 influenza virus challenge (D'Aoust et al., 2008). In a report, Shoji et al. (2008) described the transient expression of HA by agro infiltration of a tobacco mosaic virus (TMV)-based viral system in *N. benthamiana*. The purified plant-based HA elicited strong H5-specific immune responses in mice and displayed high hemaglutination inhibition and virus-neutralizing antibody titres. In a subsequent study, this plant-based HA showed full protection against challenge in immunized ferrets (Shoji et al., 2009).

Heat-labile holotoxin B from E. coli and E. coli mutant LT (LTA-K63/LTB)

Very recently, Miller et al. (2012) has reported the safety and immunogenicity of the mammalian mucosal adjuvants, *Escherichia coli* wild-type heat-labile holotoxin (LT) and *E. coli* mutant LT (LTA-K63/LTB) produced in a transgenic *Nicotiana tabacum* (NT-1) tobacco cell line (SLT102) in 1-day-old chicks and 10-day-old to 21-day-old broilers. These antigens were tested following parenteral and mucosal routes of administration. It was found that parenteral administration of *E. coli* recombinant wild-type LT did not have any adverse effect on bird health and was well tolerated at levels up to 400 µg per dose and the highest systemic LT-B-specific IgG titres were detected in birds that received three doses of SLT102-derived mutant LT by feeding. Their results demonstrate that *E. coli*-derived recombinant and wild-type LT holotoxins are not enteropathogenic and toxic in birds as those were shown to be in the mammalian species and these recombinant wild-type and mutant LT produced in transgenic NT-1 tobacco cells are safe and potent vaccine adjuvants in poultry.

Infectious bronchitis disease virus

Zhou et al., (2003, 2004) has successfully developed an edible potato-based vaccine against chicken infectious bronchitis virus (IBV). Sliced tubers expressing viral S1 glycoprotein were administered in three doses over two weeks. The chickens were challenged with IBV a week after the last administration. Orally immunized chickens developed a virus-specific antibody response and were protected against IBV.

1.2.5.2 Applications for cattle

Rindpest virus

Bovine rinderpest virus hemagglutinin was expressed in transgenic peanut and cows were fed three times with 5–7.5 g of transgenic leaf tissue. This oral vaccine was able to raise virus-specific antibodies, which also neutralized the virus in vitro (Khandelwal et al., 2003).

Bovine herpes virus

Immunogenic glycoprotein D of bovine herpes virus (BHV) was produced as a by-product in TMV (Tobacco mosaic virus)-inoculated tobacco plants, and the crude plant extract emulsified in oil and subsequently was injected into cows. The plant-based vaccine was able to raise specific humoral and cellular immune responses and the animals were protected against BHV to a similar level as cows vaccinated with the commercial vaccine (Perez Filgueira et al., 2003).

Foot and mouth disease virus (FMDV)

FMDV infects many meat- and milk-producing domestic animals, including cows. In an Argentinean laboratory, a vaccine against FMDV has been extensively developed. This vaccine is based on the viral structural VP1 protein, and expression has been reported in Arabidopsis (Carrillo et al., 1998), potato tubers (Carrillo et al., 2001), and alfalfa leaves (Wigdorovitz et al., 1999a; Dus Santos et al., 2002; Dus Santos et al., 2005). Alfalfa was chosen as a platform for oral delivery and protective immune response was reported in mice (Wigdorovitz et al., 1999a). The same authors have also developed a vaccine against bovine rotavirus infections. Epitopes of rotavirus VP4 protein were expressed with a TMV-based transient system (Perez Filgueira et al., 2004) and in transgenic alfalfa plants (Wigdorovitz et al., 2004). Immunogenicity was again determined in a mouse model. Most importantly, alfalfa-fed mice developed a virus-specific antibody response, with pups subsequently being protected against viral challenge by passive lactogenic immunity (Wigdorovitz et al., 2004).

Bovine pasteurellosis

A Canadian group has investigated a plant-based vaccine against bovine pneumonic pasteurellosis, ‘‘shipping fever’’ caused by *Mannheimia haemolytica*. Transgenic white clover (Lee et al., 2001) and alfalfa (Ziauddin et al., 2004) plants expressing a fragment of leucotoxin fused with green fluorescent protein (GFP) were generated, and the immunogenicity of this fusion protein was established in rabbits after intramuscular injection. The generated antibodies also neutralized a related leucotoxin in vitro. Recently, Dow

AgroSciences launched license agreement to produce the leucotoxin antigen it their plant-cell-based platform (Anonymous 2007a).

The same group produced the GS60 antigen of *Mannheimia haemolytica* A1 with sequences encoding a slightly shortened derivative of the GS60 antigen (GS6054) in white clover through *Agrobacterium tumefaciens* transformation (Lee et al., 2008). The antigen was purified from dried alfalfa plants and was shown to be immunogenic when injected intra-muscularly into rabbits. When the dried leaves were fed to rabbits it also induced immune response. The antigen was stable in dried transgenic plant material stored at ambient temperature for more than a year.

Bovine rotavirus

The expression of bovine rotavirus VP6 protein has been reported in transplastomic tobacco plants (Birch-Machin et al., 2004) and potato tubers (Matsumura et al., 2002).

Bovine diarrhea causing E. coli

The colonization factor of O157:H7 bovine diarrhea-causing enterohemorraghic *E. coli* (EHEC) was expressed in tobacco plants (Judge et al., 2004). Intimin was purified from plant extracts and injected intraperitoneally in mice. Alternatively, mice were fed the transgenic plant material. In the following EHEC challenge, reduced *E. coli* shedding was observed in the parenterally immunized group as well as in mice that had received an oral boost after being intraperitoneally primed (Judge et al., 2004).

Coliform Mastitis

Coliform mastitis is one of the most common forms of environmental mastitis in dairy cows (Hogan & Larry Smith 2003). Significant losses also occur in goats, sheep, and pigs. Important Gram-negative organisms in mastitis include *E. coli*, *Klebsiella pneumonia*, *Serratia marcescens*, and *Pseudomonas aeruginosa*. In the absence of effective vaccines, current control strategies rely heavily upon antibiotics and topical germicidal chemicals. The bovine CD14 antigen is a high-affinity receptor for the complex of lipopolysaccharide (LPS, endotoxin) and LPS-binding protein. A CD14 recombinant antigen was produced transiently in *N. benthamiana* (Nemchinov et al., 2006). The plant-based sCD14 was biologically active as demonstrated in vitro by induction of apoptosis and interleukin-8 production in bovine endothelial cells and in vivo in bovine bladders as shown by an increased leukocyte response in the presence of sCD14.

1.2.5.3 Applications for swine

Swine transmissible gastroenteritis virus (TGEV)

Development of a plant-based vaccine against porcine transmissible gastroenteritis virus (TGEV) has been carried out by several research groups. Neutralizing virus spike protein antigens have been expressed in the leaf tissue of transgenic tobacco (Tuboly et al., 2000) and Arabidopsis plants (Gomez et al., 1998), in potato tubers (Gomez et al., 2000), and in maize seeds (Streatfield et al., 2001 & 2002). The use of maize seeds as an edible delivery vehicle has been studied. The efficacy of a plant-based vaccine produced in transgenic maize seeds against TGEV has been presented in multiple experiments with piglets (Streatfield et al., 2001; Lamphear et al., 2002). In addition, it was found that the antigen was stable during storage in various conditions and authors were able to concentrate the antigen with milling techniques (Lamphear et al., 2002).

Enterotoxigenic E. coli (ETEC)

Enterotoxigenic *E. coli* expressing F5 fimbriae causes diarrhea in various farm animals, including pigs, chickens, and cows, whereas F4+ ETEC is pathogenic only for pigs (Van den Broeck et al., et al. 2000). The major subunit protein of F4 fimbriae have been expressed in the leaves of tobacco (Huang et al., 2003; Joensuu et al., 2004; Liang et al., 2006), alfalfa (Joensuu et al., 2006b) and in seeds of barley (Joensuu et al., 2006a) while the F5 fimbrial subunit was expressed in leaves of soybean plants (Piller et al., 2005; Garg et al., 2007). F4 subunit vaccine was shown to be immunogenic and partially protective after oral delivery to weaned piglets (Joensuu et al., 2006b). The immunogenicity of F5 ETEC vaccine was confirmed by vaccinating mice parenterally with crude leaf extracts (Piller et al., 2005). In addition to these candidate vaccines based on colonization factors, the expression of ETEC heat-labile toxin subunit B in plants has been widely studied. The LT-B encoding gene was expressed in transgenic plants including tobacco (Kang et al., 2005), lettuce leaves (Kim et al., 2007), potato tubers (Haq et al., 1995; Mason et al., 1998; Lauterslager et al., 2001), and maize (Streatfield et al., 2001; Chikwamba et al., 2002) and soybean seeds (Moravec et al., 2007). Orally administered Plant-based LT-B was able to protect mice against subsequent challenge with the LT-holotoxin and the immunogenicity of this antigen was also shown in humans (Tacket et al., 1998, 2004).

Foot and mouth disease virus in swine

VPI protein of the Foot and mouth disease virus (FMDV) in swine was expressed in plant using the chimeric virus particles (CVPs) approach (Yang et al., 2007). Several groups of two month old swine were immunized with different doses of plant-based VPI antigen by intramuscular injection and, six weeks later, boosted by the same route with the same amount. Four weeks after the boost all swine were challenged with the FMDV and monitored for symptoms. All of non-vaccinated animals showed serious symptoms of FMD, while all swine immunized with the CVPs showed no symptoms after the challenge (Yang et al., 2007).

Swine edema

Swine edema, also called gut edema is caused by certain serotypes of enterotossimic *Escherichia coli* (0138, 0139, and 0141), which can cause morbidity and mortality in piglets. F18 fimbriae produced by these *E. coli* strains have been reported to be an important virulence factor related to edema (Rippinger et al., 1995). Rossi et al. (2003) reported the isolation of the F18 fimbriae gene from genomic DNA of *E. coli* isolated from pigs that died of edema. The gene was cloned into plant transformation vectors, and transgenic tobacco seeds expressing up to 0.1% TSP of F18 adhesive fimbriae were produced. However, no animal tests were conducted in this study.

Porcine Pleuropneumonia

Actinobacillus pleuropneumoniae is the causative agent of porcine pleuropneumonia. ApxIIA is one of the virulence factors, which is a bacterial exotoxin. Lee et al., (2006) initially confirmed the immunogenicity of ApxIIA expressed in *Escherichia coli*. Then they developed transgenic tobacco plants expressing ApxIIA and tested its efficacy in mice against *A. pleuropneumoniae* infection after oral administration of the plant powder. They reported that protective immune responses were induced in mice after oral administration of the plant powder once a week for 4 weeks. The levels of antigen-specific immunoglobulin G against ApxIIA increased in mice that were fed a powder made from the transgenic plant compared to that in mice, which were fed wild-type tobacco. Additionally, mice fed the transgenic plant powder were protected from an injection of a lethal dose of *A. pleuropneumoniae*.

Min-Kyoung Shin et al., (2011) reported the production of full-size ApxIIA and a cholera toxin B (CTB)-ApxIIA fusion proteins in corn seed as subunit vaccine candidates. Both

antigens were observed to induce effective Apx-specific immune responses in forms of crude plant extracts in mice when injected subcutaneously.

All these studies performed on vaccine antigens produced in plants suggest the feasibility of plant-based vaccines either in injectable or oral forms in model animals.

1.2.6 Sub-cellular targeting: optimal yield and glycosylation

The sub-cellular environment of a plant cell-made recombinant protein influences its folding, assembly, and post-translational modification. Factors such as surrounding pH and presence of chaperones or proteases can affect antigen stability and therefore accumulation. Recombinant proteins can be directed to the secretory pathway of plants by an N-terminal signal peptide (Figure 1.1). Secreted proteins are co-translationally inserted into the endoplasmic reticulum (ER) and transported by default through the Golgi network to the apoplast. In the presence of a suitable C-terminal carboxy signal the protein may be retained in the ER or directed to the vacuole (Matsuoka & Nakamura, 1991) (Figure 1.1). If a protein is targeted to the apoplast, it can be retained by the cell wall matrix between the cell wall and cell membrane or secreted from the cell depending on the protein's size and structure. The ER is an oxidizing environment with an abundance of molecular chaperones and very few proteases. Comparative analysis with recombinant antibodies has shown that they accumulate more efficiently when targeted to the secretory pathway than to the cell cytoplasm (Schillberg et al., 1999). This has also been shown with vaccine antigens. Richter et al. (2000) targeted the synthesis of HBsAg to the apoplast or vacuoles and found 2- to 7-fold accumulation level enhancements compared with cytoplasm-targeted potato plants.

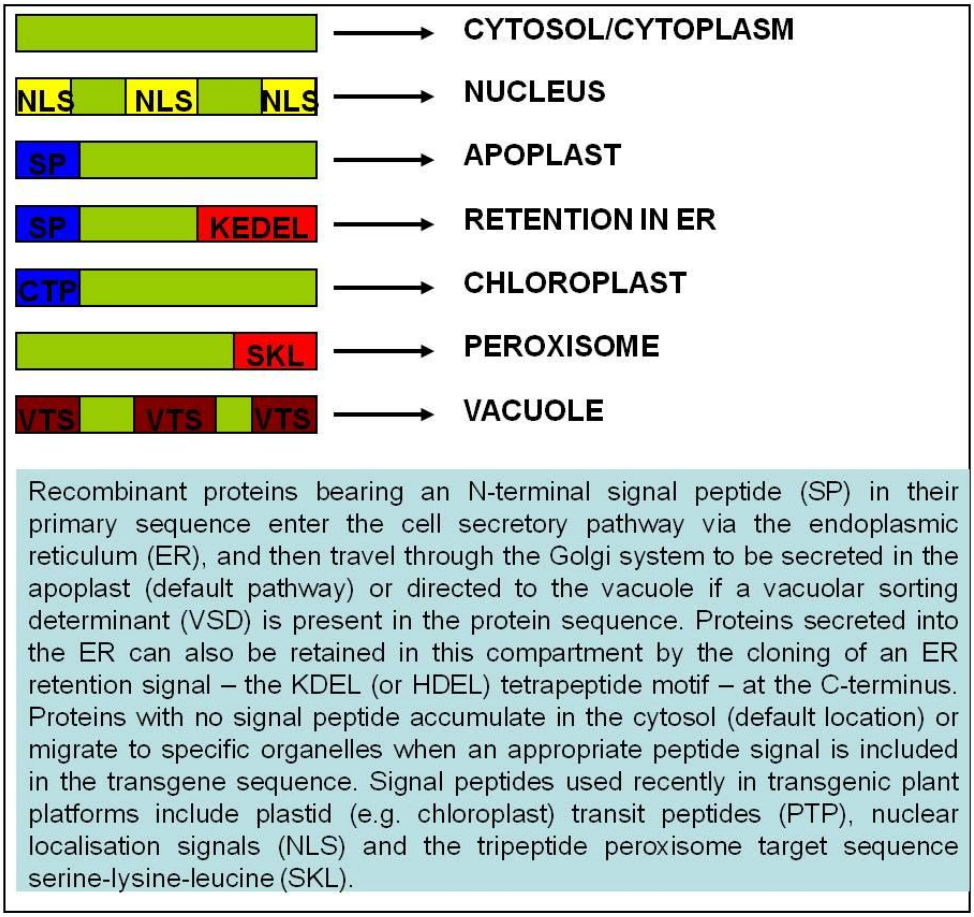

Figure 1.1 Sub-cellular localization of recombinant proteins in plant cells

Streatfield et al. (2003) studied the subcellular targeting of LT-B in maize seeds, and reported that targeting to the apoplast and vacuoles increased the expression level 3080-fold and 20,000-fold, respectively, compared to the cytoplasm. Retaining the recombinant protein in the ER though the use of a C-terminal (SE)H/KDEL peptide (Munro & Pelham, 1987) also enhances accumulation of recombinant proteins (Conrad & Fiedler, 1998). Similarly, a 4-fold increase in LT-B accumulation was shown in tobacco and potato plants when the protein was retained in the ER (Haq et al., 1996). In contrast, accumulation of LT-B in the ER of maize seeds resulted in expression of one-tenth of that when the protein was secreted (Streatfield et al., 2003). Recombinant proteins can also be targeted to organelles like the mitochondria and plastids. This can be done by adding N-terminal transit peptides, which are recognized by the organelle transport machinery delivering the proteins to organelles (Glaser & Soll, 2004). In maize seeds, targeting bacterial LT-B to the plastids led to a 7-fold increase compared with

cytosolic targeting, but did not reach the levels obtained with apoplast, ER, or vacuolar targeting (Streatfield et al., 2003). In contrary, FaeG, the major subunit of ETEC F4 fimbria accumulated higher levels in chloroplasts than cytosol, ER or apoplast (Van Molle et al., 2007). Optimal sub-cellular location of Plant-based recombinant protein therefore needs to be optimized on a case-by-case basis however studies indicate that location away from the plant cell cytosol is desirable.

Many proteins undergo some form of post-translational modification, such as the addition of carbohydrates, lipids or other functional groups. Glycosylation can greatly alter the surface of an antigen, thus affecting the shape of the antigen presented to the immune system (Ko et al., 2003). Many viral surface proteins are heavily glycosylated, whereas bacterial proteins are non-glycosylated. However, glycosylation can increase the antigenicity and stability of a protein (Faye et al., 2005). Glycosylation of a recombinant, plant-based antigen can be achieved by appropriate sub-cellular targeting. When glycosylation is desired, the antigen should be directed to the ER. The type of glycosylation can be affected by retaining the antigen peptide in the ER (simple, animal-like glycosylation) instead of being secreted through the Golgi apparatus (complex, plant-like glycosylation), where further carbohydrate groups will be added (Faye et al., 2005). Glycosylation can be avoided by targeting the accumulation of the antigen to the cytoplasm or to intracellular plant organelles such as the chloroplast or mitochondria. Alternatively, the addition of carbohydrates can be prevented by mutating the putative glycosylation sites on the antigen peptide. Mammals and plants have a similar structure of core high-mannose glycans, but some differences in glycosylation do exist. Plant glycans use a plant-specific α-1,3 fucose linkage rather than the α -1,6 fucose linkage found in mammals, have additional β-1,2 xylose linkages, and lack the sialic acid moieties typical of mammalian glycosylation (Faye et al., 2005). Completely mammalianized plant glycosylation has not been reported to date, but has been of a considerable interest (reviewed by Saint-Jore-Dupas et al., 2007).

PlpE, the outer-membrane lipoprotein of *P. multocida* was found to have several potential glycosylation sites in its amino acid sequence. There were 2 *N*- and 6 *O*-linked potential glycosylation sites identified by the software NetNGlyc1.0Server, Technical University of Denmark (Gupta et al., 2004; Julenius et al., 2005).

The biggest advantage of plant-based vaccines lies in the possibility of delivering plant-based vaccines orally (Streatfield, 2005). Oral delivery is attractive for its simplicity, and increases likelihood for local mucosal immune responses at sites of infection (Rigano et al., 2003). However, the amount of antigen needed for oral delivery is high when compared to parenteral administration (Joenssu 2008). Plant-based antigens both stably and transiently expressed in edible tissues/organs have been delivered orally and the efficiency of this immunization procedure has been evaluated, which was described in the previous pages. Though many groups have been working to make plant-based vaccines for human pathogens vaccines for animal pathogens have been able to demonstrate the most convincing proofs of efficacy for plant-based vaccines in general and for both oral and injectable versions (Rybicki, 2010). It seems to be possible that the first large-scale production of plant-based vaccines would be against pathogens of animals since it is easy to demonstrate the efficacy of the system in animal models and hosts as well as the regulatory pathway might be shorter for such vaccines as has been demonstrated by Dow with their Newcastle disease virus vaccine (Dow AgroSciences, 2008) (Rybicki, 2010).

1.3 Research Hypothesis

The research hypotheses for this project were as follows:

- "Plants can express PlpE."
- "Plant-based PlpE antigen is able to induce antibody responses in mice and chickens."
- "Plant-based PlpE vaccine is able to protect mice and chickens against *P. multocida* infections."

1.4 Aims of the Research Project

The aims of this research project were:

1. To optimize the transient expression of PlpE antigen in the leaves of *Nicotiana benthamiana* through *Agrobacterium tumefaciens* infiltrations
2. To generate stable transgenic NT1 cell lines of tobacco (*N. tabacum*) cell suspensions producing PlpE antigen
3. To immunize the mice and chickens with PlpE vaccines produced in *N. benthamiana* leaves and tobacco cell suspensions to determine the immune response
4. To perform challenge trials to determine whether the plant-based PlpE vaccines could provide protection in mice and chickens against *P. multocida* infections

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Chapter 2

Production of PlpE in plants

2.1 Introduction

Vaccination is an important part of health management in poultry industries. There are 3 main types of vaccines used in poultry flocks. Those based on live, attenuated or killed organisms (Thitisak et al., 1989). Most of these vaccines are generally fragile and heat-sensitive (Jayawardane et al., 1990). Heat-inactivation is a problem common to the delivery of any purified vaccine, human or animal, especially in tropical or subtropical countries (Spradbrow, 1992). The other concerns are administration procedures, which affects the efficacy of many vaccines and consequently the level of immunity the bird develops.

There are a number of ways that vaccines may be administered to poultry. Most methods of application require catching and handling of individual chickens (oral drop, eye-drop, intranasal application), which is time consuming and stressful to the birds and the vaccinator (Spradbrow, 1992). Vaccines that are administered by the intramuscular or subcutaneous route require well-trained staff and use of syringes and needles. Other methods involve administration via the drinking water or as an aerosol spray. However, poor distribution of live vaccine administered by mass application methods such as the water or spray route, may result in chickens being missed in parts of the house. In addition, relying on replication of the vaccine virus in chickens and then transmission of the vaccine from bird to bird is risky. This usually results in excessive reactions of long duration, increased intensity and delayed immunity in the flock. On the other hand missed birds or those vaccinated with killed and possibly attenuated vaccines results in chickens with no protection (Copland, 1987). Vaccination is also stressful. A bird inoculated with a live vaccine is actually being infected with a mild form of the disease and stress may reduce the chicken's ability to mount an effective immune response resulting in disease (Beard & Hanson, 1984). Therefore, a recombinant sub-unit antigen that is able to elicit sufficient protective immunity, while being

heat-stable, cost-effective and easily deliverable to mass poultry flock would be an ideal vaccine.

Fowl cholera, caused by the gram-negative bacterium *Pasteurella multocida* (Carter, 1967) is a severe disease causing significant economic losses to poultry industries worldwide (Boyce et al., 2004 & 2009). The pathogenicity and virulence factors behind this disease are still not well-characterized (Adler et al., 1996). The vaccines currently available against fowl choldera are bacterins (Boyce & Adler, 2001) that only provide protection against homologous serotypes, and live attenuated strains (Scott et al., 1999), which can cross-protect but may revert to virulence and cause mortality in vaccinated flocks (Glisson, 1998). Subunit recombinant vaccines cannot revert to virulence (Hansson et al., 2000) and also safe for the vaccine administering veterinary personnel. A cross-protective antigen that would provide efficient broad-spectrum protection is a prerequisite for developing a successful subunit recombinant vaccine.

Extensive efforts have therefore been made to identify cross-protective antigens of *P. multocida* (Adler et al., 1999; Hunt et al., 2000; Al-Hasani et al., 2007). Several of these antigens have been tested as candidates for a recombinant *Pasteurella* vaccine (Tatum et al., 2009; Lee et al., 2007; Sthimatee et al., 2008). However, PlpE, *Pasteurella* lipoprotein E, an outer-membrane antigen has been reported as the most efficient recombinant vaccine antigen (Wu et al., 2007b; Hatfaludi et al., 2012). This candidate antigen has been cloned from the strain *P. multocida* X-73 (serotype A:1) and expressed in *E. coli* cultures. In its soluble form, PlpE showed 100% cross-protection in chickens and 70% in mice, when injected subcutaneously (Wu et al., 2007b). However it is difficult to produce this protein from *E. coli* in the soluble form. The insoluble form of PlpE also provided 100% homologous protection in chickens and 70% in mice (Hatfaludi et al., 2012), when injected subcutaneously. However it was not as successful with heterologous strains (personal communication with John Boyce). There is still need for more effective recombinant vaccines capable of protecting against heterologous strains to control this disease.

It is widely believed that *P. multocida* infections are initiated at mucosal surfaces, specifically the lungs and upper respiratory tracts of the hosts. It is also believed that active immunity mounted against this disease is mainly humoral (Harper et al., 2006). While all the commercial vaccines available against fowl cholera are injectable, mucosal vaccines that

increase ease of delivery to large flocks of poultry and that induce a broad immune response (systemic and mucosal) may result in protection (Ogra et al., 2001) while being easier to deliver. Therefore, a recombinant subunit vaccine consisting of one or more antigenic epitopes or proteins, delivered to the mucosal immune system may provide more efficient and effective vaccines against fowl cholera.

Commercial recombinant subunit vaccines are produced in mammalian, yeast or insect cell cultures. These vaccines are heat-sensitive and require expensive media, complicated purification steps and/or parenteral delivery (Heermann et al., 1984; Levine & Stzein, 2004). The production of vaccines in plants eliminates the need for expensive fermentation, cold storage (Langridge 2000; Sparrow et al., 2007) and overcomes the risk of contamination with endotoxins and mammalian pathogens (Fisher et al., 2004). These characteristics of plant-based vaccines simplify vaccine delivery and reduce the cost of a vaccination program (Alvarez et al., 2006). Additionally, plants can correctly fold and assemble complex proteins and can perform post-translational modifications such as glycosylation, which cannot be achieved with bacterial fermentation (Streatfield & Howard, 2003; Streatfield 2005).

Foreign proteins can be expressed either transiently or stably within plant tissues. Transient expression does not involve the insertion of the gene(s) of interest into the host genome and the gene is therefore not carried through to future cell generations. Transient expression is detectable 2 to 12 days post transformation and generally lasts for 14-20 days (Marillonnet et al., 2005; Voinett et al., 2003). The gene(s) of interest are either engineered into a plant virus that transmits the DNA or RNA into the plant cell or transformed into the Transfer region of *Agrobacterium* that is then infiltration into the intra-cellular spaces of plant tissues. Stable transformation involves the insertion of the gene(s) of interest into the host nuclear of plastid genome and continuance of the gene into the following cell generations.

In general, transient production of foreign proteins in plants is a fast and flexible system that results in accumulation of high content of targeted proteins. But this ectopic protein expression might become limited and cease upon the onset of Post translational gene silencing or PTGS. PTGS is an antiviral plant defence system, which is a nucleotide sequence-specific RNA turnover mechanism. This is highly conserved among most eukaryotes (Hammond et al., 2000). It is developed in plants as an adaptive system targeted against viruses and serves as a sequence-specific viral genome inactivation system. As a

counter-defence strategy, most plant viruses have evolved proteins that suppress various steps of this plant defence mechanism. A system is available based on co-expression of a viral-encoded suppressor of gene silencing, the p19 protein of tomato bushy stunt virus (TBSV) that prevents the onset of PTGS in the infiltrated tissues and allows high level of transient expression (Voinett et al., 2003).

Vaccine antigens produced in plants either transiently or stably can be delivered orally within fresh and raw plant parts or as minimally processed vaccine diets (Ruf et al., 2001; Thanavala et al., 2005; Webster et al., 2006; Pelosi et al., 2011). The rigid walls of plant cells are predicted to protect antigenic proteins from the acidic environment of the stomach, enabling intact antigen to reach the gut-associated lymphoid tissue (Walmsley et al., 2003; Pelosi et al., 2011). Studies have proven plants capable of expressing many different antigens simultaneously or individually including viral, bacterial, enteric and non-enteric pathogen antigens as well as autoimmune antigens all of which have demonstrated immunogenicity when tested and some have been shown to provide protection in model and target animals (Carillo et al., 2001; Kong et al., 2001; Lee et al., 2001 & 2008; Lee et al., 2006; Rossi et al., 2003; Nemchinov et al., 2006; Santi et al., 2006). Plant-based vaccines have also proven to be safe in human clinical trials (Haq et al., 1998; Tacket et al., 1998, 2001).

Veterinarian vaccines are often thought a particularly desirable target for the plant-based vaccine platform since the advantages of plant-based vaccines for vaccination of humans are also advantageous for veterinary immunization programs. In addition, the potential to deliver plant-based vaccines mucosally greatly increasing ease of delivery to large flocks and herds. It is also proposed that the regulatory hurdles relating GM crops are expected to be less for veterinary purposes (Rybicki, 2010; Santi, 2009; Joensuu et al., 2008). In fact the first plant-based vaccine to be approved for commercialization was a veterinarian vaccine, a poultry vaccine for Newcastle Virus (Cardinau et al., 2004). Newcastle disease virus belongs to the *Paramyxoviridae* family (Lamb et al., 2005) and is a highly infectious virus, affecting domestic poultry and wild birds world-wide (Alexander 1988). Hemagglutinin–neuraminidase protein (HN) of NDV was expressed in tobacco cell culture. The chickens were delivered partially purified, plant-based HN antigen subcutaneously and were challenged with a highly pathogenic strain of the virus. The vaccine provided 95% over-all protection in the chickens (Mihaliak et al., 2005) and was given a license for

commercialisation by the USDA in early 2000. This case study demonstrated that PMVs could be developed within the existing regulatory framework.

Plant cells can co-express multiple transgenes and have proven safe when delivered mucosally in animals. Tobacco cell lines provide a quick and convenient model system for stable plant cell transformation. It is also the system that was used in the USDA approved plant-based Newcastle Disease vaccine. Two tobacco cell lines commonly found in cell culture are BY2 (Bright yellow 2) and NT1 (*Nicotiana tabacum* 1) (Mayo et al., 2006). The lines, derived from *Nicotiana tabacum*, have lost the ability to regenerate. *Agrobacterium* is routinely used to transform the cells while in liquid form, and then transgenic callus is regenerated from a lawn of cells plated on solid medium (Mayo et al., 2006). The features of transgenic lines are that (i) they can be identified quickly (5–8 weeks depending on the selective agent used) and amplified in liquid culture for larger batches; (ii) harvest of transgenic cells can start 1 month after seeding the culture and can continue weekly thereafter; (iii) containment is inherent; (iv) culture conditions are closed so good laboratory and manufacturing practices (GLP and GMP, respectively) are easily applied; (v) the lines have low alkaloid content so can be used for oral delivery; and (vi) the media used is inexpensive. However, gene silencing may occur (although the frequency of this is decreased by selecting lines with low transgene copy numbers) and some high expressing lines are slow growing (Rigano & Walmsley, 2005).

A project was therefore developed to produce PlpE in plant production systems as a recombinant vaccine that may prove suitable for oral delivery. In this chapter of the thesis, the expression of *plpE* in plant cells is been described. In order to overcome PTGS in expression of *plpE*, a vector carrying the *p19* protein-suppressor of gene silencing was co-expressed with the pBinPlpE vector when transiently expressed.

2.2 Materials and methods

2.2.1 Construction of a plant binary vector carrying *plpE* coding region

All bacterial strains and clones were grown in Luria-Bertani (LB) broth (Bertani, 1952) or on LB agar. Antibiotics were added as needed for selection at the following concentrations: ampicillin, 100µg/ml; kanamycin, 50µg/ml; and carbenicillin, 100µg/ml. Plasmid DNA was isolated by the Miniprep procedure (Qiagen, Valencia, Calif.). Restriction enzymes were purchased from New England Biolabs (Beverly, Mass.), and restriction enzyme digested

DNA fragments were purified by agarose gel electrophoresis and eluted from the gel with Geneclean spin columns and reagents (Bio 101, Carlsbad, Calif.). T4 DNA ligase was purchased from U.S. Biochemicals (Cleveland, Ohio). PCRs were performed with AmpliTaq (Roche, Branchburg, N.J.) using the primers described in Table 2.1.

All restriction digests were carried out in the appropriate buffer at 37^{o}C. The cloning of pBinPlpE was performed by Dr Keith Al-Hasani using the primers described in Table 2.1 and a three way ligation. The nucleotide sequence for the *plpE* gene was amplified by PCR from the Gateway cloning vector pDEST 17 using the *plpE*-plant primer pair with BamHI/KpnI sites (Table 2.1). The primers were designed to remove the native bacterial N-terminal signal peptide of 20 amino acid residues from the native *plpE* gene. LTA signal peptide was excised using an NcoI/BamHI double digest. The pTH210 plasmid (Mason et al., 1998) was digested with NcoI/KpnI restriction enzymes and the *plpE* fragment together with the LTA signal peptide was ligated into pTH210 downstream of *CaMV 35S* Promoter (Table 2.1). The pTH210 plasmid was digested with EcoRI/HindIII restriction enzymes. The 1028 kb fragment was then cloned into the EcoRI/HindIII sites of the plant binary vector pGPTV-Kan$_r$ (Becker et al., 1992). The resulting binary plasmid, pBinPlpE was transformed into *E. coli* cells by heat shock and one colony selected after colony PCR and used for glycerol stocks for storage (Figure 2.1). Plant binary vectors were isolated from *E. coli* cells by plasmid mini prep (Invitrogen) and transformed into *Agrobacterium tumefaciens* strain LBA 4404 by electroporation. The presence of pBinPlpE in *A. tumefaciens* colonies was checked by colony PCR and restriction digest.

Table 2.1 Bacterial strains, plasmid vectors and primers used in the cloning experiment

Bacterial Strains	**LBA 4404**	***Agrobacterium tumefaciens* plant transformation strain**
	DH5α	***E. coli* cloning strain**
	BL21 codon plus	***E. coli* strain**
Plasmid vectors	pGPTV- Kan$_r$	Plant binary vector with *nptII* gene for both bacterial and plant selection
	pTH210	Plant expression cassette
	pDEST17	Gateway cloning vector
	pPSp19	plasmid containing *p19*

Primers	*plpE*-Plant Forward: *plpE*-Plant Reverse:	CGCGGATCCAGCGGCGGTGGCGGTAGCGC CGGGGTACCTTATTGTGCTTGGTGACTTTTTTC
	LTA signal peptide primers*:*	CATGGTGAAGAACATCACCTTCATCTTCTTCATCCTCTTGGCA AGCCCACTCTATGCAAACG CACTTCTTGTAGTGGAAGTAGAAGAAGTAGGAGAACCGTTCG GGTGAGATACGTTTGCCTAG
	nptII forward: *nptII* reverse:	AAGATTATACCGAGGTATG CATTAGTCCATGCAAGTTT
	Sequencing primers:	**1**. CGCCATTCAGGCTGCGCA 2. CAAGGCGATTAAGTTGGGTA 3. AGGAGGTTTCCCGATATTAC 4. AAGTCTCAATAGCCCTCTG

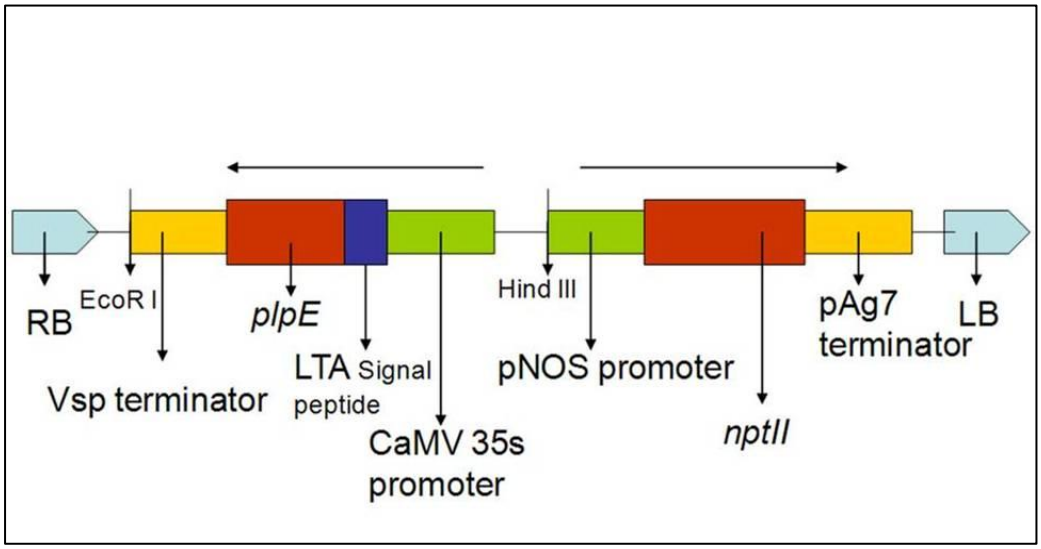

Figure 2.1 pBinPlpE Plant Binary Vector: where RB & L designate the right-hand and left-hand border of T-DNA, EcoR I & Hind III the respective restrictions sites, *plpE* the gene for *P. multocida* expressing Lipoprotein E, Vsp the vegetative storage protein terminator, LTA the plant-specific N-terminal signal peptide, CaMV35s the 35s promoter from cauliflower mosaic virus with a double enhancer, pNOS the promoter for the nopaline synthase gene from *Agrobacterium tumefaciens*, nptII the neomycin phosphor-transferase II gene, pAg7 the terminator (the 3' non-translated sequence of transcript 7 gene, which directs polyadenylation) from *Agrobacterium tumefaciens*.

2.2.2 Sequencing of the plant binary vector pBinPlpE

The pBinPlpE plasmid was sequenced to verify presence of the *plpE* and *nptII* genes and that no mutations were introduced by PCR and cloning (Appendix 1). DNA sequences were determined on an Applied Biosystems 3730S Genetic Analyser and analysed with Sequencher Version 3.1.1 (GenCodes, Ann Arbor, MI, USA).

2.2.3 Transient expression of pBinPlpE in *N. benthamiana* leaves through *A. tumefaciens* infiltration

2.2.3.1 Agrobacterium-infiltration of individual leaf by hand injection

Three *Agrobacterium* cultures, one transformed with pBinPlpE, a second with pPSp19 and an empty vector control were grown shaking in 10 ml liquid LB medium with kanamycin (50 mg/l) for 48 hours at 28°C until *plpE* and *p19* constructs reached OD600 of 0.5-0.6 and 1.0 respectively. The cultures were spun down to collect the pellets. The pellets of pPSp19 cultures were re-suspended in a 20 mM MES, 200 µM acetosyringone solutions. These resulting suspensions were then used to re-suspend the pBinPlpE or empty vector pellets. The mixed *Agrobacterium* suspensions were kept at room temperature (RT) for 1½ hours till OD600s reached around 1.5-1.6. *Nicotiana benthamiana* plants of 10-12 weeks of age were selected with moderately large and fully expanded leaves. The injection sites were at least 1 cm out from the mid-vein; and equally spaced between major lateral veins and the lower epidermis. These sites were carefully nicked with a 20G syringe needle being careful not to penetrate the upper epidermis. A 1 ml syringe without needle attached was filled with the *Agrobacterium* suspension and air bubbles removed. Supporting the leaf behind the nick with a gloved finger, the tip of the syringe was placed firmly against the leaf nick. A constant pressure was applied to the plunger. A dark patch was seen spreading away from the injection site, which indicated leaf infiltrated areas. The infiltration did not proceed past a major vein. The injected areas were marked with a permanent marker. The infiltrated plants were kept on a lab bench with plenty of light and watered regularly (Figure 2.2 A). The marked areas on the infiltrated leaves were excised 5 days after infiltration. The fresh leaves were either processed for the western blot analysis or kept in a freeze-drier for 48 hours before powdering and pooling. The resulting freeze-dried leaf powder was stored at RT.

Figure 2.2 Transient production of PlpE in *N. benthamiana* plants through *Agrobacterium* infiltration. A. In the lab through hand injected infiltration: small holes were made on the lower surface of leaf with needle; the *Agrobacterium* suspension was pushed in with a syringe without needle; plants were kept in the lab bench for 5 days with regular supply of water and light after infiltration. B. In the green-house using a vacuum infiltration device: The areal part of a plant was immersed upside down in 4 litre *Agrobacterium* suspension and then placed inside the vacuum device; plants were kept in the green-house for 5 days with regular supply of water and light after infiltration.

2.2.3.2 Agrobacterium-infiltration of whole plant using vacuum pressure

A vacuum infiltration device set up in the greenhouse was used for this process (Figure 2.2 B). *Agrobacterium* cultures for pBinPlpE, empty vector control and pPSp19 were grown according to the same procedure mentioned above except the volumes were 5 litres each and

the final volume of the co-infiltration suspension (pBinPlpE+ pPSp19/empty vector control+ pPSp19) was made up to 4 litres, with a final OD600 of 1.5-1.6. Entire *N. benthamiana* plants 10-12 weeks old were inverted so that their aerial portion was submerged in 4 litres of *Agrobacterium* suspension before being inserted into the vacuum infiltration device. A vacuum was pulled to 25 mm Hg for 30 sec, and then quickly released. The plants were kept in the greenhouse and watered regularly (Figure 2.2 B). The leaves were harvested five days after infiltration and freeze-dried for 48 hours. The freeze-dried leaves were ground using a 350 ml mini chopper (Homemaker, Kmart) and a pooled batch of powder was prepared. The powder was stored at RT.

2.2.4 Extraction of protein from *N. benthamiana* plants

2.2.4.1 Fresh leaves

Two millilitre eppendorf tubes were numbered and weighed. The same eppendorf tube was used to collect leaf discs from the infiltrated area just simply by puncturing the leaf area with the tube caps. Two discs per tube were collected and the tubes then weighed again after collecting the materials. 200 µl of phosphate buffered saline (PBS, 1X) with pH 7.4 was added to each tube. The leaf discs and the buffer were homogenized using a table-top vortex at maximum speed for 30 seconds with one 6.4 mm diameter, ceramic bead (Qbiogene, Carlasbad, CA). The homogenized solution was centrifuged at 16,438 *g* at 4°C for 10 minutes. The clear supernatant was collected into a clean tube avoiding the green pellets as much as possible and the centrifugation was repeated. The final clear supernatant was collected in a fresh 1.5 ml eppendorf tube. Everything was performed on ice. The samples were either stored at -20ºC or used directly for Western blot analysis.

2.2.4.2 Freeze-dried, pooled leaf powder

Crude protein was extracted in 2.0 ml eppendorf tube by homogenizing 20 mg freeze-dried leaf powder in 200 µl extraction buffer (1X PBS) with one 6.4mm diameter ceramice bead (Qbiogene, Carlasbad, CA) for 30 seconds in a table-top vortex at maximum speed. Insoluble material was removed by two centrifugation steps at 16,438 *g* at 4°C for 10 minutes. The clear supernatant was transferred to a 1.5 ml eppendorf tube and used for Western blot and ELISA experiments.

2.2.3 Production of PlpE in stable transgenic cell lines of NT1 (*Nicotiana tabacum*) cultures

2.2.3.1 Preparation of *Agrobacterium* culture

Agrobacterium was streaked from glycerol stock onto an LB plate with kanamycin (50 mg/l). The plate was incubated at 30°C for 48 – 72 hours (until colonies grew approximately 1mm in diameter). 3 ml YM medium containing 50 mg/l kanamycin was inoculated with a single colony and incubated slanted, in a 30°C shaking incubator at 250 rpm for two days or until OD_{600} of 0.5-0.6.

2.2.3.2 Preparation of Plant Cell Material

One-week-old NT-1 suspension culture was sub-cultured by transferring 2 ml of old NT-1 culture into 40 ml of fresh NT-1 medium (Mayo et al., 2006) and left in the dark at 23 to 25°C on a gyratory shaker at 100 rpm for 3-4 days.

2.2.3.3 Transformation

Agrobacterium culture was grown until it reached an OD600 of 0.5-0.6. Fresh acetosyringone stock was added to the 3-4-day-old NT-1 cell culture: 1 µl of a 20 mM stock solution made in 70% ethanol was added for each ml of cell culture. NT-1 cells were abraded by repeatedly pipetting (20 times) with a 10 ml-wide bore, sterile disposable plastic pipette. 100 µl of *Agrobacterium* culture was added to each of the Petri dishes (60 x 15 mm) containing 4 ml of the abraded NT-1 cell culture. Cells without *Agrobacterium* served as a non-transformed control. The plates were wrapped in parafilm and incubated in the dark on a shaker at 100 rpm at 23 – 25°C for 3 days.

2.2.3.4 Selection of transformants

The cells were transferred to a 50 ml conical centrifuge tube and diluted to a final volume of 20 ml using NTC medium (NT-1 medium containing 500 mg/l carbenicillin) (Mayo et al., 2006). The suspension was mixed gently and the cells were spun down at 112 *g* for 10 minutes in a centrifuge equipped with a swinging bucket rotor. The supernatant was pipetted off and the cells were resuspended in NTC medium. This washing step was repeated 3 times. After the final wash the cells were resuspended in 6 ml NTC medium. 2 ml of cells were placed on each of NTCK300 medium plates (150 x 15 mm disposable Petri plates) containing

NT-1 medium supplemented with 8 g/l Agar, 500 mg/l carbenicillin and 300 mg/l kanamycin. Plates were left open in the hood to allow excess liquid to evaporate and then wrapped with parafilm and cultured at 23-25°C in the dark. After 6 - 8 weeks transformed callus appeared (Figure 2.7 A). The calli were selected and subcultured to fresh NTCK300 plates. After 12 weeks the transformed callus materials were collected and used for screening by PCR, Western blotting and SDS-PAGE analysis. Small fragments (1.27 cm) of the transformed callus tissue was added into 40 ml liquid NT1 medium with kanamycin (250 mg/l) and carbenicillin (300 mg/l) in 250 ml flask and incubated in the dark on a shaker at X rpm at 23–25°C for cell suspension culture. The cell suspensions were sub-cultured every 7 days (Figure 2.7 E & F). Once cell lines were established (produced thick solutions the consistency of apple sauce after fourteen days of culture), the cells were harvested 14 days after the last subculture using a vacuum in a Bucher funnel lined with a Whatman No.1 filter disc. NT1 cells were freeze-dried for 96 hours and then made into a pooled batch of cell suspension powder.

2.2.4 Genomic DNA extraction from the transformed callus tissue using Edwards Prep (from Edwards et al., Nuc. Acids Res. 19: 1349)

Tissue (0.03 g) from individual callus was collected in a 1.5 ml eppendorf tube and ground with a disposable plastic pestle (Sigma "Pellet Pestle") for 10 to 15 seconds. The materials were always kept on ice. 400 µl of Edward buffer (200 mM Tris-HCL, pH 7.5, 250 mM Nacl, 25 mM EDTA, pH 8, 0.5% SDS, dH_2O) was added and ground briefly to get tissue off the pestle. The mixture was homogenized by vortexing for 5 seconds and then spun down for 5 minutes in a table top micro centrifuge at 16,438 *g* 300 µl of supernatant was transferred to a fresh tube and 300 µl of isopropanol was added. The extract was mixed gently and left at room temperature for 2 minutes, then spin for 5 minutes in a table top micro centrifuge at full speed and the supernatant decanted. 300 µl of 70% ethanol was added, mixed briefly and centrifuged for 5 minutes in a table top micro centrifuge at full speed. The tube was decanted and left at 37ºC for 1 hour so that the tubes could become completely dried. 40-60 µl of double distilled water was added to each tube to dissolve the DNA and kept the tubes in a fridge over night for better solubility of DNA in the solution.

2.2.5 PCR reactions

PCR amplification [95°C - 5min; 30 cycles of 94°C - 15s, 55°C (for *plpE and virD*, for *nptII* the temp. was 52 °c) - 30s, 68°C - 3 min; finally 68°C - 7 min] of DNA was performed using *Taq* DNA polymerase (Roche Diagnostics GmbH). The oligonucleotides used in this study are listed in Table 2.1. DNA sequences were determined on an Applied Biosystems 3730S Genetic Analyser and analysed with Sequencher Version 3.1.1 (GenCodes, Ann Arbor, MI, USA).

2.2.6 Extraction of protein from the callus & pooled batch of freeze-dried cell suspension powder of NT1 (*Nicotiana tabacum*) cells

Under sterile conditions 0.3 g of material was collected from each callus into a 1.5 eppendorf tube. 100 µl of PBS (1X) pH 7.4 was added to each tube and kept on ice throughout the extraction. The callus and the buffer were homogenized by vortexing for 1 minute with ceramic beads. The homogenized solution was centrifuged for 10 minutes at 16,438 *g* at 4ºC. The clear supernatant was collected into a clean tube avoiding the pellets as much as possible. This extract was either used for protein analysis or stored at -20°C for future use. The same procedure was followed to extract the protein from the dried cell suspension powder except 20 mg of powder was extracted in 500 µl PBS buffer pH 7.4.

2.2.7 Purification of PlpE from *E. coli* cells

The purification of insoluble, *E. coli* PlpE was performed according to Hatfaludi et al. (2012). The recombinant PlpE was analyzed by visualization of the protein using SDS-PAGE gel stained with Coomassie blue and Western blot. The concentration was quantified using Bradford assay (Biorad, CA, USA).

2.2.8 Production of anti-PlpE polyclonal antibody in rabbits

Two 8-10 weeks old female rabbits of New Zealand variety were supplied by Monash Animal Services (Ethics number: SOBS/M2008/02). Rabbits were kept for one week to acclimatize and provided with water, standard food pellets, hays and were monitored daily for health and condition. The animals received a 150 µg dose of insoluble *E. coli* PlpE at days 0, 15 and 28 through intradermal injections. Alhydrogel® was added as an adjuvant as 20% (v/v) of the total volume. Blood was collected from a vein in the ears at day 0 to separate the pre-immune sera. The total blood was collected at day 43 by cardiac puncture under

Nembutal anaesthesia. The animals were killed humanely on the same day by CO2 inhalation. Rabbit sera were separated from whole blood by clotting at 4°C over-night. The samples were spun down the next day in a table-top centrifuge at 4,766 *g* for 20 min at room temperature. The clear supernatant was transferred to fresh eppendorf tubes. The spinning was repeated once and the final clear sera were collected. All samples were stored at -20°C.

2.2.9 SDS-PAGE

2X SDS gel loading buffer (300mM Tris–HCl, pH 6.8, 600mM dithiothreitol, 12% SDS, 0.6% Bromophenol Blue, 60% glycerol), was added to crude plant extracts or insoluble *E. coli* PlpE and heated for 10 minutes at 99°C. The samples were run on a protein gel in 1X PAGE running buffer (10X Tris:Glycine buffer, 10% SDS). The proteins were separated on a 12% Precast Gel (Ready Gel Tris.HCl, Bio-Rad Laboratories) for 10 min at 100V and 50 min at 200V. After the proteins were separated, the gel was immersed in Coomassie blue staining solution (Coomassie Brilliant Blue R250 staining, 40% methanol, 7% acetic acid) with gentle rocking for 4 hours. The gel was then immersed over-night in de-staining solution (40% methanol, 7% acetic acid). After de-staining the gel was rinsed in distilled water and kept in fixer solution for 15 min with gentle rocking. The gel was then placed in between wet cellophane (Biorad) on a glass sheet and air-dried over-night.

2.2.10 Western blot

The separated proteins were transferred onto PVDF membrane (GE Healthcare) using a trans-blotting buffer (10X Tris:Glycine buffer, methanol, dH_2O) at 100V for one hour at RT. The membrane was then blocked with 2.5% skim-milk TBS-Tween buffer (25 mM Tris, 192 mM Glycine, 0.05% Tween-20, pH 7.5) for 1 h at room temperature on a rocking platform. The membrane was washed for 3 times (5 min each time) in TBS-Tween buffer with agitation. The membrane was then incubated with a polyclonal chicken (kindly provided by Dr. Keith Al-Hasani, Department of Microbiology, Monash University) or rabbit antibody raised against PlpE diluted at 1:200 or 1:400 in 2.5% skim milk TBS-Tween buffer on a rocking platform over-night at RT. The membrane was incubated with horseradish peroxidase conjugated, goat polyclonal, IgG antibody against chicken or goat polyclonal IgG antibody against rabbit (Sigma–Aldrich, Saint Louis, MO) for 1 hour at 37°C on a rocking platform after 4 washes (4 min each time) followed by a 5 min wash in TBS-Tween buffer. The antibody was diluted at 1:1000 in 2.5% skim-milk TBS-Tween buffer. The membrane was

then washed in TBS-Tween buffer for 4 times (5 min each time) followed by a 5 min wash in TBS buffer with agitation. 15 µl of 30% Hydrogen peroxide solution was added to 25 ml of 1X TBS buffer. 5 ml of 98% methanol was added to 0.015 mg 1, 4-chloronaphthol. This was mixed with the previous solution and finally added to the membrane to detect the protein bands. The membranes were kept on shaker with gentle rocking at 37ºC for 2-5 minutes.

2.2.11 ELISA

2.2.11.1 Direct binding ELISA for generating a standard curve for purified *E. coli* PlpE

High bind polystyrene EIA/RIA 96-well microplates (Corning, NY) were coated with10 µg/ml, purified PlpE from *E. coli* in PBS and incubated overnight at 4°C. After three washes with PBS-Tween with agitation (Phosphate buffered saline, pH 7.4, plus 0.05% Tween-20), plates were blocked with 5% skim milk powder in PBS-Tween and incubated for 2 hours at RT with agitation. After three washes with PBS-Tween, a rabbit anti-PlpE polyclonal antibody was added to the first rows of the plates at a dilution of 1:1000 and then serially diluted two-fold using skim-milk in PBS-Tween. The plates were then incubated for 1 h at 37°C with agitation before being washed three times with PBS-Tween and incubated with horseradish peroxidise (HRP) conjugated goat anti-rabbit-IgG (Sigma–Aldrich, Saint Louis, MO), at a dilution of 1:10000 in blocking buffer (0.05% skim-milk powder in 1X PBS), for 1 h at 37°C. After washing four times with PBST, the detection was performed using TMB peroxidase substrate (Bio-Rad, Hercules, CA) following manufacturer's instructions for 15 min. The reaction was stopped with 1N H_2SO_4 and then the absorbance was read at 450 nm (Figure 2.11) using the Microplate Reader Thermo Max (Molecular Devices Inc., Sunnyvale, CA).

2.2.11.2 Sandwich ELISA for plant-based PlpE

A PlpE-specific sandwich ELISA was developed. 96-well, microtitre plates were coated with 50 µl per well of chicken-anti PlpE polyclonal antibody (1:200) in phosphate buffered saline (PBS) with 0.05% Tween 20 (PBST). The plates were sealed and incubated overnight at 4°C. Plates were blocked with 5% skimmed milk PBS-Tween for 2 hours at RT with agitation. A volume of 100 µl of crude plant extracts prepared from the freeze-dried leaf powder or cell suspension powder and bacterial positive control was added per well to the first row of the plate. Samples and control were diluted to 1:10 or 1:50 using PBS and the plates were

incubated at 4°C overnight. All subsequent incubations were performed at 37°C with gentle rocking and the plates were washed in PBS-Tween three times after each incubation step. The polyclonal rabbit anti-PlpE antibody (100 µl of 1:1000 dilutions in PBS) was added to each well and incubated for an hour. Binding of this antibody was detected using a goat anti-rabbit IgG conjugated with horseradish phosphatase (100 µl of 1:5000 dilutions in PBS) by further incubation for an hour. Detection was performed using TMB Peroxidase EIA Substrate kit (Bio-Rad, Hercules, CA) according to the manufacturer's directions. The amount of PlpE expressed in the leaf or cell suspension extracts was calculated by reference to the standard curve constructed by the direct binding ELISA using purified PlpE from *E coli* (Figure 2.11). Quantified ELISA data was converted to milligram of PlpE per kilogram of dry weight (Figure2.12).

2.2.12 Use of HPLC for Pyridine alkaloid identification and quantification in tobacco tissues (Based on the method described by Saunders & Blume, 1981)

2.2.12.1 Extraction procedure

0.02g of freeze-dried leaf powder was ground in 1ml of extraction buffer (50% HPLC grade Methanol, 1M HCL, MilliQ H_2O) for a minimum of 90 sec at a speed of 2.5 using a Polytron ® 1200C homogenizer. The homogenate was transferred to a microcentrifuge tube and centrifuged at 875 g for 3 mins. The supernatant was filtered through a 0.45µm Millipore or Acrodisc ® disposable filter using a 1mL disposable syringe to remove fine particulate matter from the supernatant. For immediate use, the filtered samples were kept on ice in the dark, for longer term storage the samples were stored at -20°C until analysis using HPLC.

2.2.12.2 HPLC analysis

300 µL of sample and 100 µL of each standard were loaded into the relevant plastic inserts for samples and standards. The carousel cover was kept over samples at all times as alkaloids are light sensitive. Buffers (50% HPLC grade Methanol, H_2PO_4, MilliQ H_2O, pH 7.25 with triethylamine) were vacuum-filtered through 0.45µm regenerated cellulose Satorius® filter paper before using in HPLC analysis. 50% HPLC grade methanol was filtered first to avoid contamination. Analysis was carried out using a Varian HPLC with manual injection. The system comprises a Varian 9012 pump, a Varian 9050 U.V-Vis detector, and a DELL computer. The HPLC column used was a reversed-phase C18 (250 x 4.6 mm, 5 µm, ODS2)

from Waters. Data acquisition was done with Varian Star Software. Chromatographic separation and analysis of the alkaloids were done using a reversed-phase Nova-Pack C18 (250 x 4.6 mm, 5 µm) column (Waters,) at room temperature using a mobile phase composition of 40% or 50% HPLC grade methanol. The analysis was carried out in isocratic mode at a flow rate of 1ml/min, with column effluent being monitored at 254 nm wavelength. Alkaloids were identified by their co-elution with authentic standards of nicotine and anabasine (Sigma, Australia).

2.2.13 Deglycosylation of proteins

The crude extracts from transient and stably transgenic plant powder and purified *E. coli* PlpE (0.25 mg/ ml) were mixed with 10X glycoprotein denaturing buffer (5% SDS and 400 mM DTT) and dH_2O. The mixtures were incubated at 94°C for 10 minutes. The mixtures were placed on ice. 10X NP-40 (10% NP-40), 10X G7 buffer (500 mM Sodium Phosphate, pH 7.5), 25X BSA, dH_2O and PNGase F enzyme or Protein Deglycosylation Enzyme Mix (New England BioLabs) were added to the mixtures and incubated at 37°C for 4 hours. The samples were mixed with 3X reducing Blue Loading Buffer (4 µl of 1.25M DTT, 130 µl 3X Blue Loading Buffer) (New England BioLabs) and boiled at 94°C for 5 minutes. The protein samples were analysed by Western blotting.

2.2.14 Statistical analysis

GraphPad Prism 5 was used for statistical analyses. One-way ANOVA with Dunn's multiple comparison post test was used to determine statistical significant difference between means of the data from three groups. Results were considered statistically significant if $P < 0.05$.

2.3 Results

2.3.1 Production of anti-PlpE polyclonal antibody in rabbits

Sera from two rabbits immunized with 150 µg purified, insoluble *E. coli* PlpE elicited strong antibody response against recombinant insoluble *E. coli* PlpE in Western blot analysis, whereas the pre-immune sera remained non-responsive (Figure 2.3).

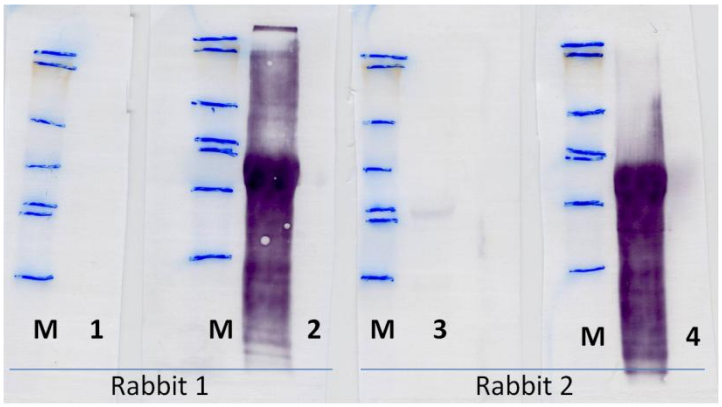

Figure 2.3 Western blot analysis showing reaction of rabbit sera produced against purified, insoluble *E. coli* PlpE. M designates the molecular mass standards (SeeBlue® Plus2, invitogen™), 1 & 3 the pre-immunized serum, 2 & 4 the immunized serum.

2.3.2 Transient production of PlpE in *N. benthamiana*

In the preliminary experiments, when leaf infiltration was performed by hand injection of individual leaves, the expression of *plpE* could not be detected by Western blot analysis. pPSp19 plasmid containing *p19*, a viral suppressor of the post-transcriptional gene silencing was co-infiltrated with pBinPlpE to check if the gene silencing was the reason behind the lack of detectable *plpE* expression. With the addition of P19, PlpE could be easily detected in the leaf extract by Western blotting, whereas no protein was detected in the leaf extract infiltrated with empty vector control (Figure 2.4, A & B). Purified insoluble PlpE from *E. coli* cells was used as the positive control. Leaves were infiltrated with a co-culture of pBinPlpE and pPSp19, pBinPlpE alone, pPSp19 alone, a co-culture of empty vector and pPSp19 or the empty vector control (Figure 2.4, A & B). The co-culture of pBinPlpE and pPSp19 was the only treatment that resulted in leaf extract with detectable PlpE using Western blot. The experiment was repeated in three plants to confirm the transient expression of *plpE* in leaves. The leaf materials were collected and extracted at 3, 4, 5 and 10 days after infiltration events. The optimum time needed for the expression of plpE in the leaves was determined to be 5 days post-infiltration (Figure 2.5). In each instance, the plant-expressed PlpE displayed a ladder-like pattern of bands with 4 prominent bands running at 44, 42, 40 and 38 kDa while the positive control of purified, insoluble or urea-solubilized *E. coli* PlpE ran at 40 kDa. Therefore, within the plant extract lanes, the third band of 40 kDa matched the

E. coli-made control while two additional bands ran higher (at 42 & 44 kDa) and the fourth lower (at 38 kDa). It was speculated that the bands running higher than the control were products of glycosylation, a post-translational modification of proteins in plants and the lower band was a specific cleavage product of plant-PlpE. Some non-specific bands running higher or lower than transient PlpE bands were observed in the lanes loaded with plant negative control materials (Figures 2.4, A & B; 2.6, B). The quantity of plant-expressed PlpE was estimated to be 200 mg per kg of freeze-dried leaf powder (Figure 2.6 A). The measurement was performed by comparing the intensity of the Western blot bands to that of a known concentration of control purified *E. coli* PlpE (urea-solubilized) by using the NIH ImageJ 1.45s software (http://imagej.nih.gov/ij). Though all 4 bands had the same intensity, to be conservative only the bands running at 40 kDa and 42 kDa were chosen for the comparison with the control *E. coli* protein band (Figure 2.6, A) since the bioactivity of the glycosylated forms and the cleavage product was not known. A PlpE-specific sandwich ELISA detected almost two fold quantity of plant-expressed transient PlpE compared to that estimated by Western blot analysis. This elevated expression would be due to the ELISA not being able to differentiate between the multiple bands observed in Western blot therefore, half of the quantity of transient plant-PlpE detected by sandwich ELISA was considered to estimate the concentration of transient plant-PlpE (Figure 2.12).

In the 2nd phase of the project, a vacuum infiltration device was used to infiltrate the *Agrobacterium* cultures into the leaf tissues. The entire aerial part of the plant was infiltrated. This vacuum infiltration device reduced the time needed for infiltration. It took 5 hours to infiltrate 40 plants by using this device compared to just 10 plants infiltrated in the same time by hand injection. Though the same banding pattern for plant-PlpE was observed on Western blot membrane the quantity of plant-expressed PlpE doubled (Figure 2.6, B) upon vacuum infiltration. It was estimated that 400 mg of PlpE was produced per kilogram of freeze-dried leaf powder. PlpE content was therefore significantly higher in vacuum-infiltrated transient plant material than that of hand-infiltrated material.

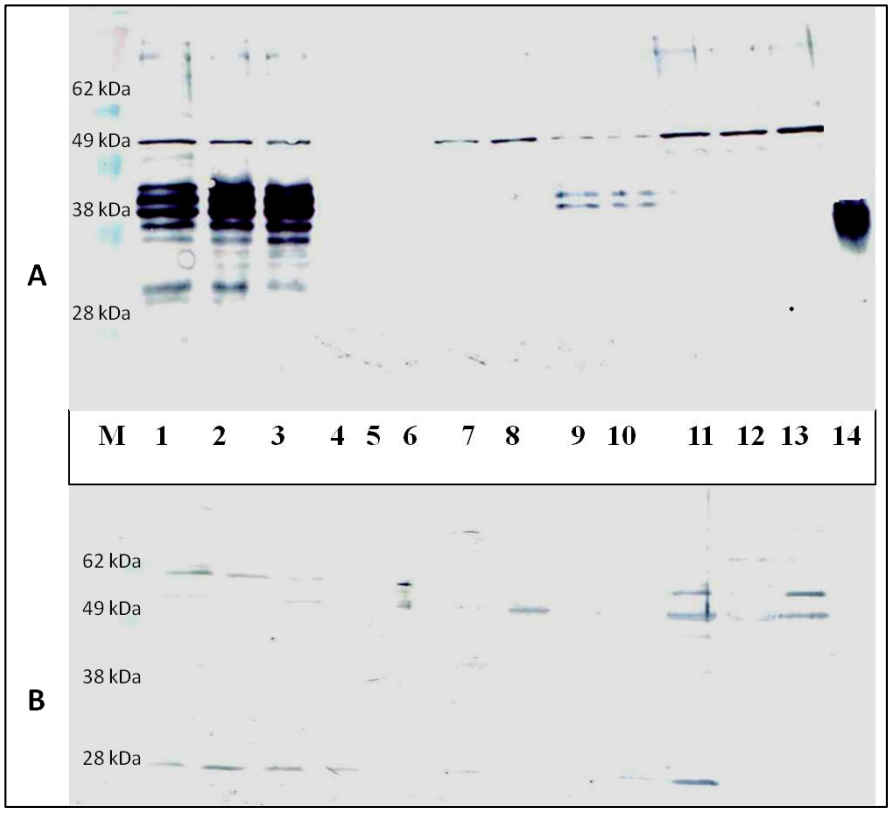

Figure 2.4 Western blot analyses showing transiently expressed PlpE in *N. benthamiana* leaves produced by hand-infiltration of *Agrobacterium*. M designates the molecular mass standards (SeeBlue® Plus2, invitogen™), lanes 1, 2 & 3 show pBinPlpE+p19 (three leaves from three plants), 4, 5 & 6 show Non-infiltrated leaf, (three leaves from three plants), 7 & 8 show empty vector control+p19 (two leaves from two plants), 9 & 10 show pBinPlpE only (two leaves from two plants), 11, 12 & 13 show p19 only, 14 shows purified insoluble PlpE from *E. coli* (200ng). A: incubated with Chicken anti-PlpE serum & B: incubated with a non-related chicken serum.

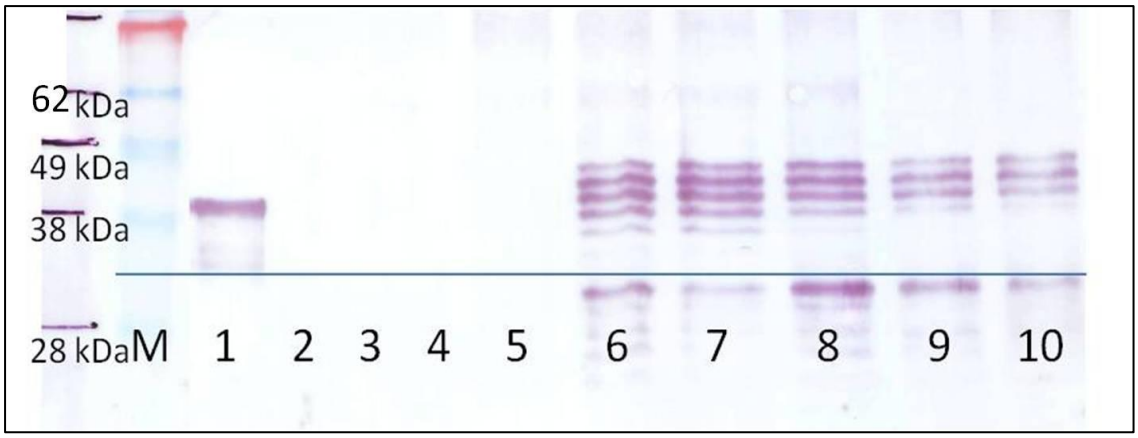

Figure 2.5 Western blot analysis showing the determination of optimum time needed for the expression of *plpE* in *N. benthamiana* leaf through agro-infiltration. M designates the molecular mass standards (SeeBlue® Plus2, invitogen™), lane 1 shows *E. coli* PlpE (100 ng), 2 & 3 show extracts from leaves collected 3 days post infiltration, 4 &5 show extracts from leaves collected 4 days post infiltration, 6, 7 & 8 show extracts from leaves collected 5 days post infiltration, 9 & 10 show extracts from leaves collected 10 days post infiltration.

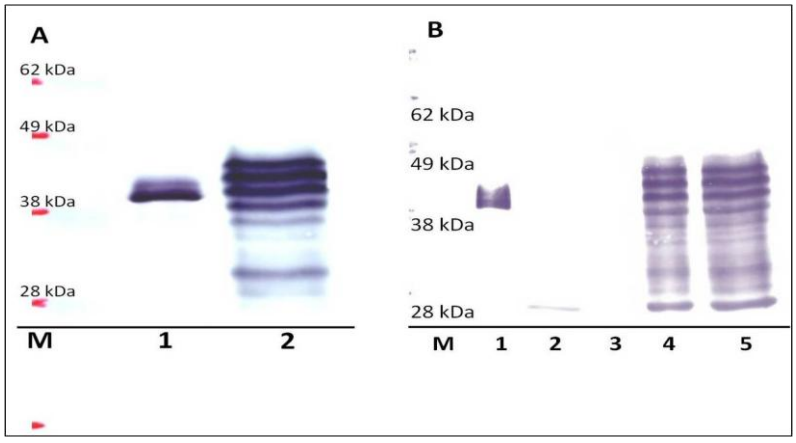

Figure 2.6 Western blot analyses showing transient plant-PlpE in freeze-dried, pooled batch of *N. benthamiana* leaves. A. Hand injected infiltration: M designates molecular mass standards (SeeBlue® Plus2, invitogen™), lane 1 shows purified *E. coli* PlpE (200 ng) & 2 shows plant-PlpE (200 ng); B. Vacuum-infiltration: M designates molecular mass standards, lane 1 shows purified *E. coli* PlpE (200 ng), 2 shows control plant material, 3 & 4 show plant-PlpE (400 ng).

2.3.3 Production of PlpE in transgenic cell line of NT1 (*Nicotiana tabacum*)

NT1 cell lines were stably transformed across four transformation experiments (Figure 2.7). A total of 43 putative transformed calli were selected and screened by PCR and Western blot analysis. The presence of *plpE* (1009 bp) and the selectable maker *nptII* (500 bp) was confirmed by PCR (Figure 2.8, A & B) and the absence of virulence gene *D* (460 bp) (Mysore et al., 1998) from *Agrobacterium* was also confirmed (Figure 2.8, C). From the original 43 putative transformants, three stable transgenic NT1 cell lines were selected and established as liquid cultures based on the quantity of PlpE expressed. The production of PlpE in these 3 transgenic NT1 cell lines was confirmed by Western blot analysis (Figure 2.9, A) and SDS-PAGE (Figure 2.9, B). The banding pattern of the transgenic plant-PlpE in NT1 cell lines was different from that of transient expression in *N. benthamiana* leaves. All three transgenic cell lines showed one PlpE-band at 42 kDa compared to the four banded ladder displayed by the transient leaf material. PlpE produced in plant cell suspension was quantified to be approximately 20-30 mg per kilogram of fresh callus tissue (comparing the intensity of the Western blot band to that of a known concentration of control purified *E. coli* PlpE by using the NIH ImageJ 1.45s software). Pooling and freeze-drying the cell suspensions, increased the quantity of PlpE to 100 mg per kilogram of dried cell suspension powder (Figure 2.10). This measure was confirmed by sandwich ELISA (Figure 2.12). PlpE content was therefore significantly higher in transient leaf material than stable transgenic cell suspension (Figure 2.12) as well as in freeze-dried powder of stable transgenic cell suspension than fresh stable transgenic callus tissue.

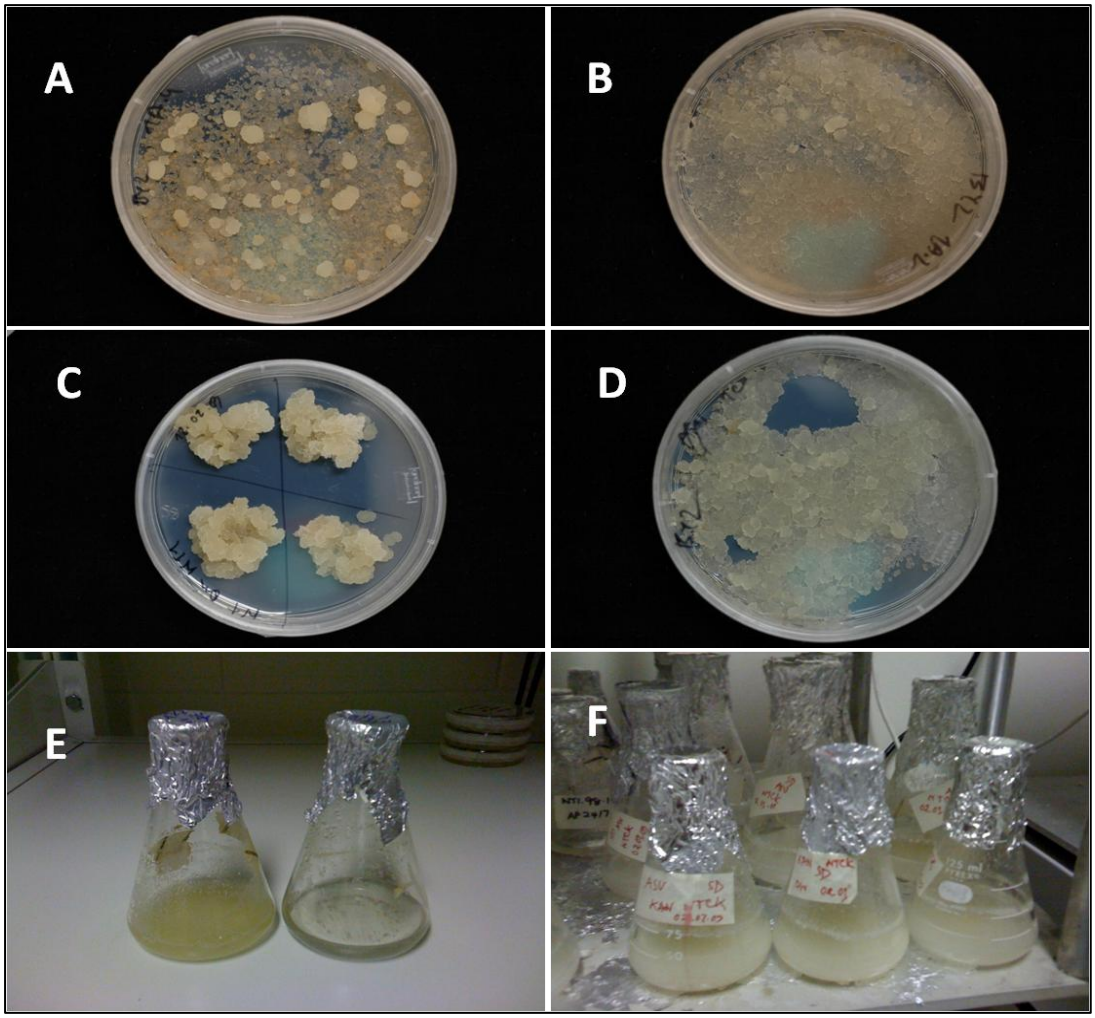

Figure 2.7 Production of stably, transgenic NT1 cell lines expressing PlpE antigen. A. PlpE expressing transformed calli on selective medium, B. Empty vector negative control on selective medium, C. wild/non-transformed NT1 calli, D. wild calli on medium with selection, E. Stable transgenic PlpE expressing NT1 cell line growing in cell suspension, F. Transgenic and control cell suspensions grown in liquid medium.

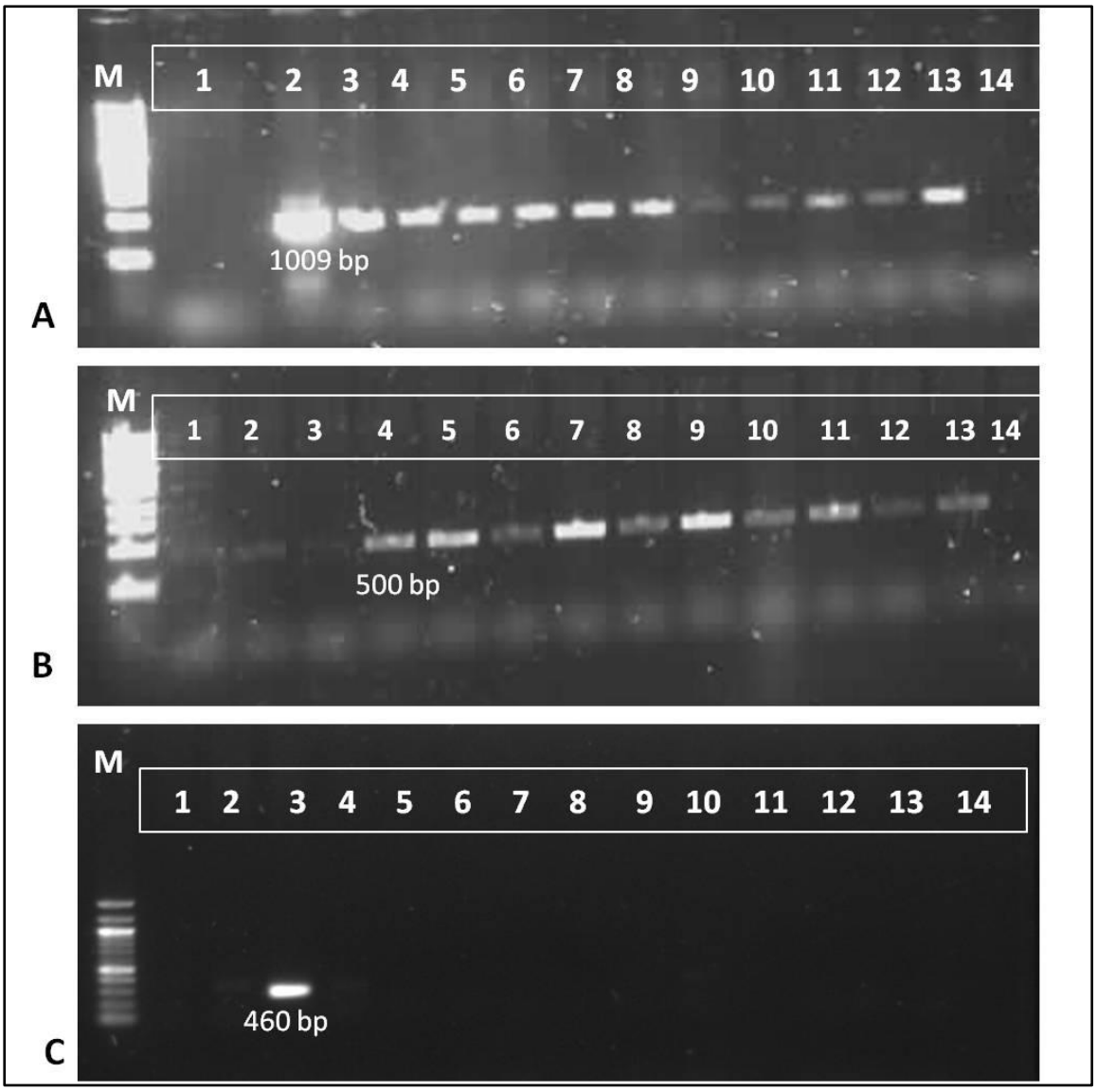

Figure 2.8 PCR analyses of callus tissues of stable transgenic NT1 cell lines. Gel A shows the PCR products for *plpE* (1009 bp), gel B shows the PCR products for *nptII* (500 bp) and gel C shows the PCR products for *VirD* (460 bp) from *Agrobacterium tumefaciens*. M designates DNA ladder (1 kb in gel A & 100 bp in gels B & C from Bio-Rad, Hercules, CA), lane 1 shows wild NT1 cell line, 2 shows NT1 cell line transformed with empty vector, 3 shows positive controls from plasmid pGPTV (gels A & B) & positive control from *A. tumefaciens* strain LBA 4404 (gel C), 4-13 show stable transgenic cell lines and 14 shows negative control with H_2O.

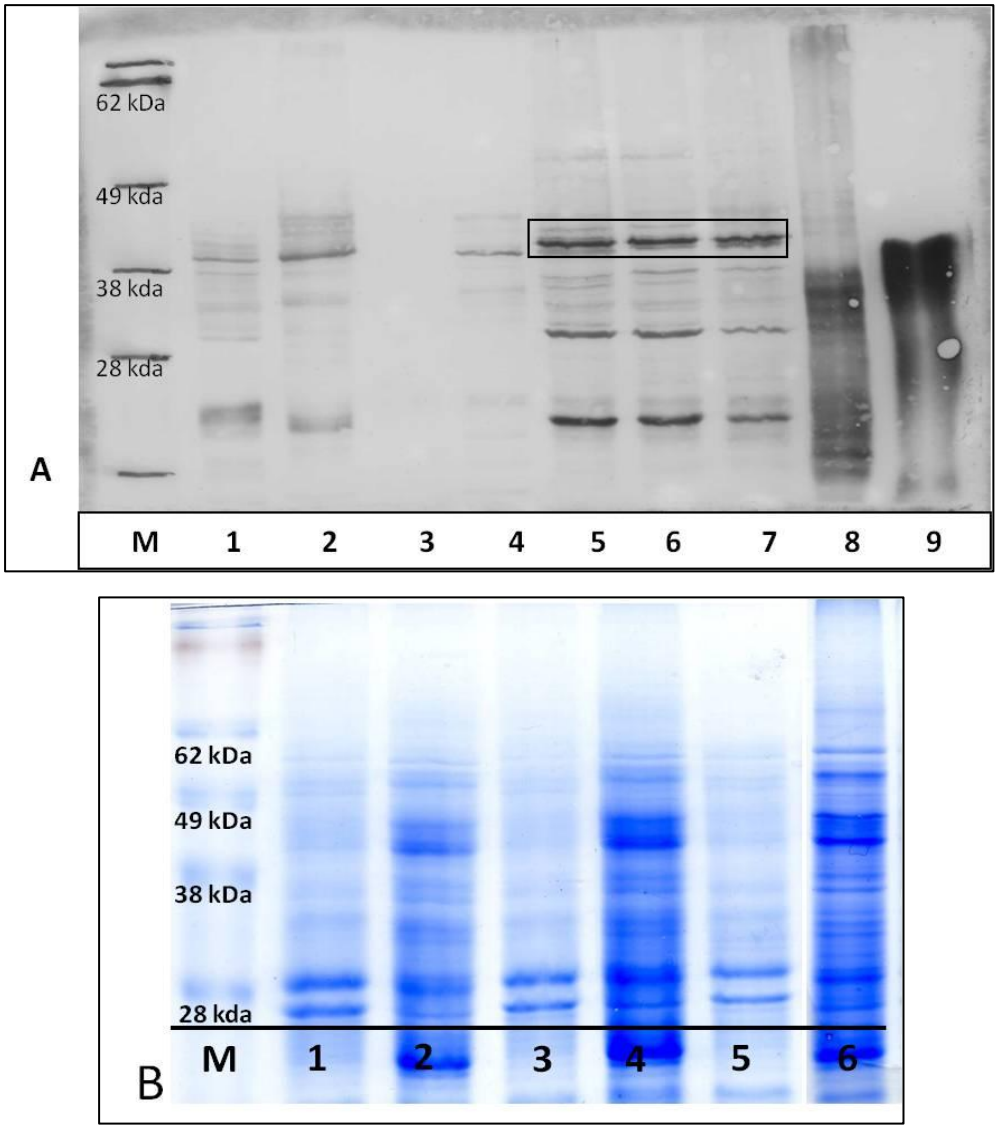

Figure 2.9 A. Western blot analysis showing plant-PlpE in stably transformed NT1 callus. M designates molecular mass standards (SeeBlue® Plus2, invitogen™), lanes 1 & 2 show NT1 cell lines transformed with *Agrobacterium tumefaciens* carrying empty vector, 3 shows Liquid media only, 4 shows wild untransformed NT1 cell line, 5-7 show 3 different stable transgenic NT1 cell lines expressing PlpE, 8 shows leaf-extract of *N. benthamiana* & 9 shows purified *E. coli* PlpE (200 ng). B. SDS-PAGE gel showing stable transgenic plant-PlpE in transgenic NT1 callus. M designates molecular mass standards; lanes 2, 4 & 6 show 3 different stable transgenic NT1 cell lines expressing PlpE; 1, 3 & 5 show 3 different wild NT1 cell lines. Crude proteins were extracted from fresh callus tissues.

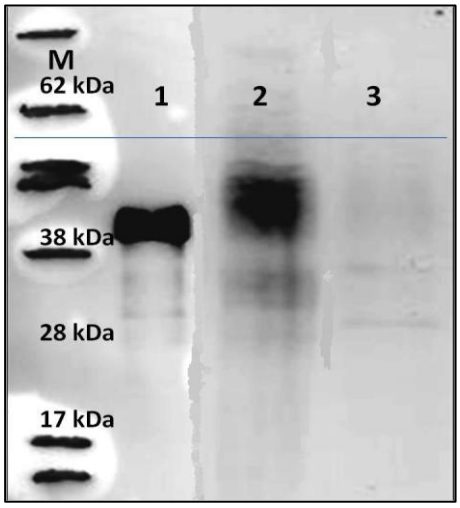

Figure 2.10 Western blot analysis showing plant-PlpE in freeze-dried cell suspension powder of stably transformed NT1 cell line. M designates molecular mass standards (SeeBlue® Plus2, invitogen™), lane 1 shows purified *E. coli* PlpE (200 ng), 2 shows stable transgenic NT1 cell line expressing PlpE (100 ng) & 3 shows NT1 cell line transformed with *Agrobacterium* carrying empty vector. Crude proteins were extracted from freeze-dried cell suspension powder.

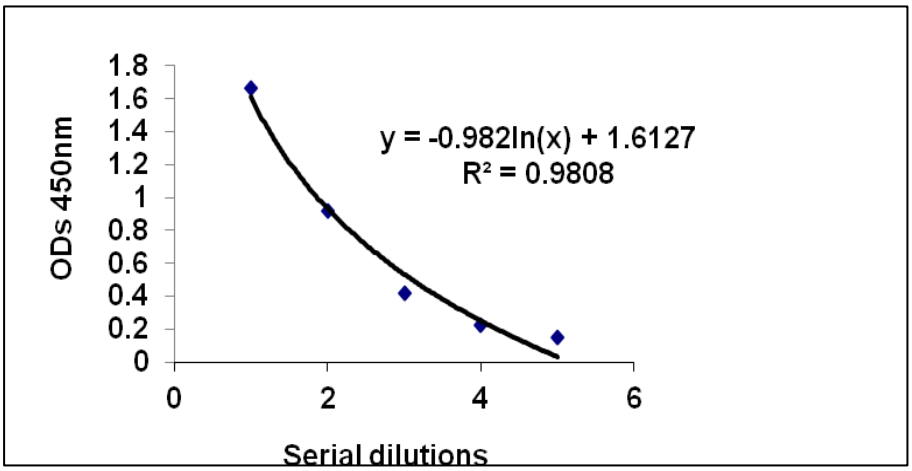

Figure 2.11 Standard curve for purified *E. coli* PlpE generated by direct ELISA

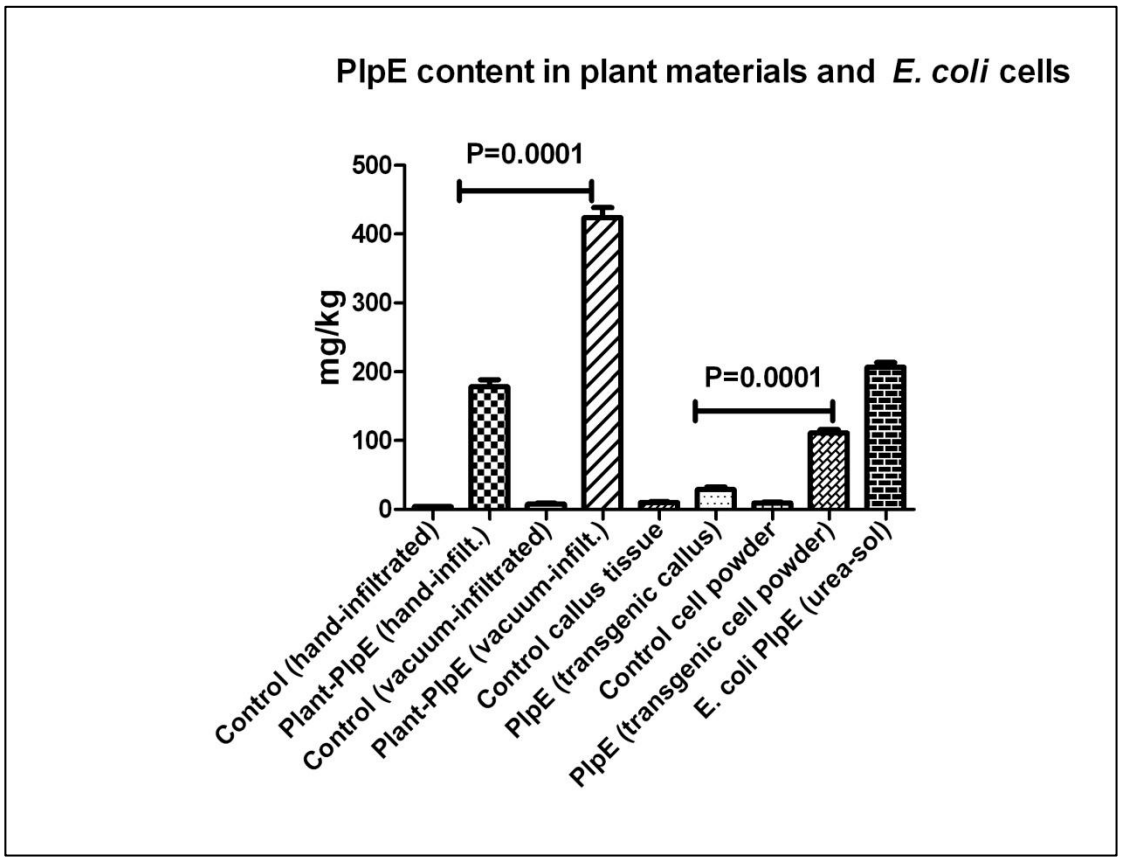

Figure 2.12 Quantification of PlpE in plant materials and *E. coli* cells by sandwich ELISA analysis. The bars represent the mean PlpE content in different materials and the error-bars indicate standard error of mean (SEM). Data represents mean of 3 replicates. ELISA readings are converted to mg/kg of dry weight of plant materials.

2.3.4 Alkaloid content in plant materials

The presence and quantification of pyridine alkaloids, anabasine and nicotine in plant materials after expressing *plpE* was determined through a HPLC analysis. The quantities of both alkaloids were significantly higher (17 mg/g and 21 mg/g respectively) in freeze-dried leaf powder of *N. benthamiana* produced by hand infiltration of *Agrobacterium* than the leaf powder produced by vacuum infiltration in a machine (4.3 mg/g and 4.5 mg/g respectively). An empty vector plant material produced by vacuum-infiltration was used as a negative control and the quantity of alkaloid content was measured to be 2.8 mg/g of anabasine and 2.9 mg/g of Nicotine (Figure 2.13).

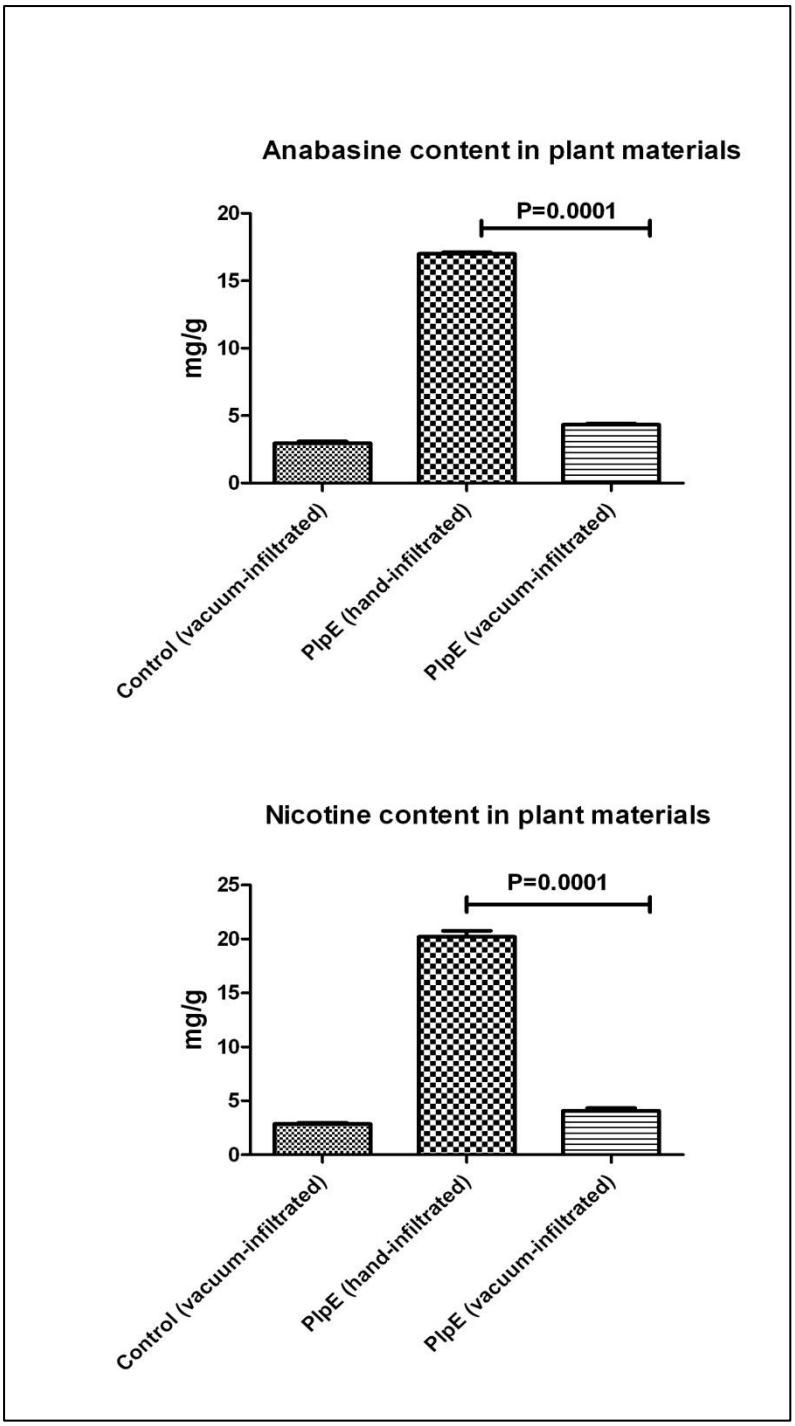

Figure 2.13 Quantification of Pyridine alkaloids (Anabasine & Nicotine) by HPLC in *N. benthamiana* leaves expressing PlpE. PlpE was expressed in those leaves through *Agrobacterium* infiltrations. Crude proteins were extracted from freeze-dried leaf powder. The bars represent the mean of 3 replicates for each material and the error-bars indicate standard error of mean (SEM).

2.2.5 Deglycosylation of proteins

Plant and *E. coli* PlpE were treated for enzymatic removal of *N*- and *O*-glycans using PNGase F or the protein deglycosylation enzyme mix. No change or shift of protein size was observed in *E. coli* PlpE in Western blot analysis after the treatment while the transient plant-PlpE was observed to reduce to a two-band pattern running at 40 and 38 kDa respectively, after treatment with PNGase F enzyme (Figure 2.14 A). The size of the stably transgenic plant-PlpE band shifted from 42 to 40 kDa after treatment with PNGase F and no further change was observed in both transient and transgenic plant-PlpE after treatment with the protein deglycosylation enzyme mix, which was composed of both PNGase F and O-glycosidase enzymes for removal of both *N*- and *O*-glycans (Figure 2.14 B).

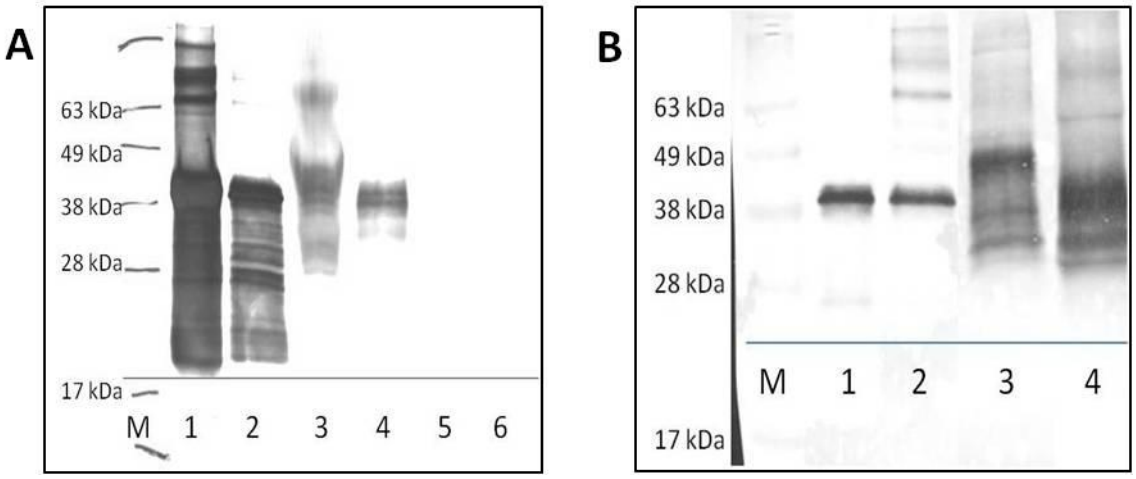

Figure 2.14 Western blot analyses showing enzymatic removals of *N*-glycans from plant-PlpE after treatment with PNGase F. A. M designates the molecular mass standards (SeeBlue® Plus2, invitogen™), lane 1 shows *E. coli* PlpE not treated with glycosylases, 2 shows *E. coli* PlpE treated, 3 shows Transient plant-PlpE not treated with glycosylases, 4 shows transient plant-PlpE treated, 5 & 6 show negative controls with H_2O; B. M designates the molecular mass standards, lane 1 shows *E. coli* PlpE not treated, 2 shows *E. coli* PlpE treated, 3 shows stable transgenic plant-PlpE not treated, 4 shows stable transgenic plant-PlpE treated. Crude plant extracts were prepared from freeze-dried leaf and cell suspension powder samples. Western blot membranes were incubated with a rabbit anti-PlpE polyclonal antibody (primary, 1:200 dilutions) and a horseradish peroxidase conjugated goat polyclonal IgG antibody against rabbit (secondary, 1:1000 dilutions)

2.4 Discussion

2.4.1 Transient expression of PlpE in *N. benthamiana* leaves

In the preliminary experiments, when pBinPlpE, the plant-PlpE construct was infiltrated into *N. benthamiana* leaves alone it could not be detected by Western blot analysis. It has been shown that post-transcriptional gene silencing (PTGS) can affect the foreign protein expression in plants (Voinett, 2001 & 2002). If the expression of PlpE was inhibited by PTGS then the silencing could be prevented by using a viral-encoded suppressor of gene silencing like p19 (Voinett et al., 2003). The p19 protein of tomato bushy stunt virus (TBSV) prevents the onset of PTGS in the infiltrated tissues and allows high level of transient expression (Voinett et al., 2003; Voinett, 2005; Sainsbury & Lomonossoff, 2008). The effect of p19 was found not to be saturated by PTGS in cells that received up to four individual T-DNAs and persisted until leaf senescence (Hammond et al., 2001). In this study, when pBinPlpE was infiltrated in combination with p19 into the leaf tissues, plpE could be detected using Western blot analysis. The post-infiltration time has been shown to be case-specific (Lombardi et al., 2009; Lindbo, 2007) and in this study, the optimum time needed for optimal expression was determined to be 5 days post-infiltration.

The quantity of PlpE expressed using transient expression increased 2-fold when a vacuum infiltration device was used on the whole aerial part of the plant. The process also greatly decreased the time needed for infiltration from 20 hours to 5 hours for infiltrating 40 plants. This proved to be a significant advantage/improvement when preparing a batch of vaccine antigen for the immunization experiments. The increase in recombinant protein produced using vacuum-infiltration may have been due to the leaves undergoing less injury and consequently placing less stress on plants than hand-injected infiltrations of individual leaves. Transiently produced plant-PlpE showed 4 bands of similar intensity to each other in Western blot analysis. One of the bands ran at a size similar to the purified *E. coli* control at 40 kDa. Two higher bands ran at 42 and 44 kDa and one ran lower than the purified insoluble *E. coli* PlpE at 38 kDa. Since an LTA signal peptide was fused with the native *plpE* gene to send the resultant recombinant protein into the ER (Endoplasmic reticulum) and by default through the Golgi complexes into the apolplast of the plant cells, it was speculated that the two higher bands were the products of glycosylation. Generally, glycans are added to an asparagine (*N*-linked) or to a serine or threonine (*O*-linked) in the secretory compartments of the eukaryotic cells (Thakur et al., 2009; Varki et al., 2009). The amino acid sequence of PlpE was run

through Glycosylation Site Prediction software (NetNGlyc1.0Server, Technical University of Denmark) (Gupta et al., 2004; Julenius et al., 2005). Two potential *N*-linked and six potential *O*-linked glycosylation sites were predicted by the software. When a deglycosylation reaction was performed on transient plant-PlpE extract with PNGase F enzyme (which removes almost all *N*-linked glycans from the peptides) the upper two bands (at 44 and 42 kDa) were no longer observed after Western blot analysis (Figure 2.14). However, no further shift in protein size was observed after enzymatic treatment with the Protein Deglycosylation Mix, which had both PNGase F and O-Glycosidase enzymes for removal of both *N*- and *O*-linked glycans. Therefore, it was confirmed that transient plant-PlpE was glycosylated and it had *N*-glycans but not *O*-glycans. The lower-most band running at 38 kDa was predicted to be a specific cleavage product of plant-PlpE due to proteases present in the plant ER. Proteolysis of recombinant proteins has also been identified as a significant problem in plant production systems as even native proteins travelling through the secretory pathway typically undergo proteolytic processing, by which targeting signal and regulatory peptides are cleaved from the maturing protein by specific proteases (Kusnadi et al., 1997). It is noteworthy that a similar banding pattern was observed when PlpE was expressed in *E. coli* cells and purified in the soluble form (Wu et al., 2007). They observed 2 bands, the major one predicted to be the mature PlpE with a size of 36.3 kDa and other one to be the full length PlpE with a size of 38.7 kDa. They concluded that some type of processing or modification of the recombinant PlpE took place inside the bacterial cells. It would be of interest to direct sequence these two plpE products to determine their identity and to direct the PlpE to additional sub-cellular locations in future studies to determine if this affects the PlpE bands produced.

As one aim of producing PlpE in plants was to make an oral vaccine against fowl cholera it was essential to check the possibly toxic alkaloid content in the infiltrated leaves. The leaf tissues of most species in the genus *Nicotiana*, including *N. benthamiana* and *N. tabacum*, can contain substantial quantities of toxic pyridine alkaloids, particularly nicotine and anabasine (Saitoh et al., 1985; Sisson & Severson 1990). The alkaloid levels increase in leaves of *Nicotiana* following any physical wounding (Baldwin, 1989; Sinclaire et al., 2004; De Boer et al., 2009, Cane et al., 2005). Though it might be possible that the presence of some of these alkaloids in crude leaf extracts have adjuvant effects when combined with antigenic proteins to improve the immune response (Ling et al., 2012; Kirk et al, 2004; Rivera et al., 2003; Song et al., 2009) high alkaloid content in crude or partially purified extracts of tobacco leaf tissues may also prove toxic. Therefore, the alkaloid content of

N. benthamiana leaves was quantified after the infiltrations. There was a significant decrease in anabasine and nicotine content in the leaves infiltrated using the vacuum device compared to the materials infiltrated by hand injections of individual leaves. This was possibly due to hand infiltration producing more stress and or injury, hence inducing alkaloid production in the plant leaves. Therefore use of a vacuum-infiltration device reduced the time required to treat large numbers of plants, increased PlpE content two fold and decreased alkaloid production in the infiltrated plant tissues. Vacuum infiltration was therefore used for PlpE production in further experiments.

2.4.2 Stable expression of PlpE in transgenic *N. tabacum* cell lines

Out of 43 putative transgenic NT1 cell lines three were selected on the basis of protein authenticity and amount of PlpE detected via Western blot analysis. Although these cell lines exhibited around 20-30 mg of PlpE produced per kilogram of fresh callus tissue, freeze-drying of these cells or cell suspension concentrated the recombinant protein to 100 mg per kilogram of dry weight. Freeze-drying has concentrated plant-based antigens produced in *Arabidopsis thaliana* (Rigano et al., 2004), tomatoes (Walmsley et al., 2003) and in potato tubers (Castañón et al., 2002).

Only one stably transformed NT1 line producing 100 mg of PlpE per milligram was chosen for further testing. This stable plant-PlpE produced one band on a Western blot membrane running at 42 kDa, higher than the recombinant *E. coli* PlpE, the positive control. As the same *plpE* gene-construct with the LTA signal peptide was used for stable transformation of the tobacco cells it was predicted that post-translational modification or glycosylation of plant-PlpE occurred inside the ER and Golgi complexes of plant cells. Stable transgenic plant-PlpE was treated with PNGase F or the protein deglycosylation enzyme mix to remove the glycans. After the enzymatic treatments the size of stable plant-PlpE shifted from 42 to 40 kDa on a Western blot membrane, which was similar to the size of the *E. coli* control protein. However, no further shift in protein size was observed after treatment with the protein deglycosylation enzyme mix. Therefore, PlpE made by stably transformed plant cells was *N*-glycosylated and not *O*-glycosylated. A similar observation has been reported by Judge et al. (2004). Intimin, the primary adhesin of *E. coli* O157:H7 was produced in transgenic tobacco cell suspension. This plant-expressed Intimin was targeted to be expressed in plant ER and shown to be glycosylated.

The different banding pattern of transient and stable plant-PlpE protein seen in Western blot analysis might be explained by the *N*-glycosylations observed in these plant-based anitigens. It is likely that different *N*-glycans were added to the transiently made PlpE to those on the stably transgenic plant-PlpE antigen as they were produced through different species and in different plant organs as found by Henquet et al. (2008). It was observed that glycosylation changes unpredictably under changing environmental conditions and that a single protein may obtain different glycosylations to yield heterogenous glycoforms (Bosch & Schots, 2010). Therefore, the different glycans could have been formed due to different environmental conditions such as pH, nutrient availability and cell status between cell culture and cells in vivo in leaves (Bosch & Schots, 2010; Brooks, SA. 2009).

The main goal of producing PlpE in plants is to produce and deliver the vaccine material in a fast, effective and inexpensive manner so that it can safely provide protective immunity in animals without the need of handling of individual animals. Further purification of this protein from the plant extract will increase the cost of immunization program and move from one of the aims of this study. Moreover, it has been shown that the products of plant glycosylation or plant *N*-glycans on plant-translated antigens could flag these antigens and facilitate their capture by antigen-presenting cells (APCs) after binding to their sugar-specific cell surface receptors (Burdin et al., 2004). Thus, plant *N*-glycans could act as new vaccine adjuvants facilitating antigen capture by APCs (Faye et al., 2005). Analysis of toxic alkaloid content of the vacuum infiltrated leaves demonstrated low, non-toxic levels of alkaloids. Therefore, further expensive purification was deemed not required. These plant materials expressing PlpE were therefore, used directly in their crude forms in future immunogenicity studies of mice and chickens.

2.5 Conclusions and future directions

PlpE, the outer-membrane lipoprotein of *Pasteurella multocida*, which provides cross-protection in mice and homologous protection in chickens against fowl cholera (Wu et al., 2007; Hatfaludi et al., 2012) was produced in plants. The antigen was expressed transiently in *N. benthamiana* leaves and stably in transgenic cell lines of *N. tabacum*. The concentration of transient plant-PlpE was quantified to be 400 mg/Kg of dry weight and stably produced plant-PlpE was 100 mg/Kg of dry weight. Both transient and stably produced plant-PlpE was

observed to be *N*-glycosylated, hence bands were observed running at higher molecular size than the recombinant *E. coli* positive control. Glycosylation might offer advantages for the stability and immunogenicity of the antigen (Burdin et al., 2004; Faye et al., 2005) therefore the plant-PlpE proteins were not deglycosylated before administering in animals. In addition, vacuum infiltration reduced toxic alkaloid levels so that further purifications of the antigen from crude plant extracts (and extra cost) were not required. Therefore, the efficacy of these plant-PlpE vaccines was tested in mice and chickens without any further purification (see chapters 3 and 4 and 5). To the author's knowledge, this is the first report of expressing any antigen from *P. multocida* in plants.

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Chapter 3

Pasteurella multocida challenge trials in mice

A subcutaneously delivered, plant-based, PlpE vaccine produced transiently in Nicotiana benthamiana leaves protected mice against Pasteurella multocida infection. Immunization of mice with a stably transformed N. tabacum plant-cell line producing recombinant PlpE vaccine induced an immune response but failed to elicit protection against P. multocida infection.

3.1 Introduction

The production of vaccines in plants offers many advantages over traditional methods of vaccine manufacturing and distribution. It does not need expensive fermentation and cold storage (Langridge 2000; Sparrow et al., 2007); plant-based vaccines eliminate the risk of contamination with endotoxins and mammalian pathogens (Fisher et al., 2004); and they have proven effective after oral immunization (Rigano et al., 2006). These characteristics of plant-based vaccines simplify vaccine delivery and reduce the cost of a vaccination program (Alvarez et al., 2006). Additionally, plants can correctly fold and assemble complex proteins and can perform post-translational modifications such as glycosylation, which cannot be achieved with bacterial fermentation (Streatfield & Howard, 2003; Streatfield 2005). The regulatory barriers for animal pharmaceuticals although considerable, are less than that for human use. Up to now, most research performed in the plant-based vaccine field has focused on veterinary diseases caused by viral infections, such as foot and mouth disease (Carrillo et al., 1998 & 2001; Wigdorovitz et al., 1999; Dus Santos et al., 2002; Dus Santos & Wigdorovitz 2005; Wigdorovitz et al., 2004), avian influenza (Mihaliak & Webb 2005), rabies (McGarvey et al., 1995; Yusibov et al., 2002; Ashraf et al., 2005), new castle disease (Zhao & Hammond 2005; Natilla et al., 2006; Berinstein et al., 2005; Hahn et al., 2007; Guerrero-Andrade et al., 2006; Yang et al., 2007) and infectious bursal disease (Wu et al., 2004). These plant-based vaccines have mainly been tested in model laboratory animals (mainly mice) however some have proven efficacious in target animals such as cattle, sheep, pigs and chickens (Lamphear et al., 2002; Zhou et al., 2004; Guerrero-Andrade et al., 2006;

Joensuu et al., 2006b; Khandelwal et al., 2003; Khandelwal et al., 2011; Loza-Rubio et al., 2012). There are a number of examples of veterinary vaccines composed of recombinant bacterial antigens produced in plants that protected the model animals (mainly mice) (Haq et al., 1995; Lauterslager et al., 2001; Huang et al., 2003; Judge et al., Joebsuu et al., 2004, 2006a & 2006b; Mason et al., 1998; Kang et al., 2003 & 2005; Lee et al., 2006; Liang et al., 2006; Yu & Langridge, 2001; Judge et al., 2004). However, no study could be found where a plant-based vaccine composed of bacterial antigen had shown to elicit protection in target animals. Only a few examples of the bacterial antigens have been produced in plants and immunogenicity tested in mice. These examples however are described in the following paragraphs.

Escherichia coli

Gastroenteritis is triggered by the release of heat labile enterotoxin (LT) produced by the enterotoxigenic *E. coli*. LT is formed by a toxic 27-kDa subunit (LTA) and a non toxic 11.6 kDa B subunit (LTB). It has been shown that mice feeding on transgenic plants expressing LTB produced antibodies that could neutralize LT (Mason et al., 1998; Kang et al., 2003 & 2005).

Bloody diarrhoea or hemorrhagic colitis is caused by the enterohemorrhagic *E. coli* O157:H7. Judge et al. (2004) reported that *E. coli* outer-membrane protein Intimin, produced in transgenic tobacco cell line elicited a protective immune response in mice against *E. coli* O157:H7, after the mice were fed with plant-Intimin along with the purified bacterial Intimin injected.

Actinobacillus pleuropneumoniae

Actinobacillus pleuropneumoniae is the causative agent of porcine pleuropneumonia. One of the virulence factors, the bacterial exotoxin ApxIIA was produced in transgenic tobacco plants (Lee et al., 2006). The immune responses induced in plant-ApxIIA fed mice proved to be protective against *A. pleuropneumonia* infection. Min-Kyoung Shin et al. (2011) reported the production of full-size ApxIIA and a cholera toxin B (CTB)-ApxIIA fusion protein in corn seed as subunit vaccine candidates. Both antigens were observed to induce effective Apx-specific immune responses in forms of crude plant extracts in mice when injected subcutaneously.

Mannheimia (Pasteurella) haemolytica

Bovine pneumonic pasteurellosis is a bovine respiratory disease complex, caused by the infection with the bacterium, *Mannheimia (Pasteurella) haemolytica* serotype A1 (Yates 1982). The leukotoxin (Lkt) is a pore-forming protein and one of the major virulence factors of *M. haemolytica* A1. A recombinant derivative of Lkt, Lkt50, lacking the putative hydrophobic trans-membrane domains, was produced in transgenic white clover (*Trifolium repens* L.). Purified Lkt prepared from clover leaf extracts were able to induce an immune response to authentic Lkt in rabbits when delivered by intramuscular injection. The resulting antibodies were able to neutralize Lkt (Lee et al., 2001). The same group produced the GS60 antigen of *Mannheimia haemolytica* A1 with sequences encoding a slightly shortened derivative of the GS60 antigen (GS6054) in white clover (Lee et al., 2008). The purified antigen from dried alfalfa plants was shown to be immunogenic when injected intra-muscularly into rabbits. When the dried leaves were fed to rabbits it also induced immune response. All these studies performed on bacterial antigens produced in plants suggest the feasibility of plant-based vaccines either in injectable or oral forms in model animals.

PlpE or Lipoprotein E of *Pasteurella multocida* is cross-protective antigen that has been produced as a recombinant antigen in *E. coli* cell culture in soluble and insoluble forms. It has also been was tested in immunological mice studies. The soluble, *E. coli*-derived recombinant PlpE vaccine candidate protected 70% of the animals when 10 µg was delivered twice (Wu et al., 2007) two weeks apart. The *E. coli*-derived derived, insoluble recombinant PlpE elicited the same level of protection (70%) in mice but using a higher dose of 150 µg (Hatfaludi et al., 2012). A soluble antigen is more convenient and preferable for vaccination as it is more available to the host immune system. Moreover, the insoluble PlpE was solubilized in 8M urea, which is not a suitable solvent for use in vaccination of animals.

Plants can produce complex, foreign proteins in soluble forms that can be delivered through systemic routes or mucosally. Since mucosal delivery has been shown to elicit both systemic and mucosal immunity it may consequently elicit a more broad-spectrum immune response against pathogens. Therefore, I sort to test the hypothesis that plant-based PlpE could induce protective immunity in mice and chickens.

In Chapter 2 of this thesis, production of PlpE antigen was described in *N. benthamiana* leaves transiently and in stable transgenic *N. tabacum* cell lines. This chapter describes two animal trials performed to investigate the efficacy of immunizing mice with candidate plant-

based PlpE recombinant proteins. In the first trial, transient plant-PlpE was delivered to mice, whereas both transient and stably transgenic plant-PlpE was delivered to mice during the second trial. Trial one also used two dose regimes of insoluble *E. coli* recombinant PlpE protein (10 & 50 µg) as positive controls and to determine the minimum amount of antigen needed for protection. By the second trial, the soluble form of PlpE from *E coli* cells was available for testing though the quantity available was not as high as the insoluble form. Additionally, a positive control group of 7 mice was administered with a whole cell killed vaccine of *P. multocida* strain X-73.

3.2 Materials and methods

3.2.1 Vaccine material preparation

3.2.1.1 Plant-based PlpE

2.5 g of freeze-dried *N. benthamiana* leaf powder (transient plant-PlpE and plant empty vector) and 5 g of freeze-dried cell suspension powder (stable transgenic plant-PlpE and plant empty vector) was resuspended in 25 ml of extraction buffer (1X PBS) by vortexing with two 6.4 mm ceramic beads (Qbiogene, Carlasbad, CA) for 2 minutes at maximum speed. Insoluble material was removed by two centrifugation steps at 47,893.3*g* at 4°C for 20 minutes in a Sorvall ultra-centrifuge using a SS34 rotor. The clear supernatant was collected in fresh a 50 ml conical tube. This extract was dialyzed over-night in 2 liters of extraction buffer (1X PBS) at 4°C through a dialyzing membrane with 10 kDa cut-off. The dialyzed plant extract was concentrated using 15 or 50 ml Amicon Ultra-4, PLGC Ultracel-PL Membrane, 10 kDa (Millipore™) at 7000 *g* and 4°C for 2 or 4 hours to reach the required concentration of 10 µg or 100 µg. The final vaccine preparation was filtered through 0.45/0.22 µm filters and 20% (v/v) Al Hydrogel® was added just before administration.

3.2.1.2 Quantification of PlpE in plant-based vaccine batches

The total soluble protein concentration of the *E. coli* PlpE was determined by Bradford Assay (Bio-Rad, Hercules, CA), using known concentrations of bovine serum albumin (BSA) as the protein standard. Plant-based PlpE concentration was determined by comparing the Western blot band intensity to that of a known concentration of control purified *E. coli* PlpE (urea-solubilised) using the NIH ImageJ 1.45s program (http://imagej.nih.gov/ij). Transient plant-PlpE produced 4 bands in Western blot analysis at 38, 40, 42 and 44 kDa. Though all 4 bands had the same intensity, to be conservative only the bands running at 40 kDa and 42 kDa were

chosen for the comparison with the control *E. coli* protein band (Figures 2.6 A, 3.1 & 3.2) since the bioactivity of the glycosylated forms and the cleavage product was not known.

3.2.1.3 *E. coli* PlpE

The purification of insoluble and soluble, *E. coli* PlpE was performed according to Hatfaludi et al. (2012). The recombinant PlpE was analyzed by visualization of the protein using SDS-PAGE gel stained with Coomassie blue and Western blot. The concentration was quantified using Bradford assay (Biorad, CA, USA).

3.2.1.4 Whole cell killed bacterins

A *Pasteurella multocida* whole cell killed vaccine was prepared from the strain X-73 (AL 848, Heddleston serotype A: 1) (Boyce & Adler, 2000). The bacteria were streaked on a HI (Heart Infusion medium) plate and incubated at 37°C overnight. The next night one well-isolated colony was inoculated in 10 ml HI medium and incubated at 37°C overnight. 50 µl from the over-night culture was transferred to 10 ml of fresh HI medium and was grown for approximately 3-4 hours until an OD_{600} of 0.45-0.55 was reached ($1x10^9$ CFU/ml). A 10^{-1} dilution ($1x10^8$ CFU/ml) of this main culture was set in a water bath at 60°C for an hour to kill the bacteria. After one hour, 100 µl of cells from this heat-treated culture were plated out on two HI plates each and incubated overnight at 37°C to confirm the absence of viable cells. A 10^{-6} dilution was prepared from the main culture and 100 µl from that was spread out on 2 HI plates each and incubated at 37°C overnight to count the viable colonies next morning as the final dose of the killed vaccine was calculated from this colony count. The preparation of heat-killed bacteria containing approximately 2.065 X 10^3 CFU in 100 µl volumes were administered to mice as described below. 20% (v/v) Al Hydrogel® was added to the vaccine just before delivery.

3.2.3 Animal handling and vaccine delivery

Specific-pathogen-free female BALB/cJAsmu mice (6-8 weeks old), supplied by Monash Animal Services were used in this study (Ethics approval number: SOBS/M2008/02 and MARP/2011/002). Mice were kept for one week to acclimatize and then grouped 3 to 4 (trial 1) and 5 (trial 2) mice per cage. They were provided with water and standard food pellets throughout the trial time. Mice were monitored daily for health and condition. In trial 1, each treatment contained 7 mice except the plant-PlpE treatment, which had 11 mice (Table 3.1).

Table 3.1 List of groups of animals in Trial 1

Treatment group	Number of mice	Vaccine dose
Transient Plant-PlpE	**11**	**8-10 µg**
Plant empty vector (control)	**7**	**N/A**
***E. coli* PlpE Insoluble with alum**	**7**	**10 µg**
***E. coli* PlpE Insoluble with alum**	**7**	**50 µg**
Adjuvant only Negative control (Al Hydrogel®)	**7**	**N/A**

N/A=Not Applicable

Trial 1: All doses were delivered subcutaneously in 100 µl volume using 1 ml syringe and 29 inch needle. The mice received 4 doses on day 0, 7, 25 and 32.

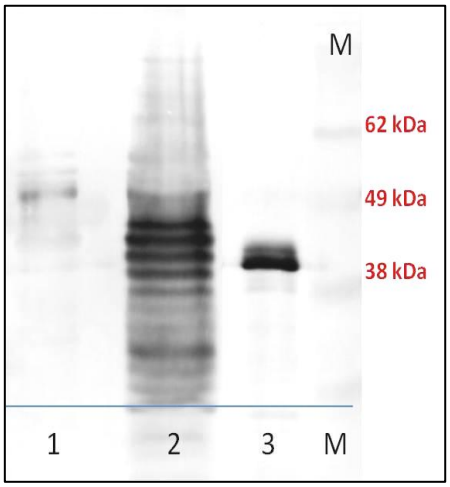


Figure 3.1 Western blot analysis showing vaccination materials. Lane 1 shows transient empty vector control, lane 2 shows transient plant-PlpE (200ng), lane 3 shows *E. coli* PlpE (200 ng) & M stands for molecular mass standards (SeeBlue® Plus2, invitogen™).

Trial 2: This trial contained 11 groups (10 mice per group except the whole cell killed bacteria, which had 7 mice, Table 3.2). The vaccines were administered on day 0, 15 and 24. Each dose was 100 µg of PlpE in 100 µl of volume of vaccine suspension. Al Hydrogel® (20% v/v) was used as the adjuvant and added, where mentioned.

Table 3.2 List of groups of animals in Trial 2

	Treatment group	Number of mice	Vaccine dose
Plant-Transient	Transient plant-PlpE with adjuvant	10	100 µg
	Transient plant-PlpE without adjuvant	10	100 µg
	Transient plant empty vector control with adjuvant	10	N/A
Plant-Stable transgenic	Stable plant- PlpE with adjuvant	10	100 µg
	Stable plant- PlpE without adjuvant	10	100 µg
	Stable plant empty vector control with adjuvant	10	N/A
E. coli	*E. coli* PlpE Insoluble with adjuvant	10	100 µg
	E. coli PlpE Soluble with adjuvant	10	100 µg
Whole cell killed vaccine with adjuvant		7	2.065 X 10^3 CFU
Adjuvant only		10	N/A

N/A=Not Applicable

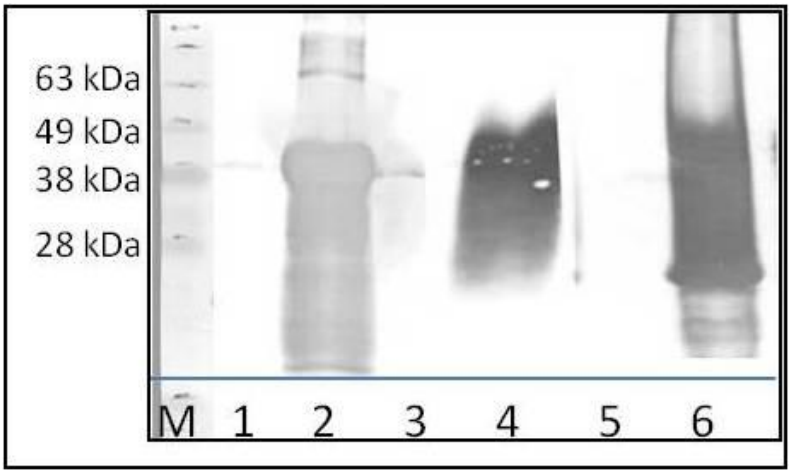


Figure 3.2 Western blot analysis showing vaccination materials. M stands for molecular mass standards (SeeBlue® Plus2, invitogen™), 2 for *E. coli* PlpE (10 µg), 4 for transgenic plant-PlpE (100 µg), and 6 for transient plant-PlpE (100 µg), samples were separated with empty lanes 1, 3 and 5.

3.2.4 Pathogen challenge trial with *Pasteurella multocida*

In both trials the mice were challenged with the *P. multocida* strain X-73 (AL848, Heddleston serotype A: 1). The bacteria was streaked on a HI (Heart Infusion media) plate and incubated at 37°C overnight. The next night, one well-isolated colony was inoculated in 10 ml HI medium and incubated at 37°C overnight. 50 µl from the overnight culture was transferred to fresh 10 ml HI medium and was grown for approximately 3-4 hours until an OD_{600} of 0.45-0.55 was reached ($1x10^9$ CFU/ml). A 10^{-6} ($1x10^3$ CFU/ml) dilution was prepared from this main culture for the animal infection trial (Boyce & Adler, 2000). 100 µl from 10^{-7} and 10^{-8} dilutions were spread out on 2 HI plates each and incubated at 37°C overnight to count the viable colonies next morning. The final infection or challenge dose was determined from this colony count.

A lethal dose of $1.3x10^3$ CFU of bacteria was delivered to the mice on day 45 in trial 1 through intra-peritoneal injections. The mice were then closely observed for 4 days. Clinical sings of the disease began with piloerection, dullness, and huddling. Sick mice did not recover and their conditions became worse gradually. Neurological symptoms like disturbances in body balance, wobbling and whirling caused by brain lesions were observed in animals (Virag et al., 2008). The surviving animals were sacrificed humanely on day 49

using a CO_2 gas chamber. In Trial 2, a dose of $1.45x10^3$ CFU were delivered to the mice on day 36 through intra-peritoneal injections. The animals were then observed for 7 days with the survivors sacrificed humanely on day 43 using a CO_2 gas chamber. Animals with signs of infection were euthanized according to the animal ethics guidelines.

3.2.5 Collection and preparation animal sera

Blood was collected from two mice from each group before first immunization on day 0 during both trials as well as from all mice from all groups before pathogen challenge on day 49 and day 43 during trial 1 and 2, respectively. Mice were eye-bled with the help of sterile Pasteur pipettes to collect blood. Mouse sera were separated from whole blood by clotting at room temperature for 2 hours. Then the samples were spun down in a Beckman fixed rotor centrifuge machine at 15,000 *g* for 20 min at room temperature. The clear supernatant was transferred to new 1.5 ml eppendorf tubes. The centrifugation was repeated before the final clear sera were collected. All samples were stored at -20°C.

3.2.6 Analysis of immunogenicity

3.2.6.1 Western blot

Insoluble PlpE purified from *E. coli* was boiled with 6× SDS gel loading buffer (300mM Tris–HCl, pH 6.8, 600mM dithiothreitol, 12% SDS, 0.6% Bromophenol Blue, 60% glycerol), for 10 minutes and placed on ice. 300 ng of this denatured PlpE was loaded on 9 lanes of an SDS-polyacrylamide gel (10.5–14% Tris–HCl, 4% stacking, Bio-Rad, Hercules, CA). The gel was electrophoresed at 100V for 10 minutes and then at 200V for 50 minutes using Tris–glycine running buffer (25mM Tris, 250mM glycine, pH 8.3, 0.1% SDS). The separated proteins were transferred to a PVDF membrane (Bio-Rad, Hercules, CA) using a Mini-Trans-Blot electrophoretic transfer cell (Bio-Rad, Hercules, CA) for 1 hour at 100V in trans-blot buffer. The membrane was then cut into lanes and blocked with 2.5% skim-milk TBS-Tween buffer (TBS buffer plus 0.05% Tween 20) for 1 h at room temperature on a rocking platform. The membranes were washed for 3 times (5 min each time) in TBS-Tween buffer with agitation. Each membrane was then incubated with a single mouse serum collected during the vaccine trials before challenge, diluted at 1:500 in 2.5% skim-milk TBS-Tween buffer on a rocking platform over-night at RT. After four 4 min washes followed by a 5 min wash in TBS-Tween buffer, the membranes were incubated with 1:1200 horseradish peroxidase conjugated goat anti-mouse polyclonal IgG antibody (Sigma–Aldrich, Saint Louis, MO) for 1 hour at 37°C on a rocking platform. The membranes were then washed in TBS-Tween buffer for 4 times (5 min each time) followed by a 5 min wash in TBS buffer with agitation. Blots

were developed colorimetrically. 15 µl of 30% hydrogen peroxide solution was added to 25 ml of 1X TBS buffer and kept in the dark. 5 ml of methanol was added to 0.015 mg of 1, 4-chloronaphthol. This was mixed with the previous solution and finally added to the membranes.

3.2.6.2 ELISA

ELISA plates (high bind polystyrene EIA/RIA 96-well microplate, Corning, NY) were coated with 10 µg/ml purified insoluble PlpE from *E. coli* in PBS and incubated overnight at 4°C. After three shaking washes with PBS-Tween (PBS, pH 7.4, plus 0.05% Tween 20), plates were blocked with 5% skim-milk powder in PBS-Tween and incubated shaking for 2 hours at RT. After three washes with PBS-Tween, mice sera were added to the first rows of the plates at a dilution of 1:100 and then serially diluted two-fold using skim-milk in PBS-Tween. The plates were then incubated shaking for 1 h at 37°C before being washed three times with PBS-Tween and incubated with horseradish peroxidise (HRP) conjugated goat anti-mouse-IgG (Sigma–Aldrich, Saint Louis, MO), at a dilution of 1:1000 in blocking buffer (0.05% skim-milk powder in 1X PBS), for 1 h at 37°C. After washing four times with PBST, the reaction was detected using TMB peroxidase substrate (Bio-Rad, Hercules, CA) following manufacturer's instructions for 15 min. The reaction was stopped with 1N H_2SO_4 and then the absorbance read at 450 nm using a Microplate Reader Thermo Max (Molecular Devices Inc., Sunnyvale, CA). Titres were estimated as the reciprocal of the maximum dilution of serum giving an absorbance reading of 0.1 units after subtraction of non-specific binding in serum from non-treated animals (negative control).

3.2.7 Statistical analyses

GraphPad Prism 6 was used for all statistical analyses. One-way ANOVA with Tukey's multiple comparisons test was used to determine statistical significant difference between means of the data from three or more groups. Log-rank (Mantel-Cox) test was used to analyze the survival rate/proportions of different groups of mice. Regardless of the test applied, results were considered statistically significant if $P < 0.05$.

3.3 Results

3.3.1 Mouse vaccination Trial 1

During Trial 1, the aim was to deliver 20 µg of plant-PlpE to the mice. However, when the first dose was delivered a violent toxic reaction was observed in the animals and three died. The plant extracts were therefore dialyzed through a 10 µm membrane before the 2nd

immunization and the quantity of PlpE was decreased to 8-10 µg. Another seven mice were added to this group to maintain the sample size so that the final treatment size was 11 mice. The above described negative reaction was not further observed in the mice and the following two doses were kept at 8-10 µg of plant-PlpE.

3.3.1.1 Antibody response

100% of the sera collected from *E. coli* and transient plant-PlpE injected groups of mice produced antibody response against recombinant *E. coli* PlpE in Western blotting while the negative control groups produced none (Figure 3.3). The mice immunized with the *E. coli* PlpE produced stronger reactions than those immunized with transient plant-PlpE (Figure 3.3).

3.3.1.2 Elisa

The Western blot results were confirmed by ELISAs developed for PlpE-specific total antibody titre detection. The PlpE-specific antibody titre was significantly higher in sera of mice immunized with 50 µg insoluble *E. coli* PlpE than that in mice immunized with 10 µg insoluble *E. coli* PlpE or 8-10 µg transient plant-PlpE (Figure 3.5, A). However, there was no significant difference between PlpE-specific antibody titres between mice immunized with 10 µg *E. coli* or transient plant-PlpE (Figure 3.5, top). PlpE-specific antibody titres increased significantly in the sera collected after the 4^{th} immunization than that collected after the 2^{nd} immunization (Figures 3.4 & 3.5, B).

3.3.1.3 Survival

After challenge, the mice from the negative control groups died within 24 hours, (Table 3.3) whereas the death of mice from the *E. coli* PlpE injected groups was delayed for 60 hours on average. Only one mouse from the 10 µg *E. coli* PlpE immunized group survived challenge while 2 survived from the group immunized with 50 µg of same PlpE. Conversely, 7 mice from the 8-10 µg transient plant-PlpE survived challenge, with the death of non-surviving mice of this group being delayed for 72 hours on average (Figure 3.7). The protection level was significantly higher in mice immunized with transient plant-PlpE compared to that in mice in negative control groups as well as in *E. coli* PlpE groups (Figure 3.7). No significant difference was found between anti-PlpE antibody titres produced in the sera of the surviving and non-surviving mice that received *E. coli* or plant-PlpE (Figure 3.6).

Table 3.3 Summarized results from the pathogen challenge trial 1 in mice with *P. multocida*

Treatment group	Challenge strain and dose	% of survival	Average hours to death	Antibody Response in Western blot analysis
Transient Plant-PlpE	X-73 1.3×10^3	64* (7/11)	72	Moderate
Transient empty vector (control)	X-73 1.3×10^3	0 (0/7)	24	none
***E. coli* PlpE Insoluble** **10 µg**	X-73 1.3×10^3	14^{ns} (1/7)	60	Strong
***E. coli* PlpE Insoluble** **50 µg**	X-73 1.3×10^3	29^{ns} (2/7)	60	Strong
Adjuvant only control (Al Hydrogel®)	X-73 1.3×10^3	0 (0/7)	24	None

*=significant, ns= not significant

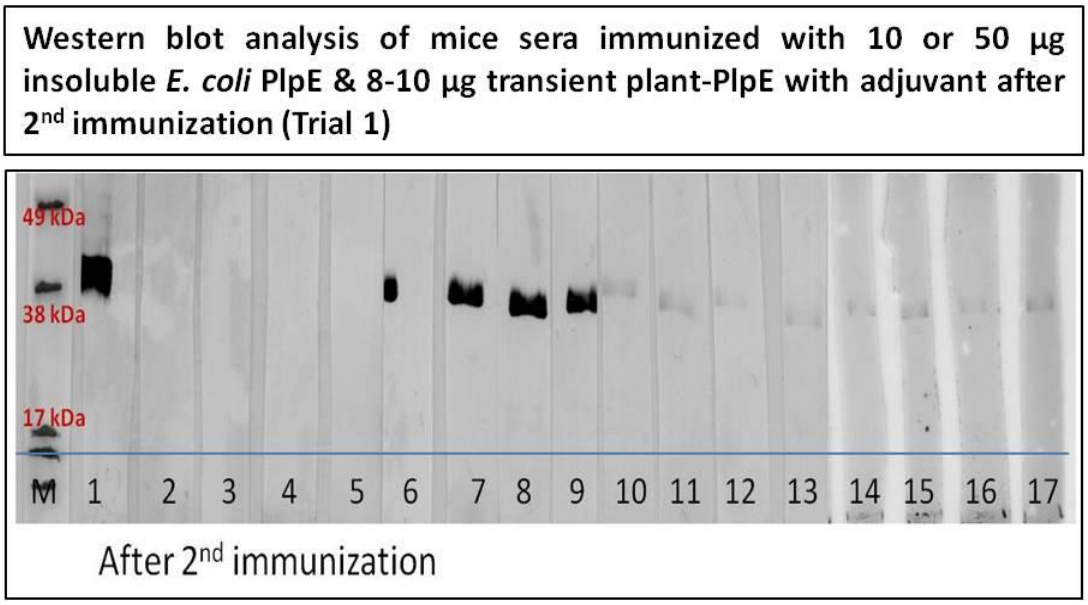


Figure 3.3 Western blot analyses showing the antibody responses in mice sera after the 2nd immunization (Trial 1). *E. coli* insoluble PlpE (300 ng/lane) was probed with mice sera collected after 2nd immunization (1:500) and with HRP conjugated polyclonal IgG goat secondary antibody (1:1200). M designates molecular mass standards (SeeBlue® Plus2, invitogen™), lane 1 represents positive control (anti-PlpE rabbit serum), lanes 2 & 3 show pre-immune sera, lanes 4 & 5 show sera from mice immunized with adjuvant only, lanes 6 & 7 show sera from mice immunized with 10 µg insoluble *E. coli* PlpE, lanes 8 & 9 show sera from mice immunized with 50 µg insoluble *E. coli* PlpE, lanes 10 to 17 show sera from mice immunized with 8-10 µg transient plant-PlpE.

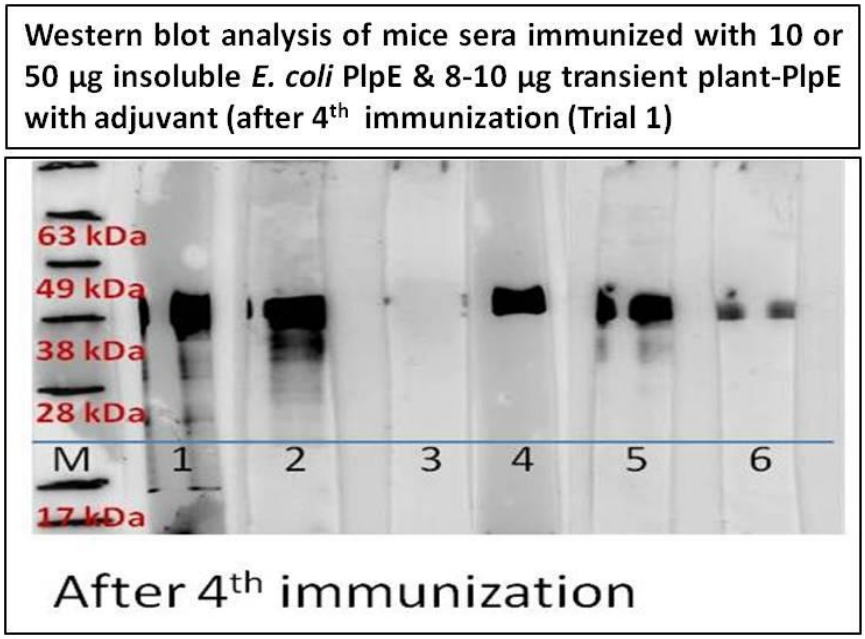


Figure 3.4 Western blot analyses showing the antibody responses in mice sera after the 4th immunization (Trial 1). *E. coli* insoluble PlpE (300 ng/lane) was probed with mice sera collected after 2nd immunization (1:500) and with HRP conjugated polyclonal IgG goat secondary antibody (1:1200). M stands for molecular mass standards (SeeBlue® Plus2, invitogen™), lanes 1 & 2 show sera from mice immunized with 10 & 50 µg insoluble *E. coli* PlpE respectively, lane 3 shows serum from mouse immunized with adjuvant only, lanes 4, 5 & 6 show sera from mice immunized with 8-10 µg transient plant-PlpE.

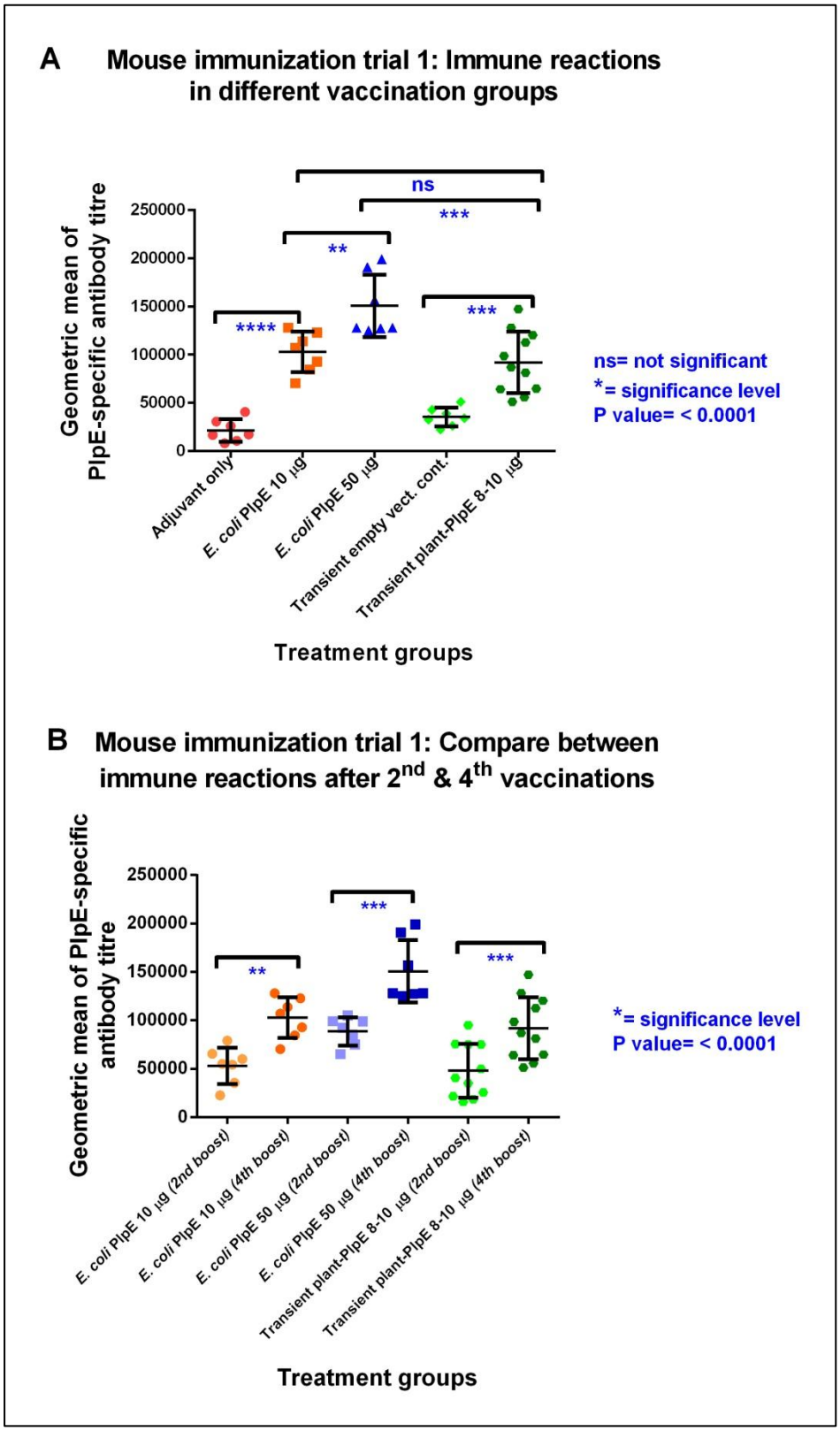


Figure 3.5 Detection of PlpE-specific antibody titres in mice sera by ELISA. A: Comparison of antibody titres in *E. coli* PlpE and transient plant-PlpE injected mice groups after the 4th or final immunization. B: Comparison of antibody titres after the

2nd and 4th immunizations in *E. coli* PlpE and transient plant-PlpE injected mice groups. The data represents the average values from 3 replications of 7-11 mice. The comparisons were performed using one-way ANOVA followed by a Tukey's Multiple Comparison test. The dots represent the average titre for an individual mouse and the black lines indicate the mean titre for each group. The error bars represent the standard error of the mean (SEM).

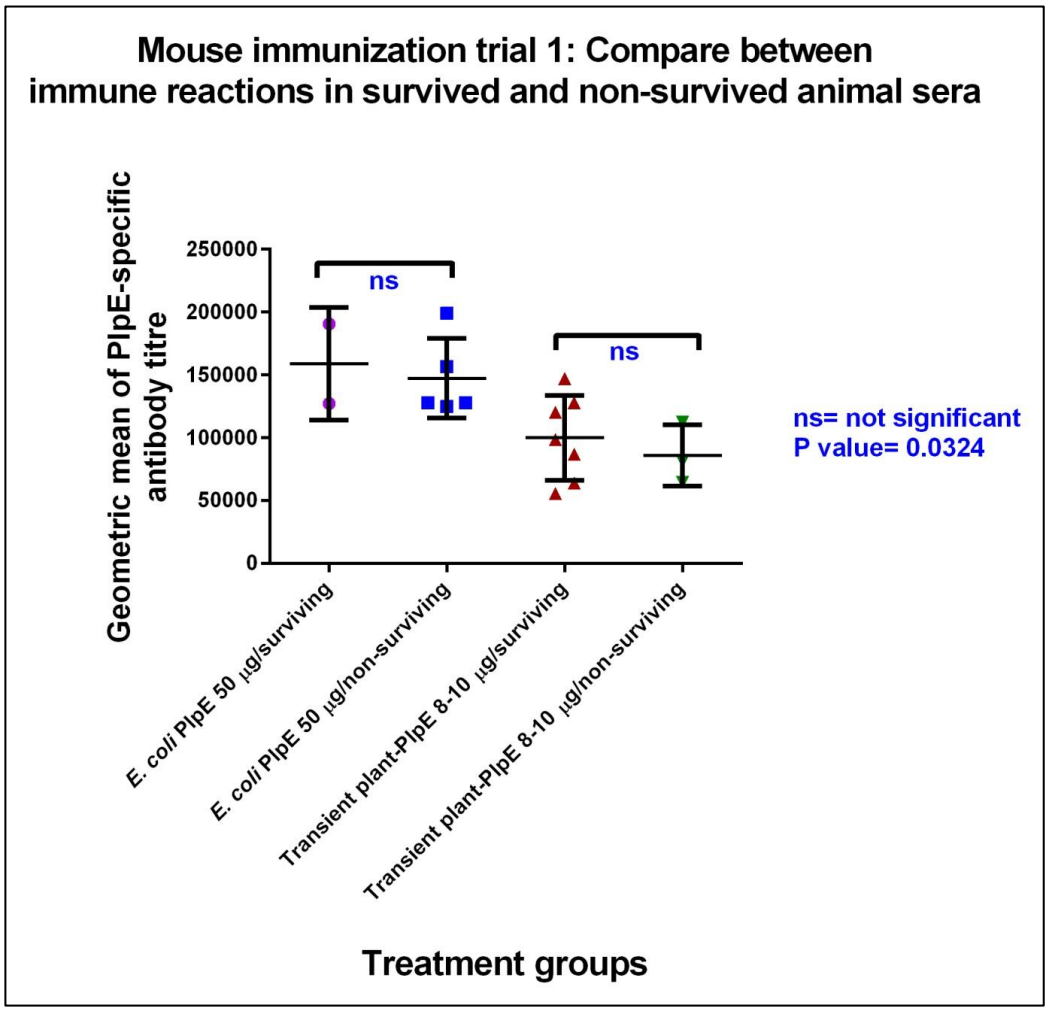


Figure 3.6 Detection of PlpE-specific antibody titres in mice sera by ELISA. Comparison of antibody titres after the 4th or final immunization in surviving and non-surviving mice delivered *E. coli* PlpE (50 µg) and transient plant-PlpE (8-10 µg). The data represents the average values from 3 replications of 7-11 individuals. The comparisons were performed using one-way ANOVA followed by a Tukey's Multiple Comparison test. The dots represent the titre for individual mouse and the black lines

indicate the mean titre for each group. The error bars represent the standard error of the mean (SEM).

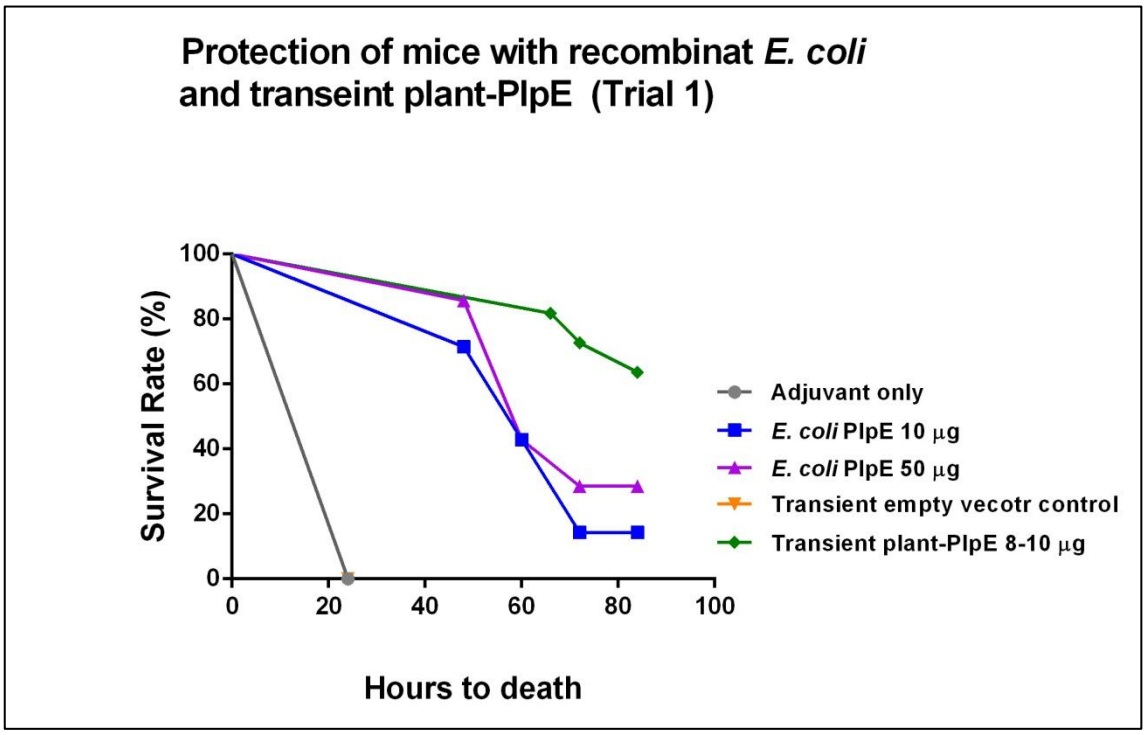


Figure 3.7 Comparisons of the survival curves in different groups of mice during immunization trial 1. Statistical analysis was performed using the Log-rank (Mantel-Cox) Test.

3.3.2 Mouse vaccination trial 2

Whole cell killed vaccine and negative control groups

Mice sera collected from the negative control groups (adjuvant only, transient empty vector and transgenic empty vector controls) produced no antibody response against recombinant *E. coli* PlpE in Western blot analysis (Figure 3.8, A). The mice immunized with the whole cell killed bacterins also did not produce any specific reaction against *E. coli* recombinant PlpE in their sera in Western blot analysis (Figure 3.8, B). These mice also did not show significantly high PlpE-specific antibody titre in ELISA compared to that in adjuvant only negative control (Figure 3.13). The animals in the negative control groups died within 24 hours of the pathogen challenge. The animals in whole cell killed vaccinated group died within 24-50 hours (Figure 3.15), which was not significantly different compared to that in animals from adjuvant only negative control group.

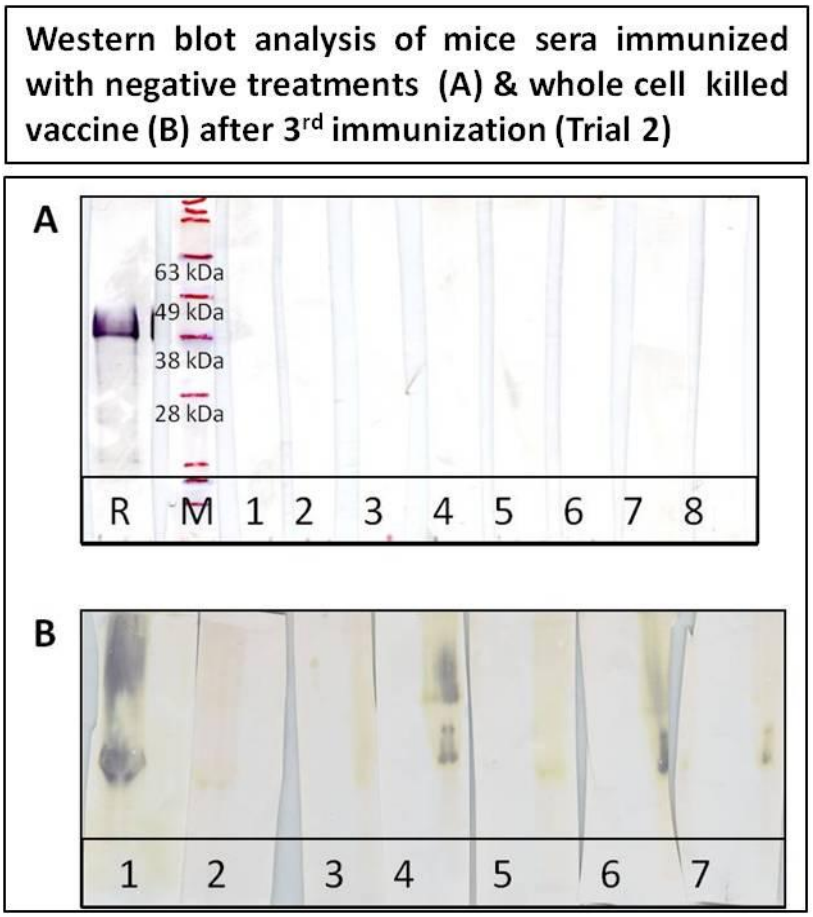


Figure 3.8 Western blot analyses showing the antibody responses in mice sera after the 3rd or final immunization (Trial 2). *E. coli* insoluble PlpE (300 ng/lane) was probed with mice sera collected after 3rd immunization (1:500) and with HRP conjugated polyclonal IgG goat secondary antibody (1:1200). A: Lane R shows positive control (anti-PlpE rabbit serum), M stands for molecular mass standards (SeeBlue® Plus2, invitogen™), lanes 1 & 2 show pre-immune sera, lanes 3 & 4 show sera from mice immunized with adjuvant only, lanes 5 & 6 show sera from mice immunized with transient plant empty vector control, lanes 7 & 8 show sera from mice immunized with transgenic plant empty vector control. B: Lanes 1 to 7 show sera from 7 mice immunized with whole cell killed bacterins.

Insoluble and soluble *E. coli* PlpE

100% of the mice injected with insoluble *E. coli* PlpE and 70% of the mice injected with soluble *E. coli* PlpE produced strong antibody response against insoluble *E. coli* recombinant PlpE during western blot analysis (Figures 3.9, A & B respectively). 90% of mice sera immunized with 100 µg insoluble *E. coli* PlpE produced strong to moderate reactions against transient plant-PlpE during western blot analysis (Figure 3.10). PlpE-specific antibody titres were found to be significantly higher in *E. coli* plpE immunized animal groups compared to that in animals immunized with adjuvant only (Figure 3.13). The survival rate was 60% and 50% in the groups of mice immunized with insoluble and soluble *E. coli* PlpE respectively (Figure 3.15), which was significantly higher than the negative control adjuvant only group. The death of the non-surviving animals was delayed 108 hours on average in these groups (Figure 3.15), which was also significantly higher compared to that in animals in negative control group.

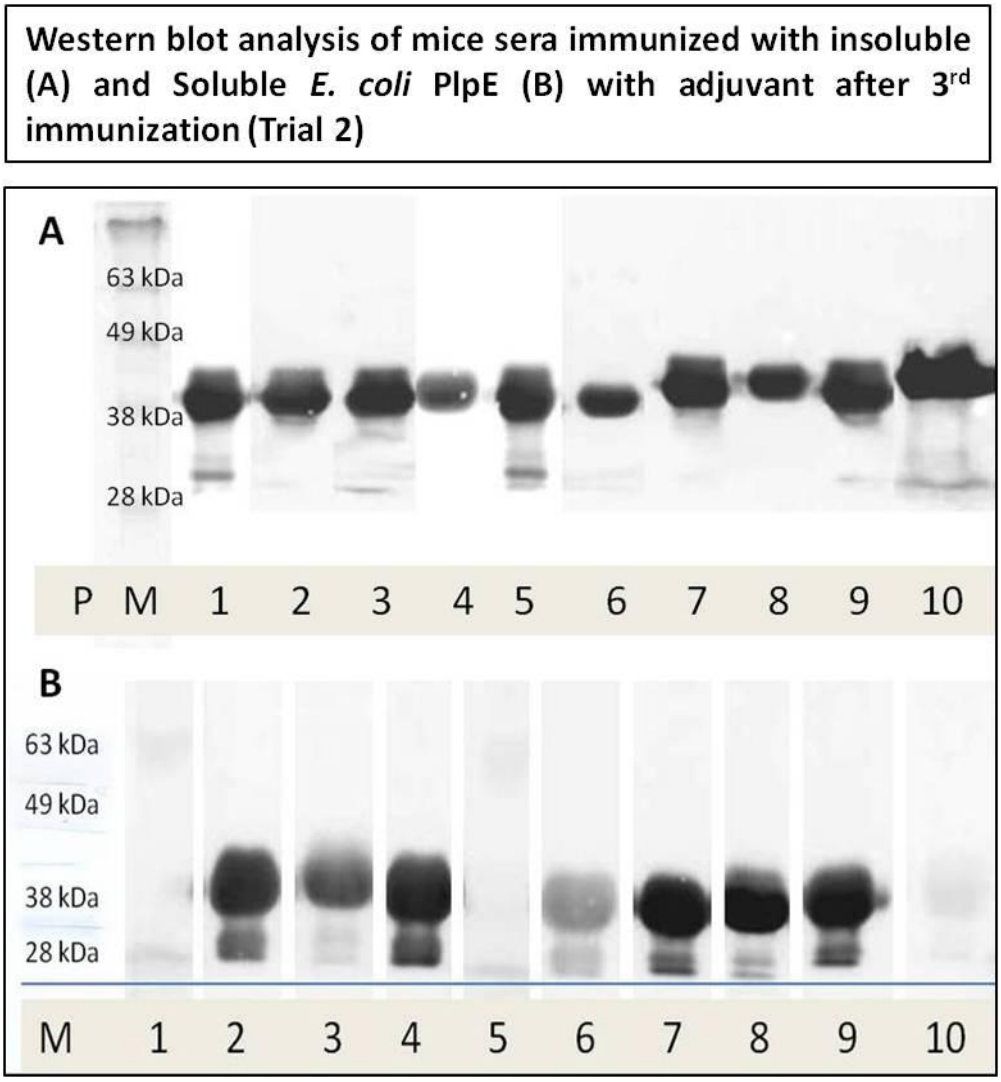


Figure 3.9 Western blot analyses showing the antibody responses in mice sera after the 3rd or final immunization (Trial 2). *E. coli* insoluble PlpE (300 ng/lane) was probed with mice sera collected after 2nd immunization (1:500) and with HRP conjugated polyclonal IgG goat secondary antibody (1:1200). A: Lane P shows serum from mouse immunized with adjuvant only, M stands for molecular mass standards (SeeBlue® Plus2, invitogen™), Lanes 1 to 10 show sera from 10 mice immunized with 100 µg insoluble *E. coli* PlpE with adjuvant. B: M stands for molecular mass standards, lanes 1 to 10 show sera from 10 mice immunized with 100 µg soluble *E. coli* PlpE with adjuvant.

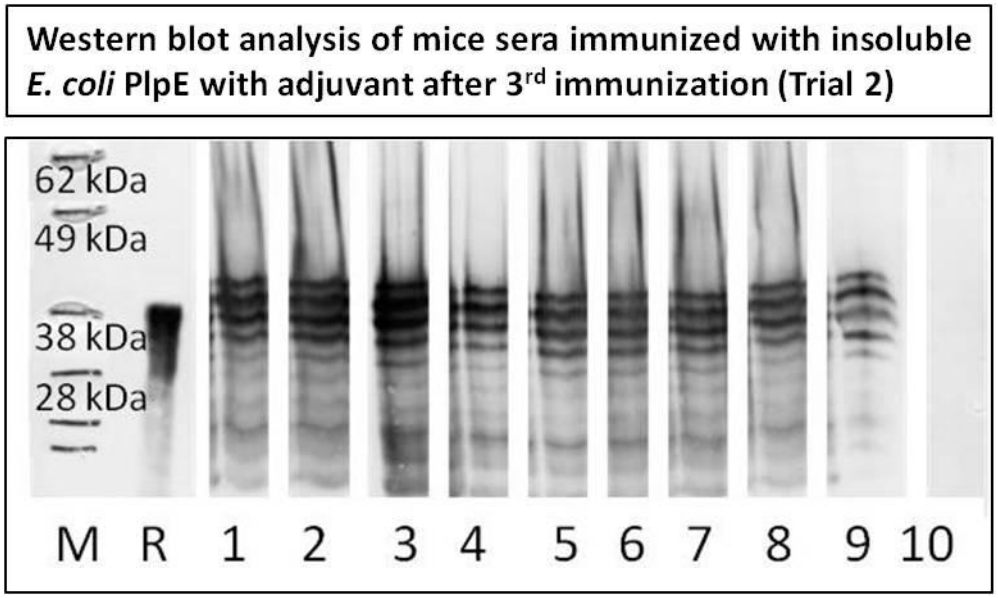


Figure 3.10 Western blot analyses showing the antibody responses in mice sera after the 3rd or final immunization (Trial 2). Transient plant-PlpE (600 ng/lane) was probed with mice sera collected after 3rd immunization (1:500) and with HRP conjugated polyclonal IgG goat secondary antibody (1:1200). M stands for molecular mass standards (SeeBlue® Plus2, invitogen™), R shows positive control (Anti-PlpE rabbit serum) and lanes 1 to 10 show sera from 10 mice immunized with 100 µg insoluble *E. coli* PlpE with adjuvant.

Stable transgenic plant-PlpE

90% of the mice injected with stably transgenic plant-PlpE with adjuvant and 60% of the mice injected with stably transgenic plant-PlpE without adjuvant produced moderate reactions against recombinant insoluble *E. coli* PlpE (Figure 3.11, A & B respectively). But the same sera did not react with transient plant-PlpE when run side by side in the same membrane (Figure 3.11, A & B respectively). The survival rate was only 10% in mice immunized with stable transgenic plant-PlpE without adjuvant whereas; no animal was protected in the group immunized with stable plant-PlpE with adjuvant (Figure 3.13). However, the average hours to death was delayed up to 70 and 66 hours respectively, which were significantly higher compared to that in animals in control groups (Figure 3.15).

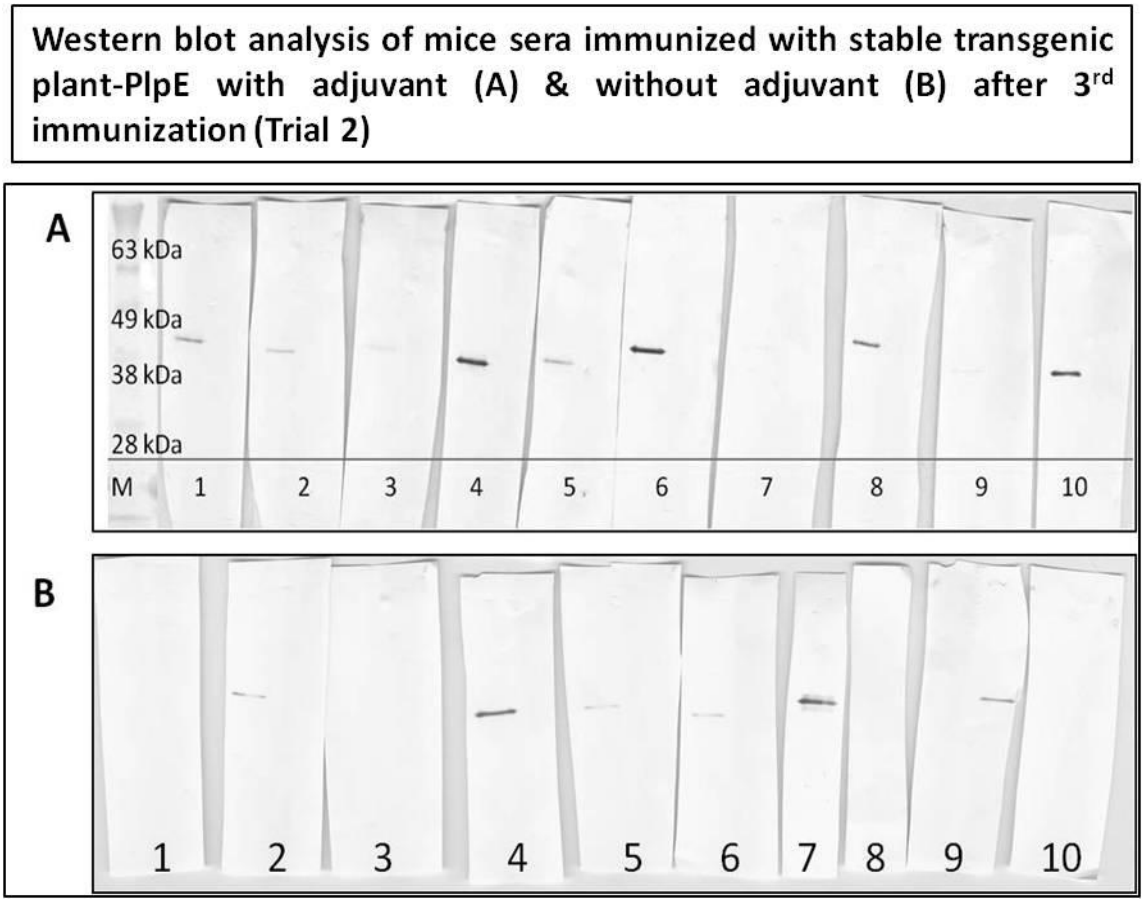


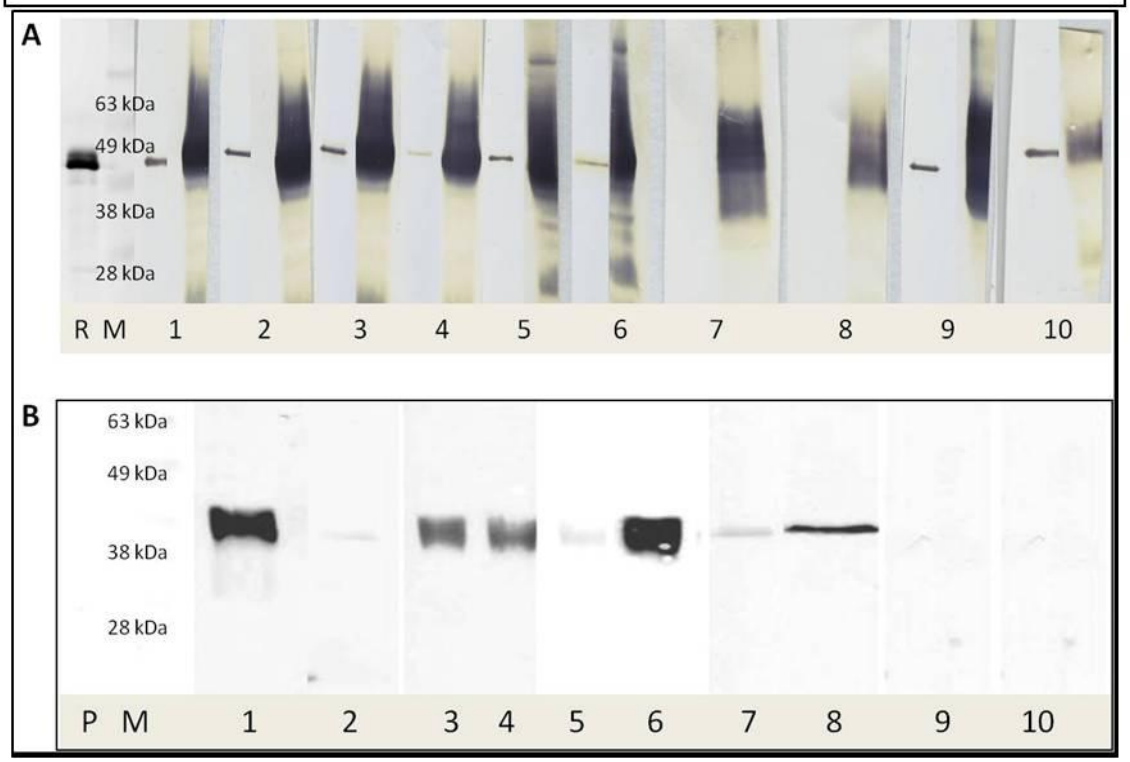
Figure 3.11 Western blot analyses of antibody responses in mice sera after the 3rd or final immunization (Trial 2). *E. coli* insoluble PlpE (300 ng/lane) and transient plant-PlpE (600 ng/lane) was probed with mice sera collected after 3rd immunization (1:500) and with HRP conjugated polyclonal IgG goat secondary antibody (1:1200). A: M stands for molecular mass standards (SeeBlue® Plus2, invitogen™), lanes 1 to 10 show sera from mice immunized with 100 µg stable transgenic plant-PlpE with adjuvant; B: lanes 1 to 10 show sera from 10 mice immunized with 100 µg stable transgenic plant-PlpE without adjuvant.

Transient plant-PlpE

100% of mice sera collected from the group administered with transient plant-PlpE with adjuvant produced strong antibody responses against transient plant-PlpE and moderate reactions against *E. coli* insoluble PlpE in Western blot analysis (Figure 3.12, A) when *E. coli* and plant proteins were run side by side in the same membrane. Moreover, 100% of mice sera collected from the same group produced strong to moderate reactions against *E. coli* soluble PlpE in Western blot analysis (Figure 3.12, C). 80% of the sera collected from the group

administered with transient plant-PlpE without adjuvant produced strong antibody response against *E. coli* insoluble PlpE during Western blot analysis (Figure 3.12, B). The PlpE-specific antibody titre was significantly higher in animal groups immunized with transient plant-PlpE (both with & without adjuvant added) compared to that in transient empty vector control group (Figure 3.13). However, no significant difference was observed in the animal sera immunized with *E. coli* or transient plant-PlpE in terms of anti-PlpE titres (Figure 3.13). 70% and 40 % mice in the groups immunized with transient plant-PlpE survived, with or without adjuvant added respectively (Figure 3.15). The average hours to death was delayed for 108 and 72 hours on average in non-surviving animals in these groups, which were significantly higher compared to that in transient plant empty vector group.

Western blot analysis of mice sera immunized with transient plant-PlpE with adjuvant (A) & without adjuvant (B) after 3rd immunization (Trial 2)



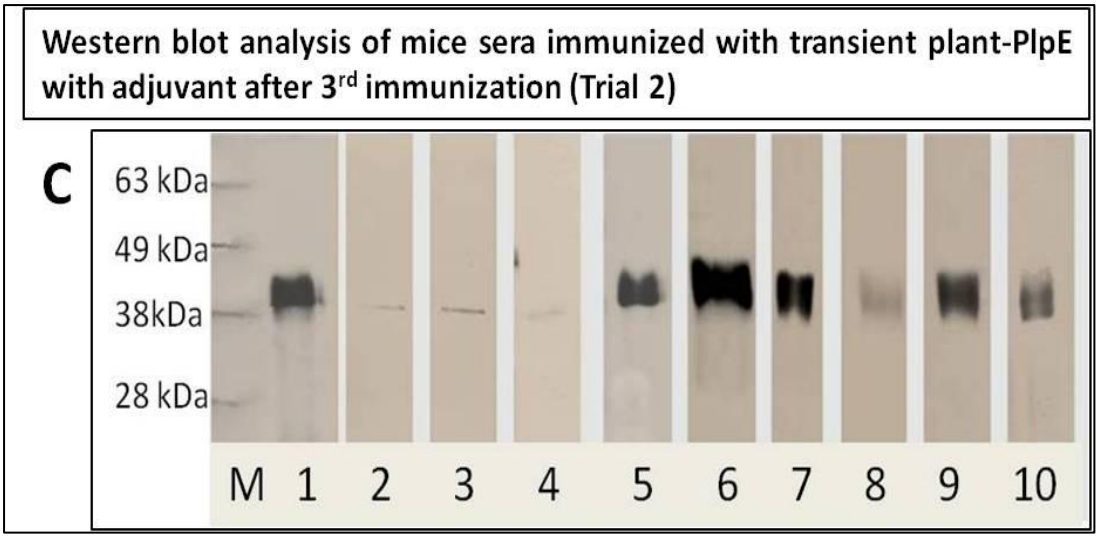


Figure 3.12 Western blot analyses showing the antibody responses in mice sera after the 3rd or final immunization (Trial 2). A: *E. coli* insoluble PlpE (300 ng/lane) and transient plant-PlpE (600 ng/lane) was probed with mice sera collected after 3rd immunization (1:500) and with HRP conjugated polyclonal IgG goat secondary antibody (1:1200). M stands for molecular mass standards (SeeBlue® Plus2, invitogen™), R shows positive control (Anti-PlpE rabbit serum), lanes 1 to 10 show sera from mice immunized with 100 µg transient plant-PlpE with adjuvant; B: *E. coli* insoluble PlpE (300 ng/lane) was probed with mice sera collected after 3rd immunization (1:500) and with HRP conjugated polyclonal IgG goat secondary antibody (1:1200). M stands for molecular mass standards, P shows serum from mouse immunized with transient plant empty vector with adjuvant (negative control), lanes 1 to 10 show sera from mice immunized with 100 µg transient plant-PlpE without adjuvant; C: *E. coli* soluble PlpE (300 ng/lane) was probed with mice sera collected after 3rd immunization (1:500) and with HRP conjugated polyclonal IgG goat secondary antibody (1:1200). M stands for molecular mass standards and lanes 1 to 10 show sera from mice immunized with 100 µg transient plant-PlpE with adjuvant.

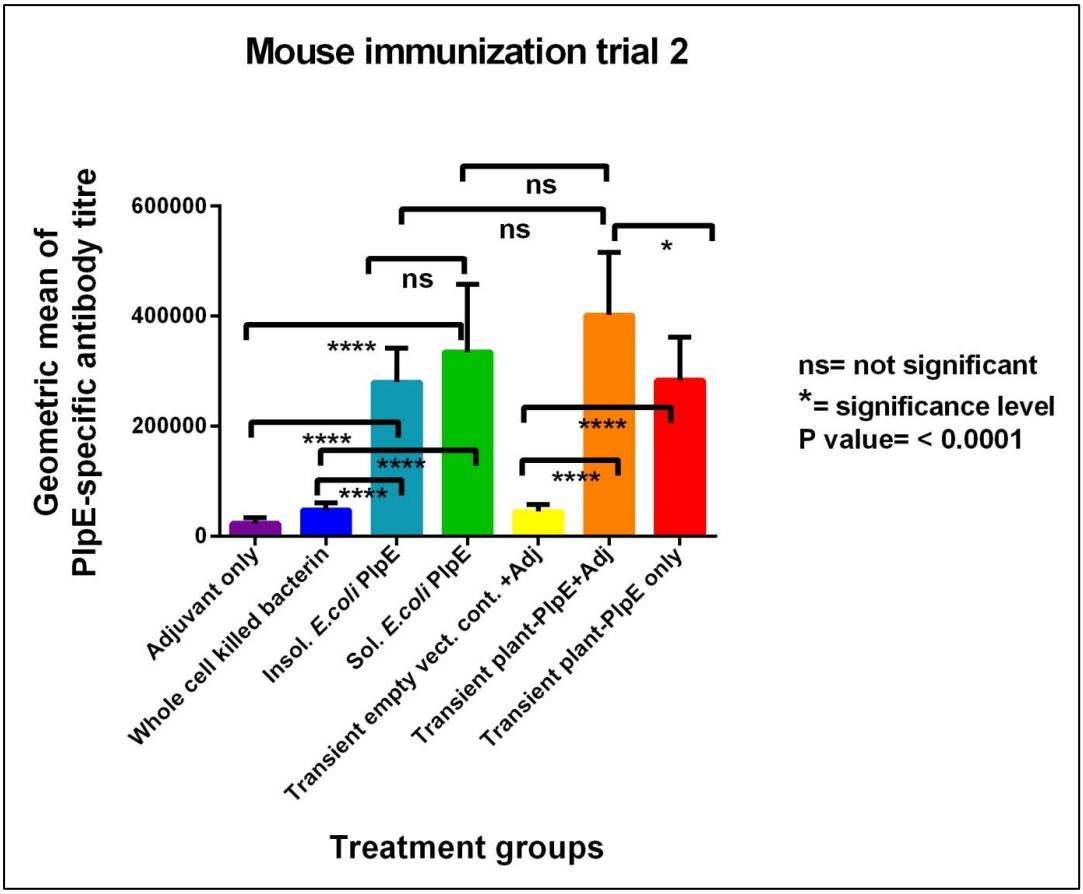


Figure 3.13 Detection of PlpE-specific antibody titres in mice sera by ELISA after 3rd or final immunization (Trial 2). Comparison of antibody titres in sera of mice immunized with 100 µg insoluble & soluble *E. coli* PlpE, 2.065 X 10^3 CFU whole cell killed vaccine and 100 µg transient plant-PlpE. The data represents the average of three replications on individual mice serum. The comparisons were performed using one-way ANOVA followed by a Tukey's Multiple Comparison test. The solid bars represent the average titre of each group of mice and the error bars represent the standard error of the mean (SEM).

No significant difference could be found in PlpE-specific antibody titres between the surviving and non-surviving mice that received *E. coli* or transient plant-PlpE (Figure 3.14). The protection level was significantly higher in groups of mice immunized with transient plant-PlpE, insoluble and soluble *E. coli* PlpE compared to that in mice in respective negative control groups (Figure 3.15). The results from Trial 2 are summarized in table 3.4.

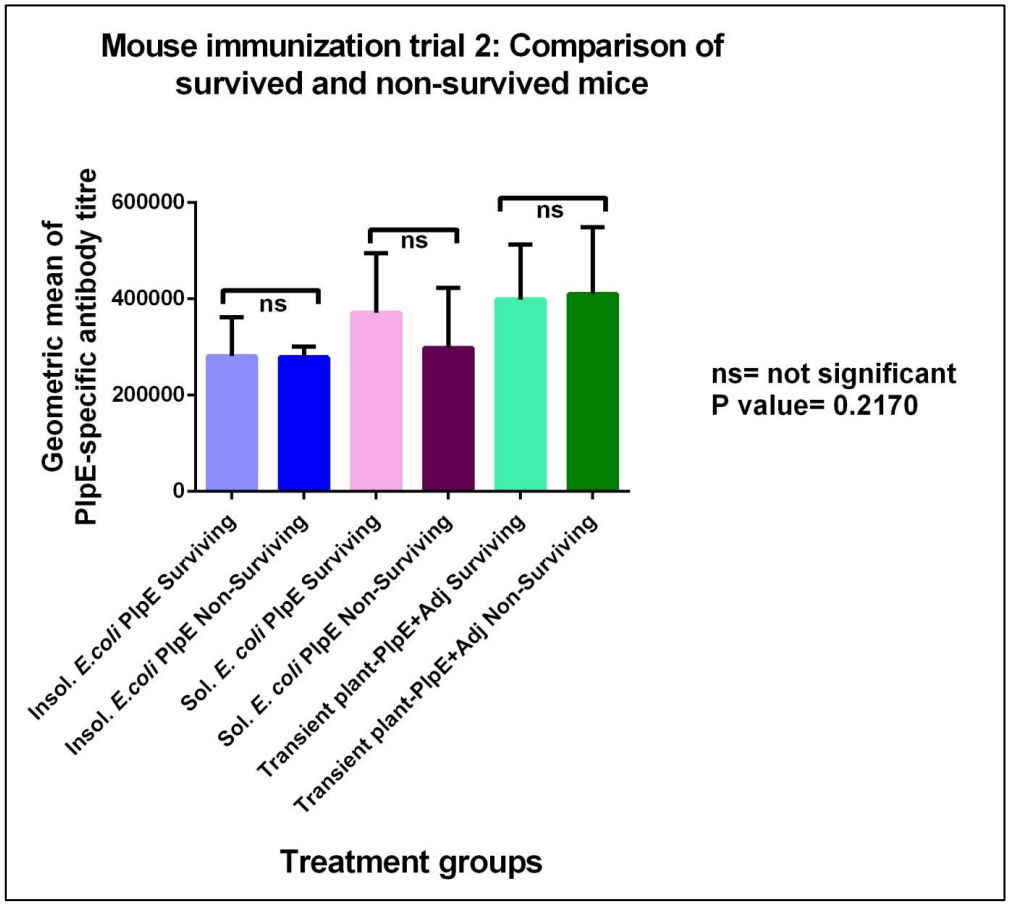


Figure 3.14 Comparison of antibody titres in sera after the 3rd or final immunization in surviving and non-surviving mice delivered insoluble & soluble *E. coli* PlpE (100 µg) and transient plant-PlpE (100 µg). The data represents the average of three replicates of individual mice serum. The comparisons were performed using one-way ANOVA followed by a Tukey's Multiple Comparison test. The solid bars represent the average titre of each group of mice and the error bars represent the standard error of the mean (SEM).

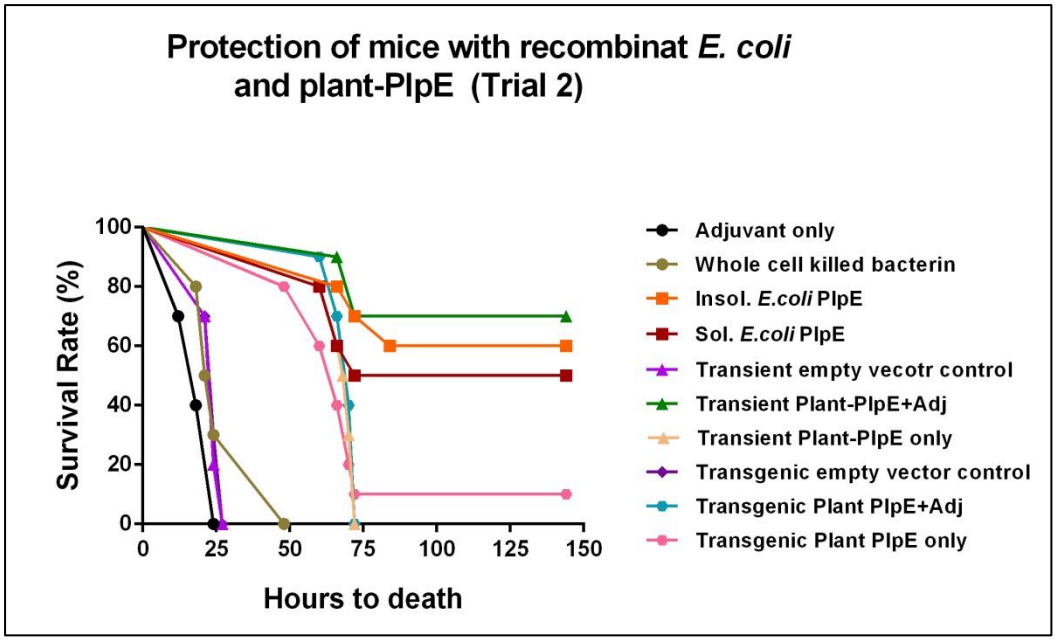


Figure 3.15 Comparisons of the survival curves in different groups of mice during immunization trial 2. Statistical analysis was performed using the Log-rank (Mantel-Cox) Test.

Table 3.4 Summarized results from the pathogen challenge trial 2 in mice with *P. multocida*

Treatment group	Challenge strain and dose	% of survival	Average hours to death	Antibody response in Western blot analysis
Transient plant-PlpE with adjuvant	X-73 1.4×10^3	**70* (7/10)**	**108**	**Strong**
Transient plant-PlpE without adjuvant	X-73 1.4×10^3	**0 (0/10)**	**70**	**Strong**
Transient empty vector control with adjuvant	X-73 1.4×10^3	**0 (0/10)**	**24**	**None**
Stable transgenic plant- PlpE with adjuvant	X-73 1.4×10^3	**0 (0/10)**	**70**	**Moderate**
Stable transgenic plant- PlpE without adjuvant	X-73 1.4×10^3	**10^{ns} (1/10)**	**66**	**Moderate**
Stable transgenic empty vector control with adjuvant	X-73 1.4×10^3	**0 (0/10)**	**24**	**None**
***E. coli* PlpE Insoluble with adjuvant**	X-73 1.4×10^3	**60* (6/10)**	**72**	**Strong**
***E. coli* PlpE Soluble with adjuvant**	X-73 1.4×10^3	**50* (5/10)**	**108**	**Strong**
Whole cell killed bacterins with adjuvant	X-73 1.4×10^3	**0 (0/7)**	**24**	**None**
Adjuvant only	X-73 1.4×10^3	**0 (0/10)**	**24**	**None**

*=significant, ns= not significant

3.4 Discussion

3.4.1 Whole cell killed vaccine

Mice immunized with a bacterin composed of 2.065×10^3 CFU of heat-inactivated X-73 failed to produce PlpE-specific antibody titres significantly higher than titres of mice immunized with adjuvant only. The animals were not protected, dying within 24-50 hours of pathogen infection. Wu et al. (2007) reported that mice immunized with a lower dose (1.25×10^7 CFU) of X-73 whole cell killed vaccine were not protected (17% survival). When a bacterin composed of 2.5×10^7 CFU of X-73 was used, and the challenge dose of X-73 was increased to 60 CFU (>20 LD50) they were moderately protected (50% survival). Therefore, in this trial the dose of 2.065 X 10^3 CFU of X-73 was not sufficient to produce protective immune response in mice and a much higher dose of whole cell killed vaccine would be required.

3.4.2 *E. coli* PlpE vaccines

During the first trial the PlpE-specific antibody titre was high in sera collected from the mice injected with 10 or 50 µg insoluble *E. coli* PlpE. However, the protection level after the pathogen challenge trial was low (only 10% and 20% respectively) and not significantly different to that of the negative control. This was not expected, as previous mouse trials performed in the laboratory resulted in a survival rate of 70% when the animals received a 150 µg dose of insoluble *E. coli* PlpE twice (Hatfaludi et al., 2012). In addition, Wu et al. (2007) showed that only 10 µg of soluble *E. coli* PlpE protected 70% of mice from challenge with *P. multocida* strain X-73. It appears that in this trial a threshold level of recombinant antigen was delivered and that the higher dose of insoluble or soluble *E. coli* PlpE was required for protection in mice.

To check the effect of increased vaccine dose on PlpE-specific antibody titre and protection level a larger sample size of 10 animals and a vaccine dose of 100 µg was used during the second mouse trial. The animals were vaccinated 3 times. This treatment resulted in the PlpE-specific antibody titres increasing significantly in sera of the mouse groups immunized with 100 µg insoluble *E. coli* PlpE compared to that in mice groups immunized with 10 or 50 µg of the same vaccine (Figure 3.16 A) with the survival rate also improving significantly (60%) compared to that in negative control group animals (Figure 3.15).

To check the effect of solubility of the antigen on protection level in mice, a soluble *E. coli PlpE* was tested during the second vaccine trial. A group of mice received 100 µg of of the soluble *E. coli* PlpE 3 times. The soluble antigen produced significantly higher antibody titres

in animal sera compared to that in non-vaccinated group and the survival rate was 50%. However, no significant difference could be detected in sera of the mice treated with insoluble and soluble *E. coli* PlpE in terms of PlpE-specific antibody titre (Figure 3.14) nor in the survival rate (50% & 60% respectively) (Figure 3.15).

It was thought that the PlpE-specific antibody titre would be positively correlated with the protection level in animals as it had been previously suggested that protection mainly comes from the humoral immune response agaisnt *P. multocida* infections (Collins, 1973; Collins et al., 1976). However, the prediction proved to be wrong as no significant PlpE-specific antibody titre difference was observed between the mice that survived and those that didn't (Figure 3.13). These results indicate that the immune mechanism that protects mice against *P. multocida* infection is not dictated by total IgG response. Survival may be due to a specific

IgG isotype and/or a cell-mediated response. Unfortunately the role of cell-mediated immunity aganist *P. multocida* infections is not well-documented in the literature. Collins (1977) suggested that the primary role of antibody against *P. multocida* infection is the inhibition of the rapid spread of the organism to the blood stream and other reticulo-endothelial organs. However, evidence for the induction of cell-mediated immunity against *P. multocida* infection in mice was reported by Hyo-ik Ryu et al. (2000). They found that mice immunized with the LPS-protein complex of *P. multocida* were able to rapidly recruit a large number of phagocytic cells at the site of infection due to release of lymphokines by T lymphocytes. The production of lymphokines by T lymphocytes indicates the induction of cell-mediated immunity, which may require significant time and appropriate antigenic stimulation (Adelman et al., 1979). They suggested that *P. multocida* may be eliminated by neutrophils and macrophages if large numbers of these cells are present at the site of infection like the other facultative intracellular parasites. Hyo-ik Ryu et al. (2000) also suggested that antibodies against the pathogen may play a role in resistance by inhibiting the rapid spread of the pathogen through the host body while certain surface material, such as the LPS-protein complex, may serve as a stimulatory agent for the later development of cell-mediated immunity. Therefore the mechanisms behind the protection of mice by PlpE antigen against *P. multocida* infection might require inducing both humoral and cell-mediated immunity.

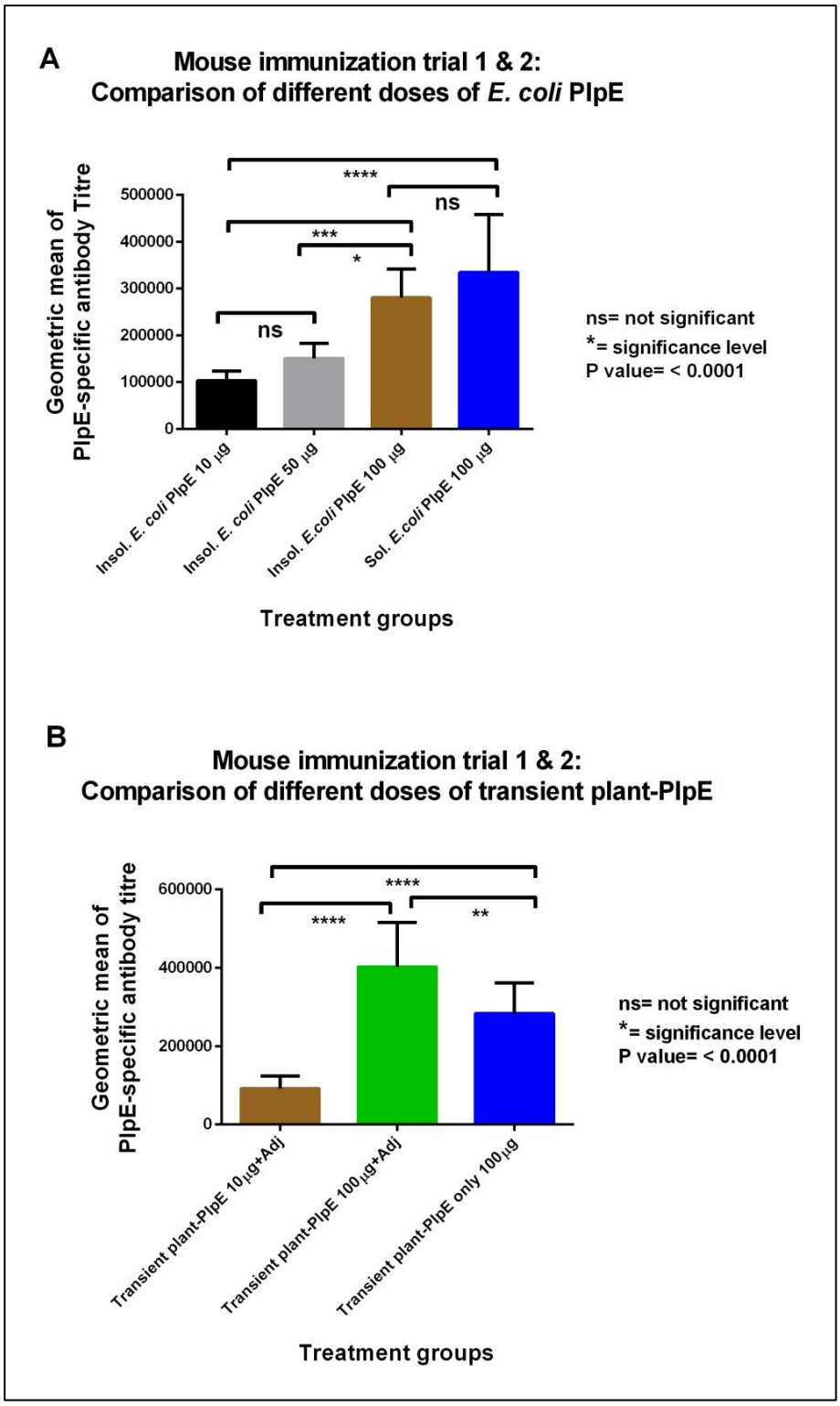


Figure 3.16 Detection of PlpE-specific antibody titres in mice sera by ELISA (Trial 1 & 2). A: Comparison of antibody titres in sera of mice immunized with 3 different doses of insoluble *E. coli* PlpE (10, 50 & 100 µg) and 100 µg soluble *E. coli* PlpE; B: Comparison

of antibody titres in sera of mice immunized with 2 different doses (8-10 & 100 µg) of transient plant-PlpE with adjuvant and 100 µg transient plant-PlpE without adjuvant. The data represents the average of three replications of individual mice serum. The comparisons were performed using one-way ANOVA followed by a Tukey's Multiple Comparison test. The solid bars represent the average titre of each group of mice and the error bars represent the standard error of the mean (SEM).

3.4.3 Plant-PlpE vaccines

The transient plant-PlpE vaccine was produced in two ways. It was produced through hand-injection and vacuum-infiltration of *Agrobacterium tumefaciens* cells carrying pBinPlpE vectors. A violent toxic reaction was observed in mice delivered with hand-infiltrated plant-PlpE and 3 animals died. It was thought that the toxic affect resulted from the alkaloids presented in the *N. benthamiana* leaf tissues. To check this prediction the vaccine material was dialyzed through a membrane with 10 kDa cut-off to get rid of the small molecules of alkaloids and then administered to animals. The outcome was no toxicity upon dialysis. In the second phase of the project, transient plant-PlpE was produced through vacuum-infiltration. The quantity of alkaloid was determined in both transient plant-PlpE preparations by a HPLC analysis and it was found that the alkaloid level was much higher in hand-injected leaf material than in vacuum-infiltrated samples (Chapter 2). Therefore, vacuum-infiltrated leaf powder was used as the vaccine material during the second trial.

Sera collected from the mice immunized with transient and stably transgenic plant-PlpE produced one band at 40 kDa in Western blot analysis, which is the molecular mass for insoluble *E. coli* PlpE protein. Therefore, the plant-based PlpE immunized sera reacted with and recongnized the *E. coli* recombinant PlpE. Sera collected from the animals immunized with transient plant-PlpE could recongnize and react with both insoluble *E. coli* and transient plant-PlpE (Figure 3.12), whereas sera collected from mice immunized with stably transgenic plant-PlpE could only react with *E. coli* PlpE and not with the transient plant-PlpE (Figure 3.11). This might be explained by the *N*-glycosylations observed in these plant-based anitigens. It is likely that different *N*-glycans were added to the transiently made PlpE to those on the stably transgenic plant-PlpE antigens as they were produced through in different species and different plant organs as found by Henquet et al. (2008). These different *N*-glycan

structures might be recongnized differently by the murine hosts (Chargelegue et al., 2000; Bardor et al., 2003; Jin et al., 2006).

The transient plant-PlpE protected 64% of the mice when only 8-10 µg was administered and protected 70% of the mice with a 100 µg dose. These protection rates were significantly higher compared to the protection afforded to the negative control groups receiving transeint plant empty vector. PlpE-specific antibody titre increased significantly in mouse sera with increased dose of transient plant-PlpE (Figure 3.16, B). However, there was no correlation between the PlpE-specific antibody titre in mice sera and survival rate or protection level (Figure 3.14). In addition, the stably transgenic plant-PlpE produced significantly higher PlpE-specific antibody titres in vaccinated mouse sera compared to that in transgenic plant empty vector control vaccinated mice sera. However, stably transgenic plant-PlpE did not elicited protection in animals with only 10% of the mice immunized with this vaccine without adjuvant survived the pathogen challenge (Figure 3.15).

Translation of the same *plpE* gene produced different banding patterns during Western blot analysis (Figure. 3.2). The different protection levels elicited by transeint and stably transgenic plant-PlpE antigens could be explained by different post-translational modificaitons of the same gene due to translation in different plant species, organs or tissues. As both antigens were observed to be *N*-glycosylated it might be possible that the carbohydrate structures added to the polypeptide chain were different or that different sites were glycosylated. It was observed that glycosylation changes unpredictably under changing environmental conditions and that a single protein may obtain different glycosylations to yield heterogenous glycoforms (Bosch & Schots, 2010). Therefore different glycans may have formed due to different environmental conditions such as pH, nutrient availability and cell status between cell culture and cells in vivo in leaves (Bosch & Schots, 2010; Brooks, 2009) and this could have affected immunogenicity and/or level of protection.

During the second mouse trial plant-based PlpE antigens were delivered to mice both with and without adjuvant. This was to test if plant cells alone could act as adjuvants and offer some benefit to immunization. Differing glycosylation states and alkaloids have been shown to affect immunogenicity (Faye & Chrispeels, 1988; Faye et al., 2005; Ling et al., 2012). Plant *N*-glycans or alkaloids could act as new vaccine adjuvants facilitating antigen capture by APCs (Burdin et al., 2004). Plant-based vaccines providing their own adjuvants could reduce cost through not requiring addition of other adjuvants along with the vaccines.

However, this hypothesis was not supported by the data obtained from this second mouse trial. It was observed that plant-PlpE antigens alone could not elicit sufficient protection in animals and Al Hydrogel® added some benefits with transient plant-PlpE administered to mice eliciting 70% protection as opposed to no protection without adjuvant.

3.5 Conclusions and future directions

PlpE or lipoprotein E is an outer membrane lipoprotein and a cross-protective antigen of *Pasteurella multocida*. A recombinant form was produced in *E coli* cell culture and reported to be 70% protective in mice and 100% in chickens against a lethal dose of *P. multocida* infection when administered sub-cutaneously (Wu et al., 2007; Hatfaludi et al., 2012). PlpE was produced in plants, transiently in *N. benthamiana* leaves and stably in *N. tabacum* cell lines. These plant-based PlpE antigens in the form of crude plant extracts were tested in mice through the systemic route to check if sufficient immunity could be induced to protect against challenge. Transient plant-PlpE adjuvanted with Al Hydrogel® (20% v/v) elicited 64% protection when 8-10 µg was administered to mice and 70% protection when 100 µg was delivered with adjuvant. These levels were significantly higher than the protection levels elicited by the negative controls (transient plant empty vector material and adjuvant only) as well as the positive control of whole cell killed vaccine. The whole cell killed vaccine most likely didn't work due to insufficient dose. The protection level elicited by 8-10 µg of transient plant-PlpE was also significantly higher than that elicited by 10 or 50 µg insoluble *E. coli* PlpE and the protection level elicited by 100 µg of transient plant-PlpE (70%) was higher than that elicited by 100 µg insoluble (60%) or soluble *E. coli* PlpE (50%). As both *E. coli* antigens were purified and the plant-PlpE was in crude plant extract the crude extract might offer some advantage even in injectable form in terms of cost, down-stream processing, heat-stability and handling of the vaccine. Stably produced plant-PlpE did not show the same efficacy as the transient form providing only 10% protection in mice. A possible reason for this difference could be the different type of *N*-glycosylation occurred to stable transgenic plant-PlpE in cell culture.

Another important finding of this study is that protection elicited by the lipoprotein E or PlpE does not originate from the PlpE-specific total IgG antibody titre produced in the mice as no significant difference could be found between the PlpE-specific antibody titres in sera of mice that survived and mice that did not. It is suggested that a specific IgG isotype and/or a combination of humoral and cell-mediated immunity is required to be elicited by PlpE to

protected mice from *P. multocida* challenge. This is the first report of any plant-based *P. multocida* vaccine significantly protecting mice from challenge with a virulent strain.

3.6 References

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Chapter 4

A plant-based oral PlpE vaccine against fowl cholera

A plant-based PlpE vaccine elicited PlpE-specific antibody responses in mice when fed orally but failed to protect them against a Pasteurella multocida infection.

4.1 Introduction

Fowl cholera is a systemic infection caused by *P. multocida*, which is believed to initiate infection at the mucosal surfaces of the lungs and upper respiratory airways (Glisson, 1998). It may be that the respiratory mucosa is more easily accessed by *P. multocida* than that of the gastro-intestinal tract (Olson & Schlink, 1986), but the significance of infection via the gastro-intestinal tract is unknown (Wilkie et al., 2000). Though injectable vaccines against fowl cholera are currently commercially available, it is proposed that an oral mucosal vaccine should provide a better protective immune response as the local protective immunity could prove as important as the systemic immune response (Scott et al., 1999). Oral vaccines deliver antigens to mucosal surfaces and can induce humoral and cell-mediated responses of the mucosal and systemic immune systems (Rigano et al., 2006). These vaccines cause less pain and discomfort than parenteral delivery, reduce the need for trained veterinary personnel and needle-associated risks (Holmgren, Czerkinsky, 2005).

There are few examples of oral vaccines against *P. multocida*; all produced from live attenuated *P. multocida* strains. The Clemson University (CU) strain elicited excellent humoral and cell mediated cross-protection in turkeys when given orally but was less efficient in chickens (Dua, Maheswaran, 1978, a & b). A live streptomycin-dependent *P. multocida* (Serotype 3) vaccine was found to protect turkeys orally and parentally against homologous challenge (Chengappa et al., 1978). The live attenuated strains of M2283 and P1580 were reported to induce average to low systemic immunity (Humoral and cell-mediated) in turkeys when administered in drinking water (Dua & Maheswaran, 1978a).

Not knowing the molecular basis for attenuation, it is of concern that the attenuated strains could revert to virulence. A recombinant oral vaccine against fowl cholera would circumvent this issue. Subcutaneous injection of PlpE, an outer-membrane lipoprotein of *P. multocida*, produced in *E. coli* has been found to be 100% protective in chickens and 70% protective in mice in both soluble and insoluble forms against the lethal dose pathogen challenge (Wu et al., 2007; Hatfaludi et al., 2012). However the immunogenicity of this recombinant antigen has not yet been tested using oral delivery to animals.

Plants can be an ideal platform for producing recombinant oral vaccines as the rigid walls of the plant cells would protect antigenic proteins from the acidic environment of the stomach and enable intact antigen to reach the gut associated lymphoid tissue (Rigano et al., 2003). Recently, an outer-membrane antigen, GS60 of *Mannhymia haemolytica* was produced in transgenic alfalfa and tested in animal models. It induced antigen-specific immune responses in rabbits when injected intramuscularly and delivered orally (Lee et al., 2008). The same tests have been performed in calves and results are being analysed (Reggie Lo, Personal communication, 2012). *M. haemolytica* is a very close relative of *P. multocida* and is also a respiratory pathogen (Pandher et al., 1998). *Actinobacillus pleuropneumoniae* is another pathogenic bacterium of the same family Pasteurellaceae, which causes severe pneumonia in pigs. Apx, a bacterial toxin was produced in transgenic tobacco plants and the purified extract of that antigen was given orally to mice by gavage. The plant-derived Apx elicited immune response in mice and protected them after challenging with the lethal dose of the bacteria (Lee et al., 2006).

Based on the above mentioned facts, it was hypothesized that our plant-based *P. multocida* outer-membrane lipo-protein, PlpE (Chapter 2) would induce protective immunity when tested in animal trials. Plant-based PlpE proved protective in mice when delivered subcutaneously (Chapter 3). This chapter therefore investigates the oral immunogenicity of plant-based PlpE. *N. benthamiana* leaves transiently transformed with pBinPlpE constructs were orally administered to mice and the induced immunity analysed. A pathogen challenge was also issued using *P. multocida* strain X-73 to test the protection level elicited by this plant-based PlpE. To author's knowledge, this is the first report of an orally delivered, plant-based fowl cholera vaccine being tested in challenge trials.

4.2 Materials and methods

4.2.1 Vaccine materials

4.2.1.1 Plant-PlpE

Two oral delivery trials were performed using two types of transient plant-PlpE produced by hand-injected and vacuum infiltration. During the first and second feeding trials hand-injection infiltrated and vacuum-infiltrated leaf powder containing PlpE were used, respectively (Chapter 2). Transient plant empty vector (negative controls) materials were produced by vacuum infiltration during both trials.

4.2.1.2 *E. coli* PlpE

The purification of insoluble bacterial PlpE was performed according to Hatfaludi et al. (2012). The recombinant PlpE was analyzed by visualization of the protein using SDS-PAGE gel stained with Coomassie blue and Western blot.

4.2.1.3 Whole cell killed bacterins

A *Pasteurella multocida* whole cell killed vaccine was prepared from the strain X-73 (AL 848, Heddleston serotype A: 1) as per chapter 3 (Boyce & Adler, 2000). The final vaccine dose was determined to be $4x10^7$ CFU per feeding for each mouse.

4.2.2 Quantification of PlpE in vaccine batches

The total soluble protein concentration of the bacterial *E. coli* PlpE was determined by Bradford Assay (Bio-Rad, Hercules, CA), using known concentrations of bovine serum albumin (BSA) as the protein standard. Plant-based PlpE concentration was determined by comparing the Western blot band intensity to that of a known concentration of control purified *E. coli* PlpE (urea-solubilised) using the NIH ImageJ 1.45s program (http://imagej.nih.gov/ij). Transient plant-PlpE produced 4 bands in Western blot analysis at 38, 40, 42 and 44 kDa. Though all 4 bands had the same intensity only the band running at 40 kDa was chosen for the comparison with the control bacterial protein band (Figures 3.1 & 3.2) since it was speculated that the bands running higher than the control were products of glycosylation and the lower band was a specific cleavage product of plant-PlpE.

4.2.3 HPLC analysis of plant materials

Pyridine alkaloids were identified and quantified in *Agrobacterium*-infiltrated *N. benthamiana* leaf materials as per chapter 2.

4.2.4 Animals

Specific-pathogen-free female BALB/CJAsmu mice (6-8 weeks old) were used in this study (Ethics number: SOBS/M2008/02 and MARP/2011/002). The animals were kept for one week to acclimatize and then grouped 3 to 4 (trial 1) or 5 (trial 2) mice per cage. They were provided with water and standard food pellets except when fed the test vaccine diets (Figures 4.1, A & B). Mice were monitored daily for health and condition.

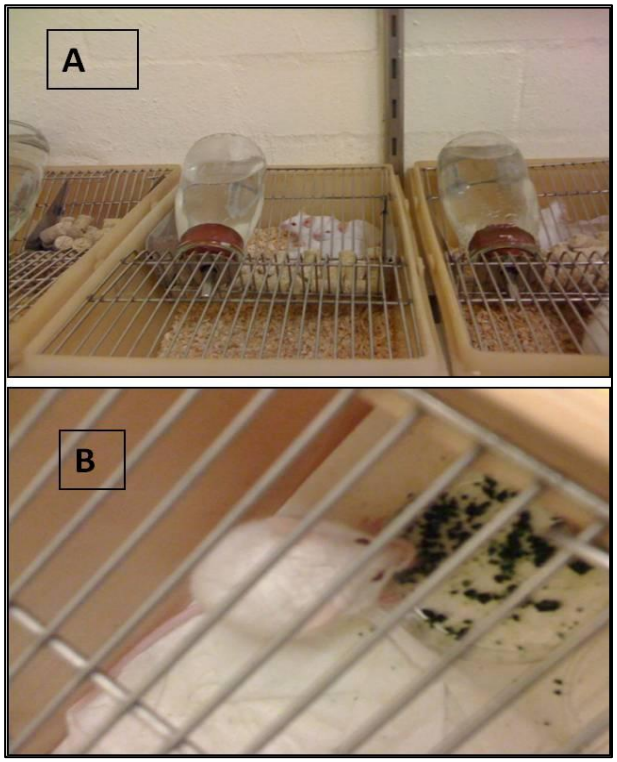

Figure 4.1 Female BalbC mice used in the feeding trials; A. 3-4 Mice were grouped per cage and provided with regular mice food and water, B. Single mouse in individual cage while feeding on the vaccine diet.

4.2.5 Preparation of test diet and vaccination

The plant materials (PlpE and empty vector as negative control) and the whole cell killed bacteria were mixed into a paste of peanut-butter and peanut-butter oil (1 part peanut butter to 3 parts peanut oil) immediately before administration (Pelosi et al., 2011). 10 mg of food-grade saponin powder (Sigma-Aldrich, Australia) was added to each dose of the vaccine diets during the second trial. One drop of caramel essence was added to the paste each time to remove the strong odour of plant materials and also to bring a sweet flavour to the diet (Figure 4.1B).

4.2.5.1 Mouse feeding trial 1:

Groups of seven mice were fed the plant-PlpE and plant empty vector (negative control) test diets on days 0, 7, 15 and 21. For feeding the test diet, mice were separated and housed in individual cages with a supply of water. The regular mice food was removed 4 hours before the vaccine diets were supplied each time. The vaccine diets were left in the cages for 4 hours. The total mass (plant material + formulation media) of treatments at each immunisation was 1.14g. Mice receiving test treatments were fed enough plant material to deliver an antigen dose of 50 µg PlpE. The left-over of test diets were weighed to determine the amount of PlpE antigen consumed by the mice within that time-frame.

4.2.5.2 Mouse feeding trial 2:

Groups of ten mice were fed the vaccine treatments three times. The treatments were plant-PlpE, plant empty vector (negative control) and whole cell killed bacterial (positive control). The mice were fed the test diets on day 0, 8 and 16. As in Trial 1, the mice were separated and housed in individual cages with supply of water before the test diet was provided. The regular mice food was removed 16 hours before the vaccine diets were supplied each time. The vaccine diets were left in the cages for 16 hours. The total mass (plant material/killed bacteria + formulation media) of treatments provided to the mice at each immunisation was 4g. Mice receiving test treatments were fed enough plant material to deliver an antigen dose of 100 µg PlpE or approximately $4x10^7$ CFU of whole cell killed bacteria. The test diet remaining after 16 hours was weighed to determine the amount of PlpE antigen and whole cell killed bacteria consumed by the mice within that time-frame. Another group of 10 mice were included in this trial as a positive control and they received subcutaneous injections of 100 µg purified insoluble *E. coli* PlpE with 20% (v/v) Alhydrogel™ on day 0 and day 15.

4.2.6 Pathogen challenge trial with *Pasteurella multocida*

P. multocida strain X-73 (AL848) was prepared for pathogen challenge trial as per chapter 3. Mice were challenged on day 28 with a dose of $1.3x10^3$ CFU during trial 1 and $1.45x10^3$ CFU during trial 2 and observed closely for the next 4 days. Clinical sings of the disease began with piloerection, dullness, and huddling. Sick mice did not recover and their conditions became worse gradually. Neurological symptoms like disturbances in body balance, wobbling and whirling caused by brain lesions were observed in animals (Virag et al., 2008). Animals with signs of infection were euthanized according to the animal ethics guidelines. The surviving animals were sacrificed humanely on day 32 using a CO_2 gas chamber. Both trials are summarized in table 4.1 and 4.2.

Table 4.1 Summary of mouse feeding trial 1

Treatment group	Number of mice	Vaccine dose	% of average vaccine diet consumption	% of average antigen uptake (µg)	Pathogen challenge strain & dose	% of survival
Plant-PlpE	7	50 µg per feeding	54.6	45.1	X-73 $1.3x10^3$	0 (0/7)
Plant empty vector (control)	7	N/A	83.6	N/A	X-73 $1.3x10^3$	0 (0/7)

N/A=Not Applicable

Table 4.2 Summary of mouse feeding trial 2

Treatment group	Number of mice	Vaccine dose	% of average vaccine diet consumption	% of average antigen uptake (µg)	Pathogen challenge strain & dose	% of survival
Plant-PlpE with saponin	10	100 µg per feeding	95.8	95.8	X-73 $1.4x10^3$ CFU	0 (0/10)
Plant empty vector with saponin	10	NA	95.3	NA	X-73 $1.4x10^3$ CFU	0 (0/10)
Whole cell killed bacterins with saponin	10	$4x10^7$ CFU	96.5	96.5	X-73 $1.4x10^3$ CFU	0 (0/10)
Insoluble *E. coli* PlpE with adjuvant	10	100 µg Injected (SC)	NA	NA	X-73 $1.4x10^3$ CFU	70 (7/10)

SC=Subcutaneous, N/A=Not Applicable

4.2.7 Collection and preparation of sera

Mice were eye-bled with sterile Pasteur pipette to collect blood. Blood was collected from two mice from each group on day 1 and from all mice of each group on day 28 before challenge. Mice sera were separated from whole blood and prepared as per chapter 3 and all samples were stored at -20°C. Sera isolated from whole blood were analyzed by Western blot and ELISA.

4.2.8 Analysis of immunogenicity of vaccines delivered

4.2.8.1 Western blot

Western blot analysis was performed as per chapter 3 to detect the PlpE-specific antibody response in vaccinated mice sera (Figures 4.4 & 4.5).

4.2.8.2 ELISA

ELISA was performed as per chapter 3. Titres were estimated as the reciprocal of the maximum dilution of serum giving an absorbance reading of 0.1 units after subtraction of non-specific binding in serum from non-treated animals (negative control).

4.2.9 Statistical analyses

GraphPad Prism 5 was used for all statistical analyses. Two-tailed T test and One-way ANOVA with Dunn's multiple comparison post test were used to determine statistical significant difference between means of the data from different groups. Log-rank (Mantel-Cox) test was used to analyze the survival curves of different groups of mice during trials 1 and 2. Regardless of the test applied, results were considered statistically significant if $P < 0.05$.

4.3 Results

4.3.1 HPLC analysis of plant materials

The presence and quantification of pyridine alkaloids, anabasine and nicotine in plant materials was determined through a HPLC analysis. The quantities of both alkaloids were significantly higher (17 mg/g and 21 mg/g respectively) in freeze-dried leaf powder of *N. benthamiana* produced by hand infiltration of *Agrobacterium* than the leaf powder produced by vacuum infiltration in a machine (4.3 mg/g and 4.5 mg/g respectively). An empty vector plant material produced by vacuum-infiltration was used as a negative control and the quantity of alkaloid content was measured to be 2.8 mg/g of anabasine and 2.9 mg/g of Nicotine (Figure 4.2).

4.3.2 Mouse feeding trial 1

During the first trial only two groups of mice were tested, one group was fed with plant-based PlpE vaccine and the other group was fed with the plant negative control material carrying the empty vector. It was calculated that on average 45% of the plant-PlpE vaccine diet was consumed by the mice and the same pattern of feeding was observed over all 4 immunizations.

No PlpE–specific immune reaction could be detected in the pre-vaccinated sera using Western blot analysis. Sera collected from the mice fed with plant-PlpE did not react with the insoluble *E. coli* PlpE in Western blot analysis (Figure 4.4) and there was no significant difference found between the PlpE–specific antibody titres in mice sera from treatments fed with plant-PlpE and plant control material (Figure 4.6 A). However, the titres increased significantly in the sera of the mice fed with plant-PlpE after the final or 4^{th} feeding compared to that after the second feeding (Figure 4.6 B).

4.3.3 Mouse feeding trial 2

During the second feeding trial the dose of plant-PlpE received by the mice was increased to 100 µg per feeding. Additionally saponin, an oral adjuvant was given to the animals with the vaccine diets. During this trial both plant-PlpE and plant empty vector control vaccine materials were prepared through vacuum infiltration. No significant difference was found between the amount of plant-PlpE and control vaccine diets consumed by the mice (Figure 4.3 B). Moreover, there was a positive control group, fed with a whole cell killed bacterial vaccine formulated with plant materials and peanut butter in the same manner as the plant-

PlpE and plant empty vector, that consumed an amount that was not significantly different from animals in the other two groups (Figure 4.3 B).

Sera collected from three out of ten mice fed with plant-PlpE reacted with the insoluble *E. coli* PlpE in Western blot analysis (Figure 4.5) while rest of them did not. No immune response was detected in mice sera of treatments fed with the negative plant control or whole cell killed bacterial treatments (Figure 4.5). PlpE-specific antibody was detected in the sera of the 10 mice fed with plant-PlpE and a significant difference was found between plpE specific antibody titres in the sera of mice fed plant PlpE and those fed the negative control plant material (Figure 4.7 A). The PlpE-specific antibody titres detected in the sera of mice fed with whole cell killed bacterial vaccine was not significantly higher than that of mice fed with plant-PlpE or plant negative control material (Figure 4.7 A). There was an additional positive control group consisting of 10 mice that were subcutaneously injected with 100 µg of purified insoluble *E. coli* PlpE two times. When the sera of these injected positive controls were analyzed through Western blot, all showed strong or moderate immune reactions against the bacterial PlpE (Figure not shown). Moreover, PlpE-specific antibody could be detected in all these sera through an ELISA and the titres were significantly higher compared to those fed with plant-PlpE (Figure 4.7 B).

4.3.3 Survival analysis for both trials

All the mice were challenged with *Pasteurella multocida* (Strain X-73) in both trials to determine the level of protection provided by the vaccines fed and injected. There was 70% survival in the injected group of mice that received purified *E. coli* PlpE vaccine subcutaneously (Figure 4.8 B) but none of the animals survived in any of the fed groups in either trial (Figures 4.8 A & B). However, when the survival curves were analyzed, significant delay was found in mean hours to death for the groups of mice fed with plant-PlpE materials compared to those fed the negative controls (Figures 4.8 A & B). This occurred in both trials. The mice fed with the whole cell killed bacterial vaccine did not show any significant delay to death compared to that of the mice in both plant-PlpE and plant negative control fed groups (Figure 4.8 B).

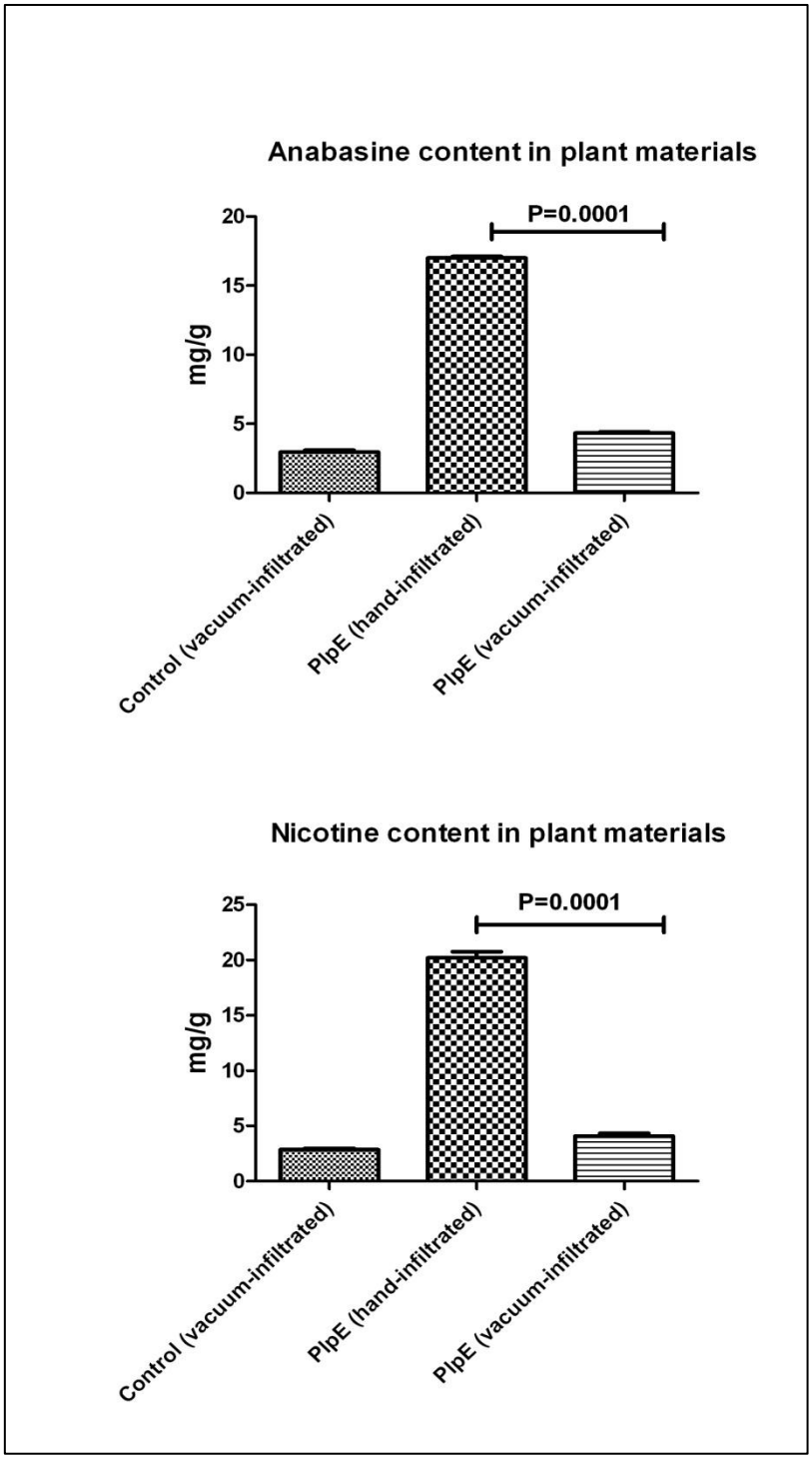

Figure 4.2 Quantification of Pyridine alkaloids (Anabasine & Nicotine) by HPLC in *N. benthamiana* leaves expressing PlpE. PlpE was expressed in those leaves through *Agrobacterium* infiltrations. Crude proteins were extracted from freeze-dried leaf powder. The bars represent the mean of 3 replicates for each material and the error-bars indicate standard error of mean (SEM).

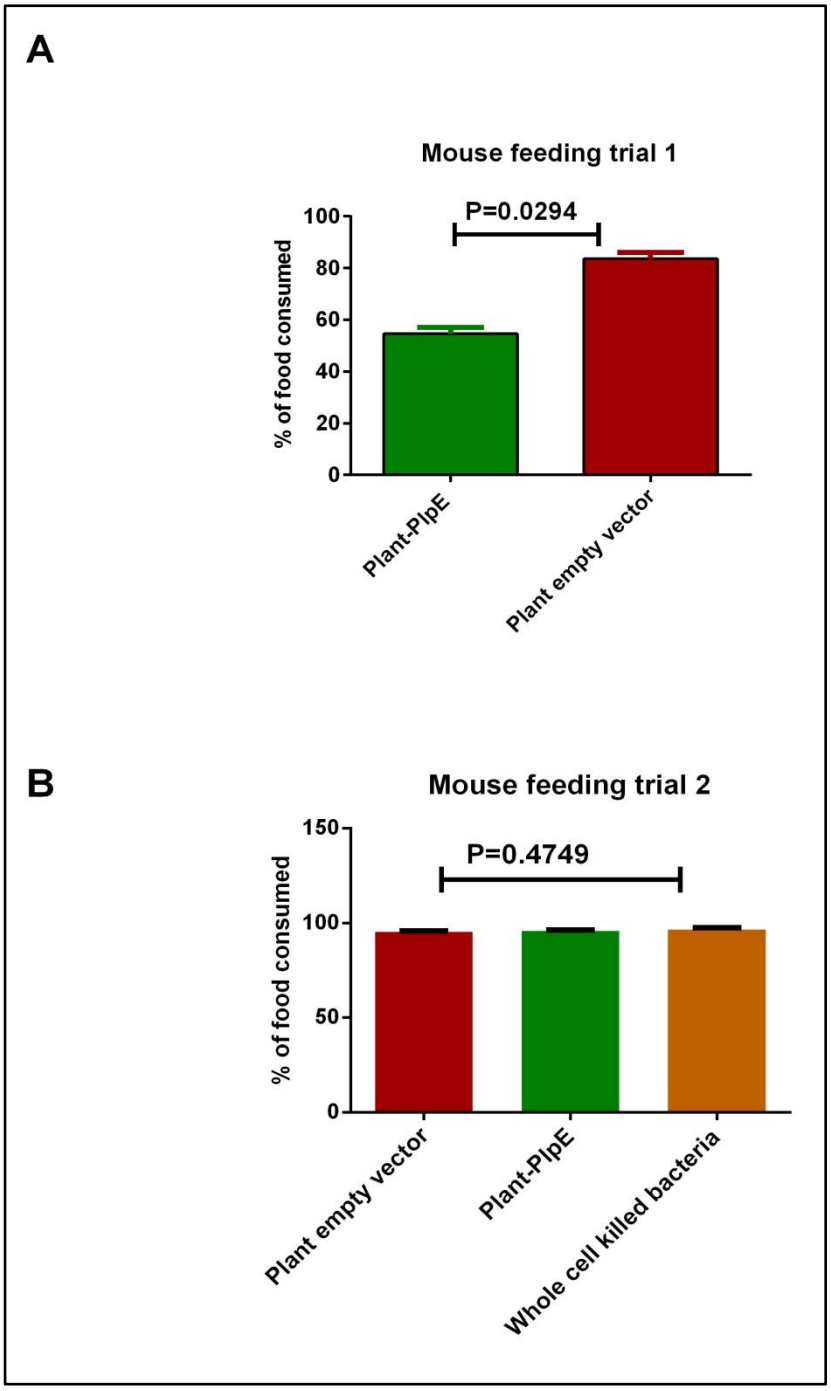

Figure 4.3 Comparison of different vaccine diets consumed by the mice. A: Trial 1, comparison was performed using a two-tailed T test and B: Trial 2, comparison was performed using one-way ANOVA. The bars represent the mean consumption by each group and the error bars indicate the standard error of the mean (SEM).

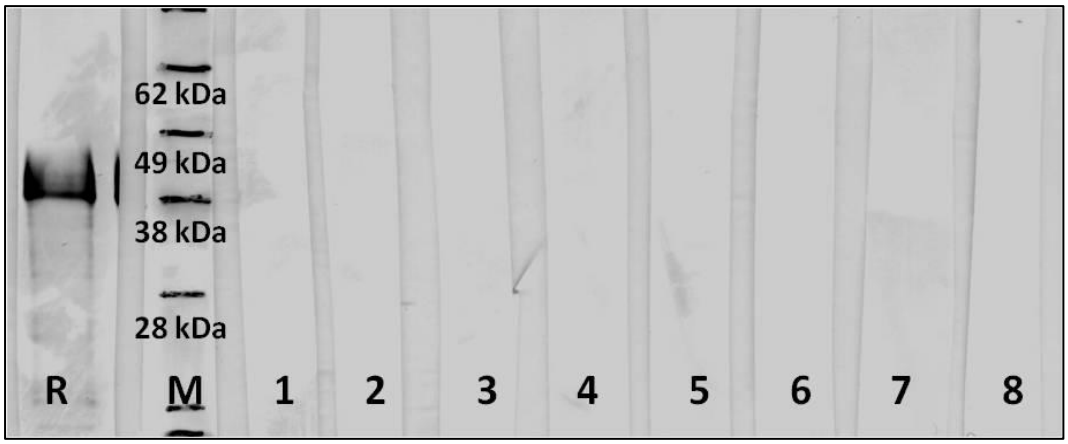

Figure 4.4 Western blot analyses showing antibody responses in individual mouse serum after the 4th immunization during the feeding trial 1. *E. coli* insoluble PlpE (500 ng/lane) was probed with individual mouse serum collected after 4th immunization (1:500) and with HRP conjugated polyclonal IgG goat secondary antibody (1:1200). M represents molecular mass standards (SeeBlue® Plus2, invitogen™), R represents rabbit anti-PlpE serum (positive control), lanes 1-6 show sera collected from mice fed with plant-PlpE, 7 shows pre-immunization serum and 8 shows serum collected from mouse fed with control plant material.

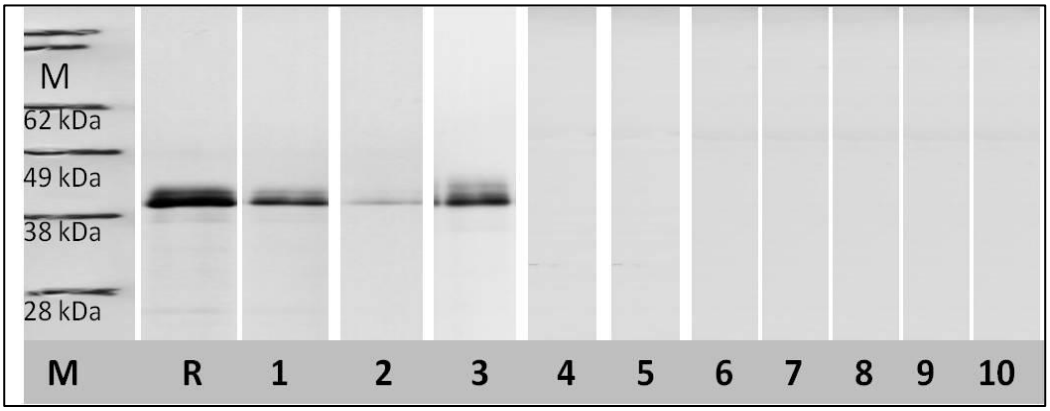

Figure 4.5 Western blot analyses showing antibody responses in individual mouse serum collected after the 3rd immunization during feeding trial 2. *E. coli* insoluble PlpE (500 ng/lane) was probed with individual mouse serum collected after 3rd immunization (1:500) and with HRP conjugated polyclonal IgG goat secondary antibody (1:1200). M represents molecular mass standards (SeeBlue® Plus2, invitogen™), R represents

rabbit anti-PlpE serum (positive control), lanes 1-5 show sera collected from mice fed with plant-PlpE with saponin, lanes 6 -8 show sera collected from mice fed with whole cell killed vaccine with saponin, lanes 9 & 10 show sera collected from mice fed with control plant material with saponin.

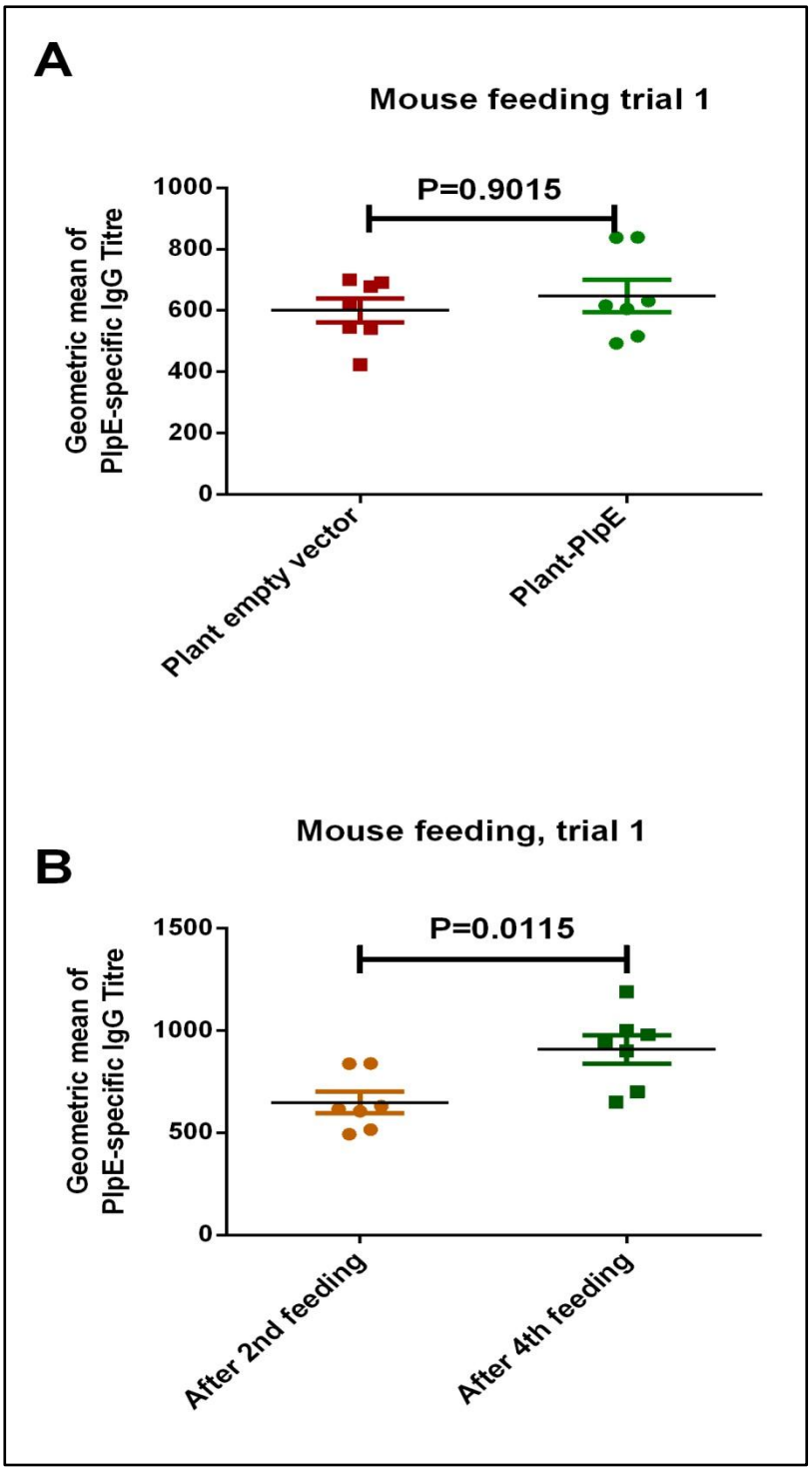

Figure 4.6 Detection of PlpE-specific antibody titres in fed mice sera by ELISA (Trial 1). A: Comparison of test and control groups after final feeding using a two tailed t-test. B: Comparison of PlpE fed groups after 2nd and 4th feeding. The dots represent the titre for individual mouse (Average of three independent replications) and the black lines

indicate the mean titre for each group. The error bars represent the standard error of the mean (SEM).

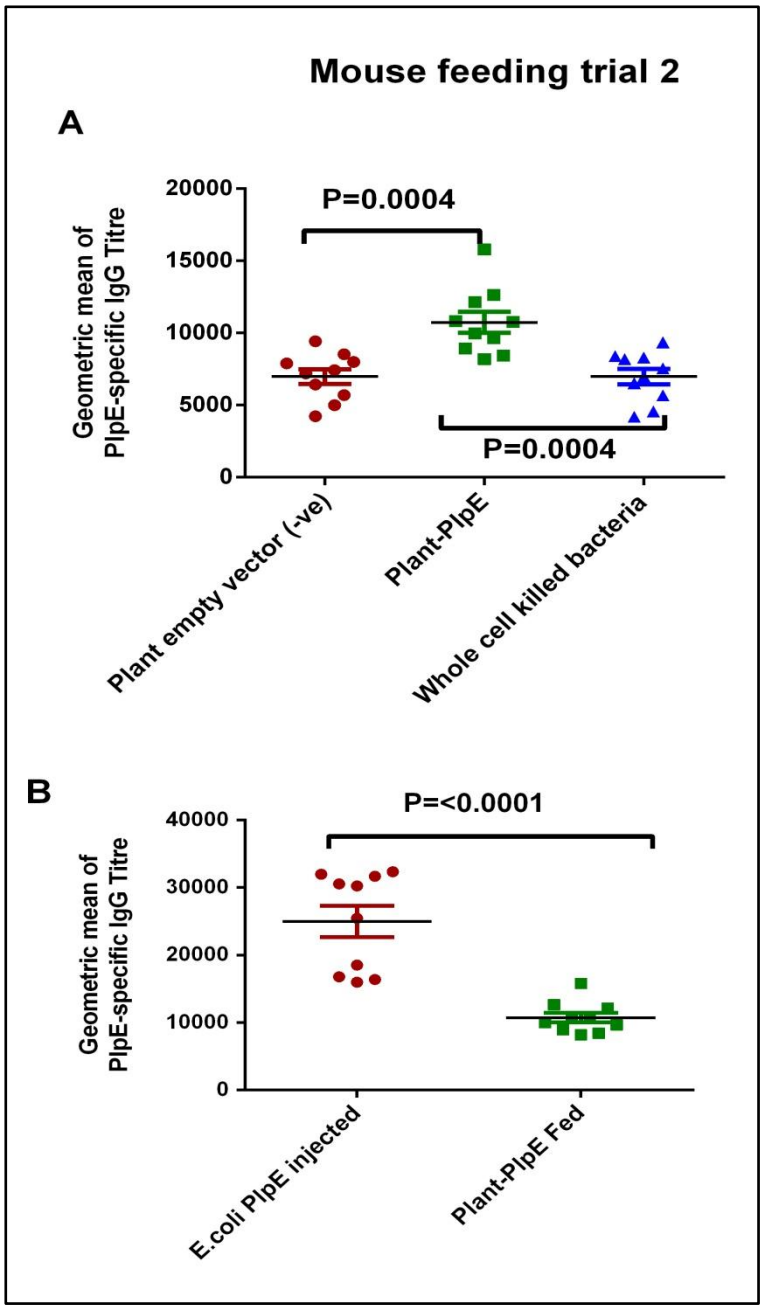

Figure 4.7 Detection of PlpE-specific antibody titres in fed and injected mice sera by ELISA (Trial 2). A: Comparison of test and control groups after the final feeding using one-way ANOVA. B: Comparison of *E. coli* PlpE injected and plant-PlpE fed groups after final immunization/feeding using One-way ANOVA. The dots represent the titre for individual mouse (Average of three independent replications) and the black lines

indicate the mean titre for each group. The error bars represent the standard error of the mean (SEM).

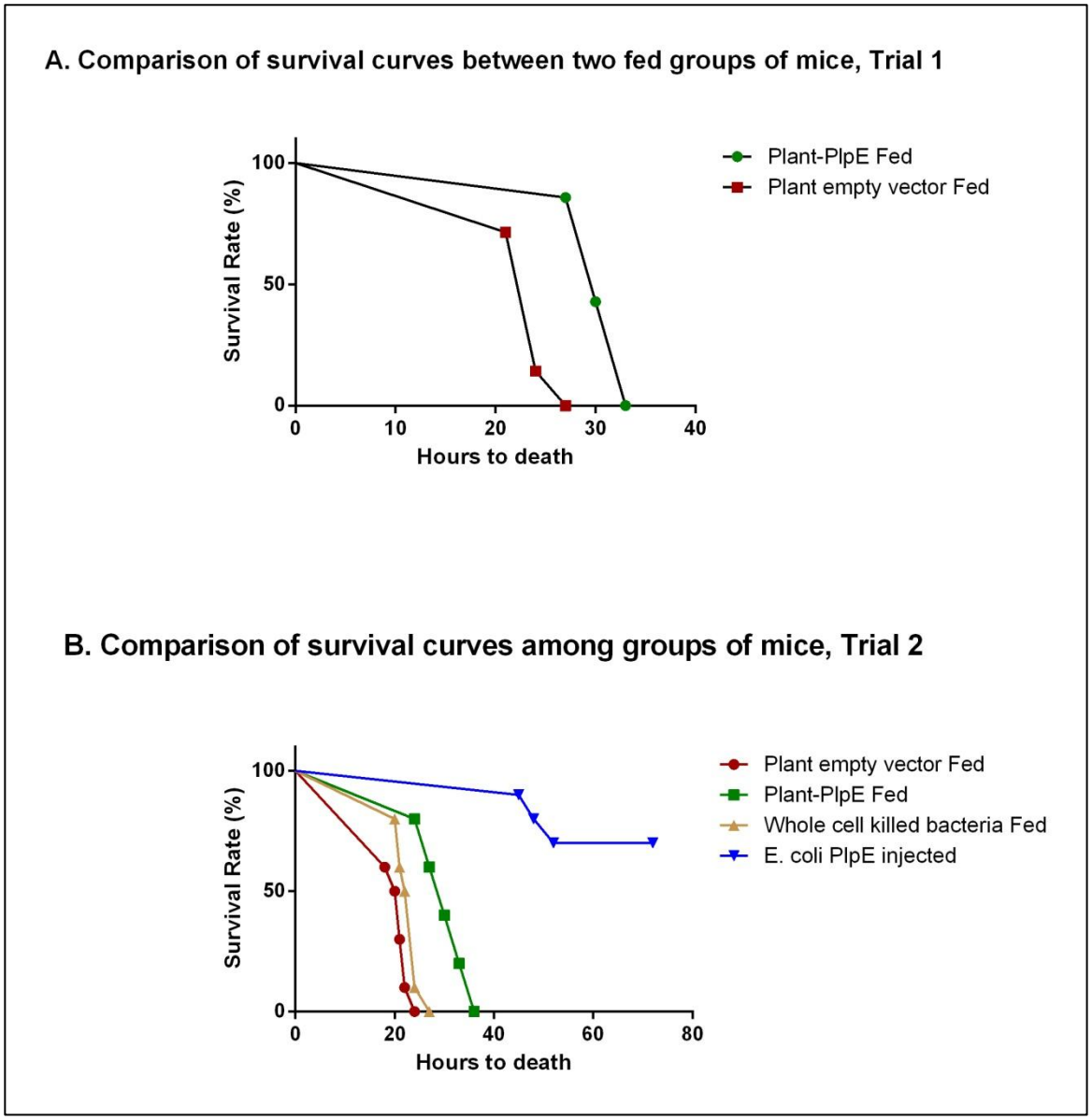

Figure 4.8 Comparisons of the survival curves among different groups of mice during feeding trials 1 and 2. Statistical analysis was done following Log-rank (Mantel-Cox) Test.

4.4 Discussion

4.4.1 Plant-based PlpE vaccine against *P. multocida* and mucosal vaccination

Although non-replicating antigens are often relatively inefficient in yielding strong and long-lasting mucosal antibody responses it has been established that plant cells expressing recombinant proteins can be ideal vehicles to orally deliver protective antigens (Haq et al., 1995; Mason et al., 1996; Streatfield et al., 2002). The production of antigens in plant materials offers the advantage of natural encapsulation when delivered orally, and such encapsulation can potentially guard against rapid and complete degradation of the antigen, thus allowing antigens to reach effector sites in the GALT (Gut-associated lymphoid tissue), lining the gastrointestinal tract (Walmsley & Arntzen, 2005). PlpE, an outer-membrane lipo-protein of *P. multocida*, was produced in *E. coli* cells as a recombinant vaccine antigen and has shown to provide 100% protection in chickens and 70% protection in mice when delivered through subcutaneous injections and in both soluble and insoluble forms (Wu et al., 2007; Hatfaludi et al., 2012). The aim of this study was to produce PlpE in plants and deliver that plant-based vaccine antigen to animals orally to induce mucosal immunity and better protection.

4.4.2 Choice of host plant and production system

N. benthamiana plants were used as the production platform as it is a non-crop species and very well-studied plant. *N. benthamiana* is also very easy to cultivate in the green-house and it contains comparatively less alkaloid content than the other members of the *Nicotiana* family (De Boer et al., 2009; Heal et al., 2001). In addition, the leaves of *N. benthamiana* plants are very broad and suitable for use in the agro-infiltration purpose. Though the vaccine diets used in these feeding trials did not seem to produce any sickness or uncomfort in the animals, other plant species could be tested in future to increase the quantity and quality of the vaccine antigen. Producing the vaccine in crop plants corn, rice, lettuce, carrot, potato or tomato might make it more palatable for animal feeding (Tacket et al., 1998 & 2004; Chikwamba et al., 2002; Zhou et al., 2006; Alvarez et al., 2006; Walmsley et al., 2003a; Webster et al., 2006; Kim et al., 2009) but introduces the need to keep the vaccine plants separate from the normal food chain.

The plant vaccine used in these trials was transiently produced by plants, which means the bacterial gene of *plp*E was expressed inside the plant cells using plant's transcriptional machinery but it did not incorporate into the plant's genome. The same antigen was expressed in stable transgenic cell lines of tobacco but the quantity of the antigen was not sufficient to be tested in animals orally (Data shown in chapters 2 and 3). It's well documented that the level of foreign protein produced in transgenic plants obtained by stable nuclear transformation is commonly low (about 0.1% of the total soluble protein) and not always sufficient to be tested in animal models (Dorokhov et al., 2006). On the other hand, foreign proteins produced in plants transiently accumulate at a very high concentration and subsequently made the testing of antigens in animals possible and feasible (Santi et al., 2006).

There are different methods to infiltrate the *Agrobacterium* cells in plants during transient transformation and this can affect the quality of plant materials produced. Two types of transient plant materials were used in these two feeding trials. During the first trial, PlpE-plant material was produced using hand-injected infiltration and the control plant material was produced though vacuum infiltration. It was observed throughout the trial that mice ate less of the PlpE-plant material compared to the control plant material. It was hypothesized that the reason behind their preference for the control plant diet could be the different agro-infiltration methods, which may alter the alkaloid content of these two leaf materials. Infiltrating the leaves individually with needle and syringe may result in more wounding of the plants and consequently more stress and more alkaloid secretion. The agro-infiltration with vacuum pressure would leave the plants with less wound and stress hence less alkaloid content. The alkaloids present in the leaves could alter the taste of test and control diets, with increased presence increasing the bitterness and decreasing palatability. To test the hypothesis a HPLC test was run to identify and quantify the pyridine alkaloids in both types of leaf materials. The hand-infiltrated leaves had a significantly higher quantity of anabasine and nicotine alkaloids compared to that in the vacuum-infiltrated leaves. During the second feeding trial both PlpE and control plant materials were produced through vacuum infiltration and no significant difference was observed between the amount consumed of the test and control feeding materials by the animals. Therefore, the hypothesis that the difference in the alkaloid content of the feeding materials produced through different methods could make a difference in the rate of consumption of vaccine diets by the animals was proved right. It could be predicted from the results of these two oral immunization trials in mice that reducing the alkaloid content in the plant materials would improve the delivery of oral

vaccine to animals since this in turn affects how much antigen is taken in and up by the animals.

4.4.3 Poor mouse protection by a plant-based oral PlpE vaccine

No significant total IgG anti-PlpE immune response could be detected in the mice sera after the first feeding trial though the titres increased with further dosing. The lack of significant immune response in the first trial was most likely due to the small amount of plant-PlpE taken up by the mice. Moreover, no oral adjuvant was delivered with the vaccine in this trial. In the second feeding trial a crude saponin powder (Walmsley et al., 2003b) was added to the vaccine diets and the antigen dose was increased 2-fold. These changes explain the detection of a significant amount of antigen specific IgG titres in the sera of three mice fed with plant-PlpE in the second trial and a PlpE-specific 40 kDa band detected in sera of these mice by Western blot analysis. However, the remaining seven mice in the plant-PlpE with adjuvant treatment did not show a detectable response. This was perhaps due to the antigen dosage being on the brink of what is required to induce an immune response.

No orally immunized animals were protected after the pathogen challenge. This lack of protection might be explained by insufficient dose of plant-PlpE antigen and/or the use of an inappropriate route of mucosal vaccination. Although mucosal vaccination induces a common immune response (Neutra & Kozlowski, 2006), that is, an immune response is induced at all mucosal surfaces, the strength of the common immune response varies between the mucosal locations depending on the route of immunization (Neutra and Kozlowski, 2006). Oral immunization induces the strongest response at enteric mucosal surfaces (Holmgren & Czerkinsky, 2005) while intranasal immunization induces a stronger immune response at the mucosal surfaces of the respiratory tract (Ogra et al., 2001). To protect against fowl cholera intranasal vaccination may be a better route to induce a protective immune response as the primary response would be at the mucosal surfaces of the respiratory tract, the preferable location for *P. multocida* colonisation (Wilkie et al., 2000).

However, intranasal delivery of a non-replicating, subunit vaccine may still require immunization of individuals and thus prove labour intensive. Oral delivery may therefore prove a more practical immunization route. Oral delivery historically requires larger doses of antigen to induce immune responses (Ogra et al., 2001). Although we did observe immune responses in mice after oral delivery of plant-based PlpE, it may be that a larger dose is required to induce a protective immune response. Therefore it is suggested that mucosal

immunization is further investigated through delivering higher doses of plant-based PlpE through the oral route and also attempting the intranasal route.

4.4.4 Prime-boost strategy and oral immune-tolerance

Alternatively, priming with parenteral injection followed by boosting with plant-based oral vaccines has shown to increase immune response significantly (Lauterslager et al., 2001; Van der Heijden et al., 1989). It is predicted that priming of the immune system parenterally may promote successful reaction between the gut-associated lymphoid tissue and the small amounts of antigen delivered during oral immunization. Judge et al (2004) demonstrated that parenteral priming of mice with *E. coli* outer-membrane protein intimin purified from transgenic tobacco plant cells assisted in the development of an intimin-specific immune response when the animals were subsequently boosted with oral feeding of the same intimin-expressing transgenic plant material. Moreover, there was a statistically significant decrease in the duration of colonization by wild type *E. coli* 0157:H7 upon challenge (Judge et al., 2004). Therefore, it might be an alternative strategy to test plant-based PlpE vaccine in animals by priming them with purified plant-based PlpE and then boost orally with the same PlpE–expressing plant material. This combination of vaccination strategies with PlpE antigen produced in and delivered by transgenic plants might elicit specific immune response sufficient for protection of the animals against *P. multocida* infections. In addition, it may reduce the possibility of inducing oral immune-tolerance to plant-based and other orally delivered vaccine antigens (Van der Heijden et al., 1989).

4.4.5 Glycosylation and plant-based vaccines

Transient plant-PlpE was observed to be *N*-glycosylated (Chapter 2). Correct glycosylation can be a prerequisite for the immunogenicity of vaccine antigens (Faye et al. 2005). Though proteins of bacterial origin are not generally glycosylated (Joensuu et al., 2008) there is a report of *E. coli* outer-membrane protein Intimin being *N*-glycosylated when produced in transgenic *N. tabacum* cell culture and that glycosylated protein was not immunogenic in mice when fed orally (Judge et al., 2004). However, the plant-based Intimin produced in another tobacco cell line and not-glycosylated was observed to elicit protective immunity in mice when fed orally (Judge et al., 2004). They concluded that glycosylation of Intimin protein inside plant cells adversely affected the immunogenicity of the antigen and resulted in aberrant immune response that failed to protect the animals. Therefore, plant-based PlpE might have failed to elicit sufficient protection in mice when delivered orally due to being

glycosylated. Plant-PlpE that is not glycosylated should be delivered orally to animals to check this possibility.

4.5 Concluding remark and future directions

The preliminary results from the mice feeding trials are promising since an immune response was indeed induced after oral delivery of the plant-based PlpE and mean hours to death significantly increased. This response was not protective but did significantly extend the time until mouse death. To the author's knowledge, this is the first report of an oral vaccine produced in plants against fowl cholera pathogen *Pasteurella multocida* however this thesis represents the first steps taken with regards to a plant-based fowl cholera vaccine and there is ample scope for improvement. Firstly, intranasal delivery of plant-PlpE should be tested as an alternate vaccination route since nasal immunization induces a more broad-spectrum mucosal response than oral immunization (Ogra et al, 2001) and the primary response is at the site of *P. multocida* entry and colonization (Wilkie et al, 2000). Secondly, due to the ease of oral delivery, increased dose of orally delivered antigen should also be investigated to determine if a protective immune response can be induced. Thirdly, a prime-boost vaccination strategy that primes the animals with purified plant-PlpE followed by an oral boosting with the same plant-PlpE (Judge et al., 2004; Lauterslager et al., 2001) should be tried to increase the immune response and guard against oral tolerance. Fourthly, the immunogenicity of plant-based PlpE antigen should be compared in animals in its glycosylated and deglycosylated forms. In addition alternative adjuvants could be tried such as co-delivery with LTB or CTB. These adjuvants could improve mucosal immunity (Arakawa et al., 2001; Mason et al., 1998). The ultimate goal should be to test mucosal delivery of the vaccine in chickens, the natural host of fowl cholera.

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Chapter 5

Immunization trials in chickens with plant-based PlpE

Both transient and stably transformed plant-based PlpE vaccines elicited immune responses in chickens when injected subcutaneously, but failed to provide sufficient protection against Pasteurella multocida infection

5.1 Introduction

Fowl cholera, caused by infection with *Pasteurella multocida*, is a disease of many avian species; all domestic poultry is susceptible to this highly infectious disease (Glisson, 1998). Chickens, turkeys, ducks, and quail are the most important domestic avian species involved and the disease causes significant economic losses. Vaccination to prevent fowl cholera is an important aspect of controlling the disease, particularly in broiler breeders and turkeys (Janmaat & Morton, 2010). There are mainly two types of vaccines available against fowl cholera, whole-cell inactivated vaccines and live attenuated vaccines. Both killed and live vaccines have limitations of eliciting only homologous protection or reversion to disease, respectively (Rimler & Glisson, 1997). Therefore, the search for cross-protective antigens and the production of subunit vaccines composed of those cross-protective antigens are key targets for researchers and for the poultry industry. Up to now, only one *P. multocida* antigen, PlpE or lipoprotein E has been reported by two different groups to be cross-protective in mice and chickens when injected sub-cutaneously (Wu et al., 2007b; Hatfaludi et al., 2012). Since *P. multocida* is widely believed to enter chickens through the upper respiratory tract a mucosal vaccine against this pathogen might elicit better protection in chickens by inducing both systemic and mucosal immune systems. Plants have been shown to be ideal platforms for producing recombinant subunit antigens for veterinary use via both systemic and oral administration. The efficacy of plant-based subunit vaccines has been proved in target animals such as cattle, sheep, pigs and chicken (Khandelwal et al., 2003; Khandelwal et al., 2011; Loza-Rubio et al., 2012; Joensuu et al., 2006 a & b; Lamphear et al., 2002 & 2004;

Guerrero-Andrade et al., 2006). As described in the introduction (Chapter 1) however, there have been relatively more plant-based vaccination and challenge studies completed in poultry. Vaccination and challenge trials are reported for Infectious Bursal Disease Virus (Miller et al., 2004; Wu et al., 2004 & 2007a), Infectious Bronchitis Disease Virus (Zhou et al., 2004), Newcastle Disease Virus (Cardineau et al., 2004; Yang et al., 2007) and avian influenza virus (Mihaliak & Webb, 2005; D'Aoust et al., 2008; Shoji et al., 2008 & 2009). All these reports described plant-based vaccines against viral diseases in poultry, but no study has been published yet on a plant-based vaccine against a bacterial disease of poultry.

Therefore, it was hypothesized that PlpE could be produced in plants and that plant-based PlpE vaccine would be immunogenic and protective in mice and/or chickens. PlpE was produced in plants, both transiently and stably (Chapter 2). Plant-based PlpE vaccines have been evaluated in mice, both by systemic and oral administration routes (Chapter 3 and 4). A sub-cutaneous delivery of transient plant-PlpE vaccine elicited significant protection (70%) in mice after pathogen challenge experiments, whereas oral feeding of the same plant vaccine did not protect the animals despite their production of significantly high serum antibody titres against PlpE. The efficacy of these plant-based PlpE vaccines was tested in chickens. In this chapter, two immunization trials with plant-based PlpE vaccines and pathogen challenge trials with *P. multocida* performed in chickens are described. During the first trial both transient and stably transgenic plant-based PlpE vaccines were delivered to chickens, whereas only transient plant-PlpE vaccine was delivered during the second trial. Both soluble and insoluble *E. coli*-derived PlpE vaccines were used as positive controls, while transient and stably stable plant empty vector materials and adjuvant only were used as negative controls. To the author's knowledge this is the first investigation of a plant-based vaccine against fowl cholera as well as against a bacterial disease of poultry.

5.2 Materials and methods

5.2.1 Vaccine material preparation

5.2.1.1 Plant-based vaccines

2.5 g of freeze-dried *N. benthamiana* leaf powder (transient plant-PlpE and plant empty vector) and 5 g of freeze-dried cell suspension powder (stable transgenic plant-PlpE and plant empty vector produced in *N. tabacum* cell lines) was resuspended in 25 ml of extraction buffer (1X PBS) by vortexing with two 6.4 mm ceramic beads (Qbiogene, Carlasbad, CA) for 2 minutes at maximum speed. Insoluble material was removed by two centrifugation steps at

47,893.3 *g* at 4°C for 20 minutes in a Sorvall ultra-centrifuge using a SS34 rotor. The clear supernatant was collected in fresh a 50 ml conical tube. This extract was dialyzed over-night in 2 liters of extraction buffer (1X PBS) at 4°C through a dialyzing membrane with 10 kDa cut-off. The dialyzed plant extract was concentrated using 15 or 50 ml Amicon Ultra-4, PLGC Ultracel-PL Membrane, 10 kDa (Millipore™) at 7000 *g* and 4°C for 2 or 4 hours to reach the required concentration of 10 µg or 100 µg. The final vaccine preparation was filtered through 0.45/0.22 µm filters and 20% (v/v) Al Hydrogel® was added just before administration.

5.2.1.2 *E. coli* PlpE

The purification of insoluble and soluble, *E. coli* PlpE was performed according to Hatfaludi et al. (2012). The recombinant PlpE was analyzed by visualization of the protein using SDS-PAGE gel stained with Coomassie blue and Western blot. The concentration was quantified using Bradford assay (Biorad, CA, USA).

5.2.2 Quantification of PlpE in vaccine batches

The total soluble protein concentration of the *E. coli* PlpE was determined by Bradford Assay (Bio-Rad, Hercules, CA), using known concentrations of bovine serum albumin (BSA) as the protein standard. Plant-based PlpE concentration was determined by comparing the Western blot band intensity to that of a known concentration of control purified *E. coli* PlpE (urea-solubilised) using the NIH ImageJ 1.45s software (http://imagej.nih.gov/ij). Transient plant-PlpE produced 4 bands in Western blot analysis at 38, 40, 42 and 44 kDa. Transient plant-PlpE produced 4 bands in Western blot analysis at 38, 40, 42 and 44 kDa. Though all 4 bands had the same intensity, to be conservative only the bands running at 40 kDa and 42 kDa were chosen for the comparison with the control *E. coli* protein band (Figure 2.6, A) since the bioactivity of the glycosylated forms and the cleavage product was not known.

5.2.3 Preparation of *P. multocida* for challenge trial

The birds were challenged with the *P. multocida* strain X-73 (AL848, Heddleston serotype A:1) or strain VP161 (Heddleston serotype A:1). The bacteria was streaked on an HI plate and incubated at 37°C overnight. The next day one well-isolated colony was inoculated in 10 ml HI broth and incubated at 37°C overnight. Next morning 50 µl from the overnight culture were transferred to 10 ml of fresh HI broth and grown for approximately 3-4 hours until an OD_{600} of 0.45-0.55 was reached ($1x10^9$ CFU/ml). A 10^{-6} dilution, corresponding to $1x10^3$ CFU/ml, was prepared for animal infection (Boyce & Adler, 2000). 100 µl from 10^{-7} and 10^{-8} dilutions were spread plated onto two HI plates each and incubated at 37°C overnight to

determine the viable count next morning. The final challenge dose was determined from this colony count.

5.2.4 Animal handling and vaccine delivery

Groups of 10 week old commercial Hyline layer hens were kept for one week to acclimatize prior to vaccination. The animals provided with water and standard food pellets throughout the trial and monitored daily for health and condition. The vaccination groups are described in Table 5.1

Table 5.1 Groups of chickens used in trial 1

Treatment group	Number of chickens	Vaccine dose
Transient plant-PlpE with adjuvant	10	100 µg
Transient plant-PlpE without adjuvant	10	100 µg
Transient empty vector control with adjuvant	10	N/A
Stable plant- PlpE with adjuvant	10	50 µg
Stable plant- PlpE without adjuvant	10	50 µg
Stable empty vector control with adjuvant	10	N/A
***E. coli* PlpE insoluble with adjuvant**	10	50 µg
***E. coli* PlpE soluble with adjuvant**	10	50 µg
Adjuvant only	15	N/A

N/A=Not Applicable

Pre-immunization sera were collected from 3 birds in each group. All vaccine doses were delivered subcutaneously in a volume of 500 µl on day 0, 14 and 21. Chickens were then

challenged intramuscularly with $1.4x10^3$ CFU of *P. multocida* strain X-73 on day 32 and subsequently with a dose of $1.3x10^5$ CFU of strain VP161 on day 34. They were kept for post-infection observation for 4 days. All birds that developed signs of acute fowl cholera were euthanized in accordance with animal ethics requirements. Serum was collected from all birds prior to challenge.

For the second chicken trial there were 5 groups and 10 chickens per group, as detailed in Table 5.2.

Table 5.2 Groups of chickens used in trial 2

Treatment group	Number of chickens	Vaccine dose
Transient Plant-PlpE with adjuvant	**10**	**100 µg**
Plant empty vector (control) with adjuvant	**10**	**N/A**
E. coli PlpE insoluble with adjuvant	**10**	**100 µg**
E. coli PlpE insoluble with adjuvant	**10**	**100 µg**
Adjuvant only negative control (Alhydrogel®)	**10**	**N/A**

N/A=Not Applicable

Pre-immunization sera were collected from 3 birds in each group. All vaccine doses were delivered subcutaneously in 500 µl volume. Hens were vaccinated on days 0, 14 and 22 and then challenged intramuscularly with a dose of $3x10^3$ CFU of *P. multocida* strain VP161 on day 28. The animals kept for post-infection observation for 3 days. All birds that developed signs of acute fowl cholera were euthanized in accordance with animal ethics requirements. Serum was collected from all birds prior to challenge.

5.2.5 Preparation of chicken sera for analysis

Chicken sera were separated from whole blood by clotting at room temperature for 4 hours. Then the sera were separated from the blood cells in a Beckman fixed rotor centrifuge

machine at 15,000 *g* for 20 min at room temperature. The clear supernatant was transferred to new 15 ml eppendorf tubes. The spinning was repeated once and the final clear sera were collected. All samples were stored at -20°C.

5.2.6 Analysis of immunogenicity of PlpE vaccines

5.2.6.1 Western blot

Insoluble PlpE purified from *E. coli* was boiled with 6× SDS gel loading buffer (300mM Tris–HCl, pH 6.8, 600mM dithiothreitol, 12% SDS, 0.6% Bromophenol Blue, 60% glycerol), for 10 minutes and placed on ice. 600 ng of this denatured PlpE was loaded on 9 lanes of an SDS-polyacrylamide gel (10.5–14% Tris–HCl, 4% stacking, Bio-Rad, Hercules, CA). The gel was electrophoresed at 100V for 10 minutes and then at 200V for 50 minutes using Tris–glycine running buffer (25mM Tris, 250mM glycine, pH 8.3, 0.1% SDS). The separated proteins were transferred to a PVDF membrane (Bio-Rad, Hercules, CA) using a Mini-Trans-Blot electrophoretic transfer cell (Bio-Rad, Hercules, CA) for 1 hour at 100V in trans-blot buffer. The membrane was then cut into lanes and blocked with 2.5% skim-milk TBS-Tween buffer (TBS buffer plus 0.05% Tween 20) for 1 h at room temperature on a rocking platform. The membranes were washed for 3 times (5 min each time) in TBS-Tween buffer with agitation. Each membrane was then incubated with a single chicken serum collected during the vaccine trials before challenge, diluted at 1:200 in 2.5% skim-milk TBS-Tween buffer on a rocking platform over-night at RT. After four 4 min washes followed by a 5 min wash in TBS-Tween buffer, the membranes were incubated with 1:1000 horseradish peroxidase conjugated donkey anti chicken immunoglobulin (Sigma–Aldrich, Saint Louis, MO) for 1 hour at 37°C on a rocking platform. The membranes were then washed in TBS-Tween buffer for 4 times (5 min each time) followed by a 5 min wash in TBS buffer with agitation. Blots were developed colorimetrically 15 µl of 30% hydrogen peroxide solution was added to 25 ml of 1X TBS buffer and kept in the dark. 5 ml of methanol was added to 0.015 mg of 1, 4-chloronaphthol. This was mixed with the previous solution and finally added to the membranes.

5.2.6.2 ELISA

ELISA plates (high bind polystyrene EIA/RIA 96-well microplate, Corning, NY) were coated with 10 µg/ml purified PlpE from *E. coli* in PBS and incubated overnight at 4°C. After three washes with PBS-Tween with shaking (PBS, pH 7.4, plus 0.05% Tween 20), plates were blocked with 5% skim-milk powder in PBS-Tween and incubated for 2 hours at RT with shaking. After three washes with PBS-Tween, mouse sera were added to the first rows of the

plates at a dilution of 1:100 and then serially diluted twofold using skim-milk in PBS-Tween. The plates were then incubated for 1 h at 37°C with shaking before being washed three times with PBS-Tween and incubated with horseradish peroxidise (HRP) conjugated donkey anti-chicken-IgY (Sigma–Aldrich, Saint Louis, MO), at a dilution of 1:1000 in blocking buffer (0.05% skim-milk powder in 1X PBS), for 1 h at 37°C. After washing four times with PBS-Tween, reactions were detected with TMB peroxidase substrate (Bio-Rad, Hercules, CA) following manufacturer's instructions for 15 min. The reaction was stopped with 1N H_2SO_4 and then the absorbance was read at 450 nm using a Microplate Reader Thermo Max (Molecular Devices Inc., Sunnyvale, CA). Titres were estimated as the reciprocal of the maximum dilution of serum giving an absorbance reading of 0.1 after subtraction of non-specific binding in serum from non-treated animals (negative control).

5.2.7 Statistical analyses

GraphPad Prism 6 was used for all statistical analyses. One-way ANOVA with Tukey's Multiple Comparisons test was used to determine statistical significance between means of the data from three or more groups. Log-rank (Mantel-Cox) test was used to analyze the survival curves of different groups of birds during trials 1 and 2. Results were considered statistically significant if $P < 0.05$.

5.3 Results

5.3.1 Chicken immunization trial 1

The majority (90-100%) of the sera collected from *E. coli* and plant-PlpE injected groups of chickens reacted with recombinant *E. coli* PlpE (Figure 5.1, 5.2, 5.3, 5.4 & 5.5). The chickens immunized with the *E. coli* PlpE produced stronger reactions than those immunized with plant PlpE. The Western blot results were confirmed by ELISA. The PlpE-specific antibody titres were significantly higher in chickens immunized with 50 µg of soluble or insoluble *E. coli* PlpE, than in chickens that received adjuvant only (Figure 5.6). Likewise, the PlpE-specific antibody titres were significantly higher in chickens immunized with 50 µg transient plant-PlpE, with or without adjuvant, than in chickens injected with transient plant empty vector control with adjuvant (Figure 5.6). Similarly, the PlpE-specific antibody titres were significantly higher in chickens immunized with 50 µg stable plant-PlpE, with or without adjuvant, compared to those in chickens injected with stable plant empty vector control (Figure 5.6). However, there was no significant difference in PlpE-specific antibody titres between chicken groups immunized with: 50 µg of soluble or insoluble *E. coli* PlpE, (Figure

5.6); transient or stable plant PlpE, with or without adjuvant; or soluble or insoluble *E. coli* PlpE and transient and stable plant-PlpE (Figure 5.6).

The birds were first challenged on day 29 with *P. multocida* strain X-73 with a normally lethal dose of $1.4x10^3$ CFU. None of the negative control birds showed any disease signs within 48 hours. It was therefore concluded that the strain had lost virulence. Accordingly, the birds were challenged for a second time with strain VP161 with a lethal dose of $1.3x10^5$ CFU. At the end of the trial still seven, six and six birds remained alive from the adjuvant only control, transient empty vector control and stable empty vector control groups respectively. On the other hand, all ten from the group immunized with soluble *E. coli* PlpE remained healthy and survived, whereas birds in the group immunized with insoluble *E. coli* PlpE all showed signs of disease, with seven surviving to the end of the trial. Only 4 birds survived from the transient plant-PlpE group that had adjuvant in the vaccine and 6 survived from the same treatment without adjuvant. Six birds survived from the stable plant-PlpE group without adjuvant and seven survived from the same treatment with adjuvant. As the negative control group birds did not show the disease symptoms at the expected time frame and more than 50 percent of them survived after challenge with two different strains of the bacteria, the results from this infection trial were not conclusive. The results are summarized in table 5.3.

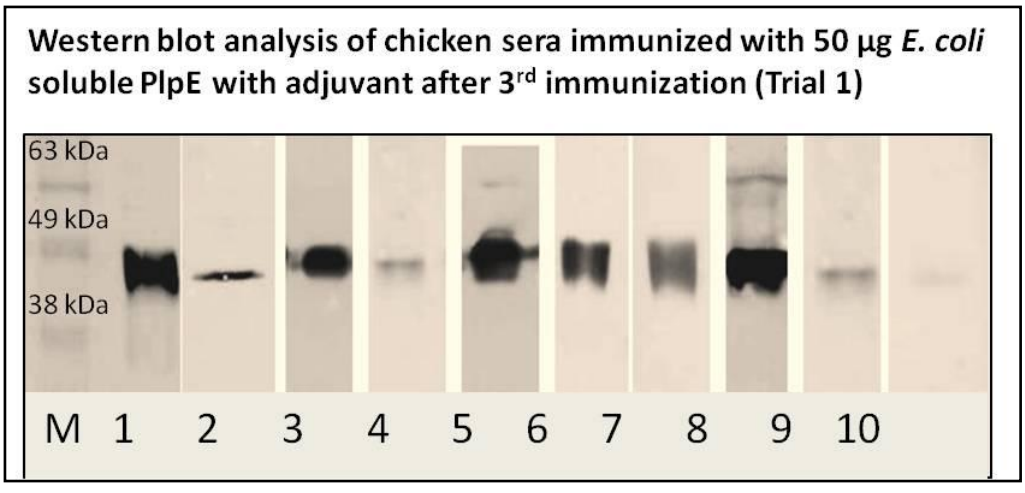

Figure 5.1 Western blot analyses showing the antibody response in chicken sera after the 3rd immunization (Trial 1). *E. coli* insoluble PlpE (600 ng/lane) was probed with chicken sera collected after 3rd immunization (1:200) and with HRP conjugated polyclonal IgY donkey secondary antibody (1:1000). Lane M: molecular mass standards

(SeeBlue® Plus2, invitogen™). Lanes 1 to 10: show sera from 10 chickens immunized with 50 µg soluble *E. coli* PlpE with adjuvant.

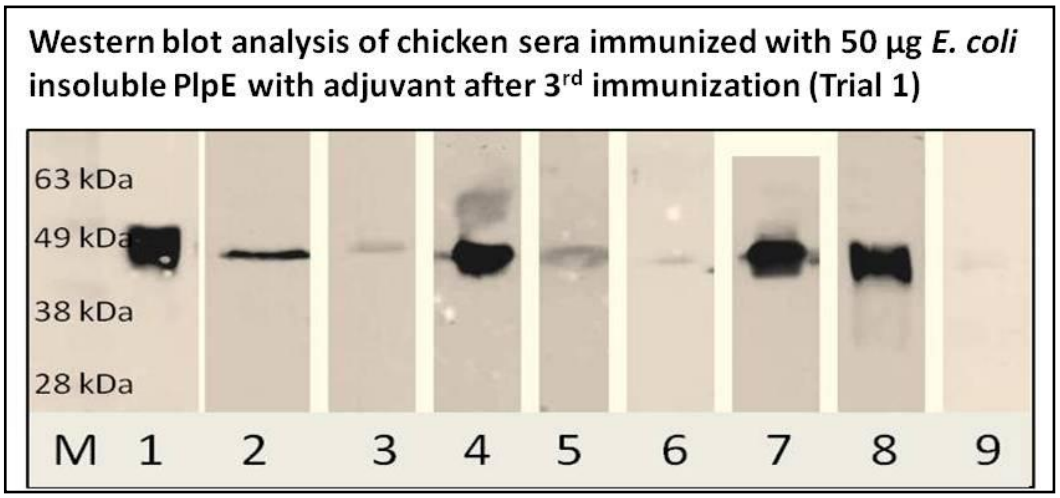

Figure 5.2 Western blot analyses showing the antibody response in chicken sera after the 3rd immunization (Trial 1). *E. coli* insoluble PlpE (600 ng/lane) was probed with chicken sera collected after 3rd immunization (1:200) and with HRP conjugated polyclonal IgY donkey secondary antibody (1:1000). Lane M: molecular mass standards (SeeBlue® Plus2, invitogen™). Lanes 1 to 10: show sera from 10 chickens immunized with 50 µg insoluble *E. coli* PlpE with adjuvant.

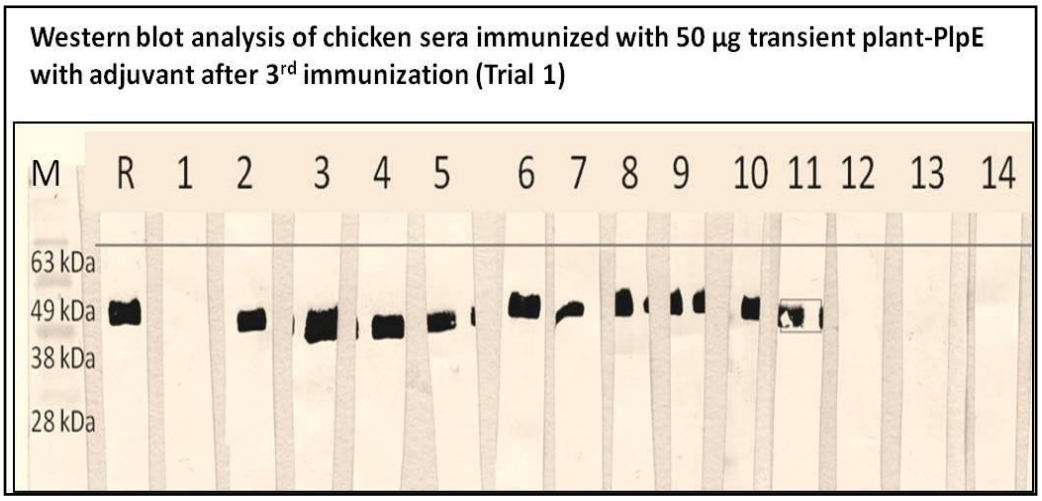

Figure 5.3 Western blot analyses showing the antibody response in chicken sera after the 3rd immunization (Trial 1). *E. coli* insoluble PlpE (600 ng/lane) was probed with chicken sera collected after 3rd immunization (1:200) and with HRP conjugated polyclonal IgY donkey secondary antibody (1:1000). Lane M: molecular mass standards

(SeeBlue® Plus2, invitogen™). Lanes 1 to 10: show sera from 10 chickens immunized with 50 µg transient plant-PlpE with adjuvant.

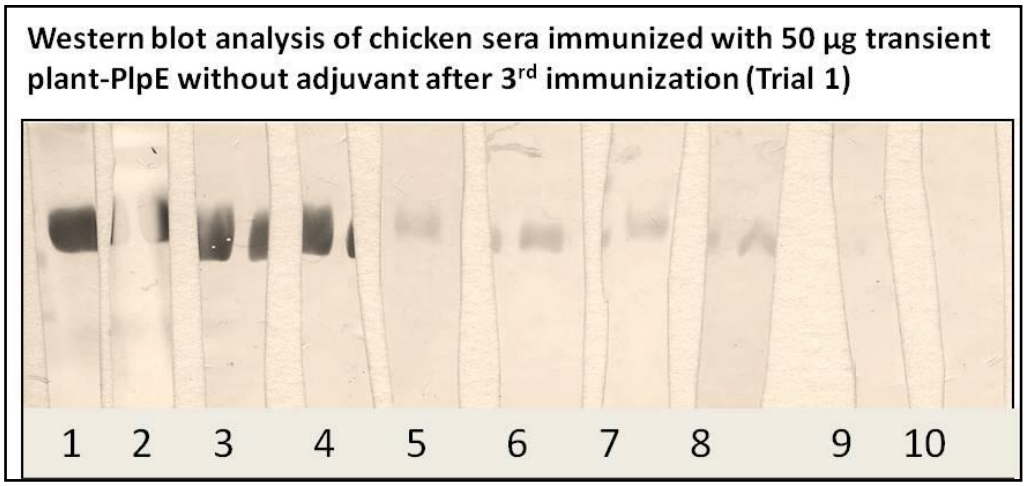

Figure 5.4 Western blot analyses showing the antibody response in chicken sera after the 3rd immunization (Trial 1). *E. coli* insoluble PlpE (600 ng/lane) was probed with chicken sera collected after 3rd immunization (1:200) and with HRP conjugated polyclonal IgY donkey secondary antibody (1:1000). Lane M: molecular mass standards (SeeBlue® Plus2, invitogen™). Lanes 1 to 10: show sera from 10 chickens immunized with 50 µg transient plant-PlpE without adjuvant.

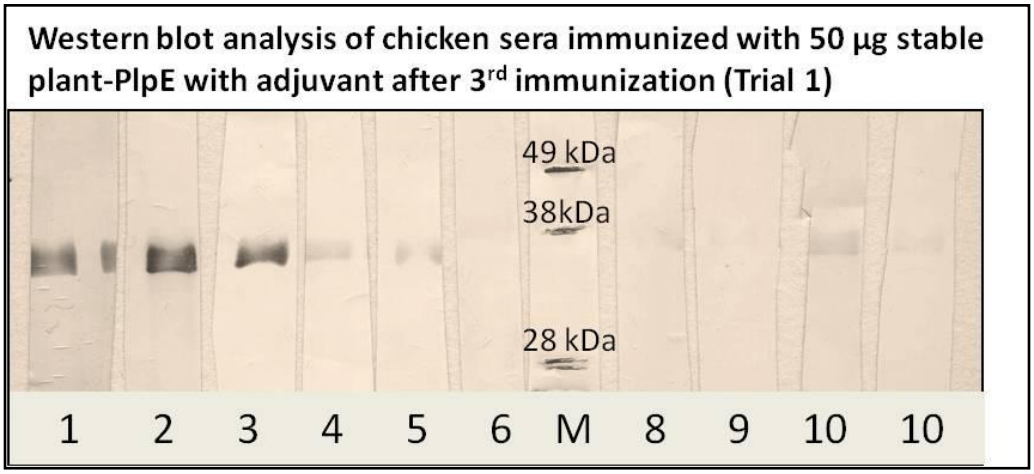

Figure 5.5 Western blot analyses showing the antibody response in chicken sera after the 3rd immunization (Trial 1). *E. coli* insoluble PlpE (600 ng/lane) was probed with chicken sera collected after 3rd immunization (1:200) and with HRP conjugated polyclonal IgY donkey secondary antibody (1:1000). Lane M: molecular mass standards

(SeeBlue® Plus2, invitogen™). Lanes 1 to 10: show sera from 10 chickens immunized with 50 µg stable plant-PlpE with adjuvant.

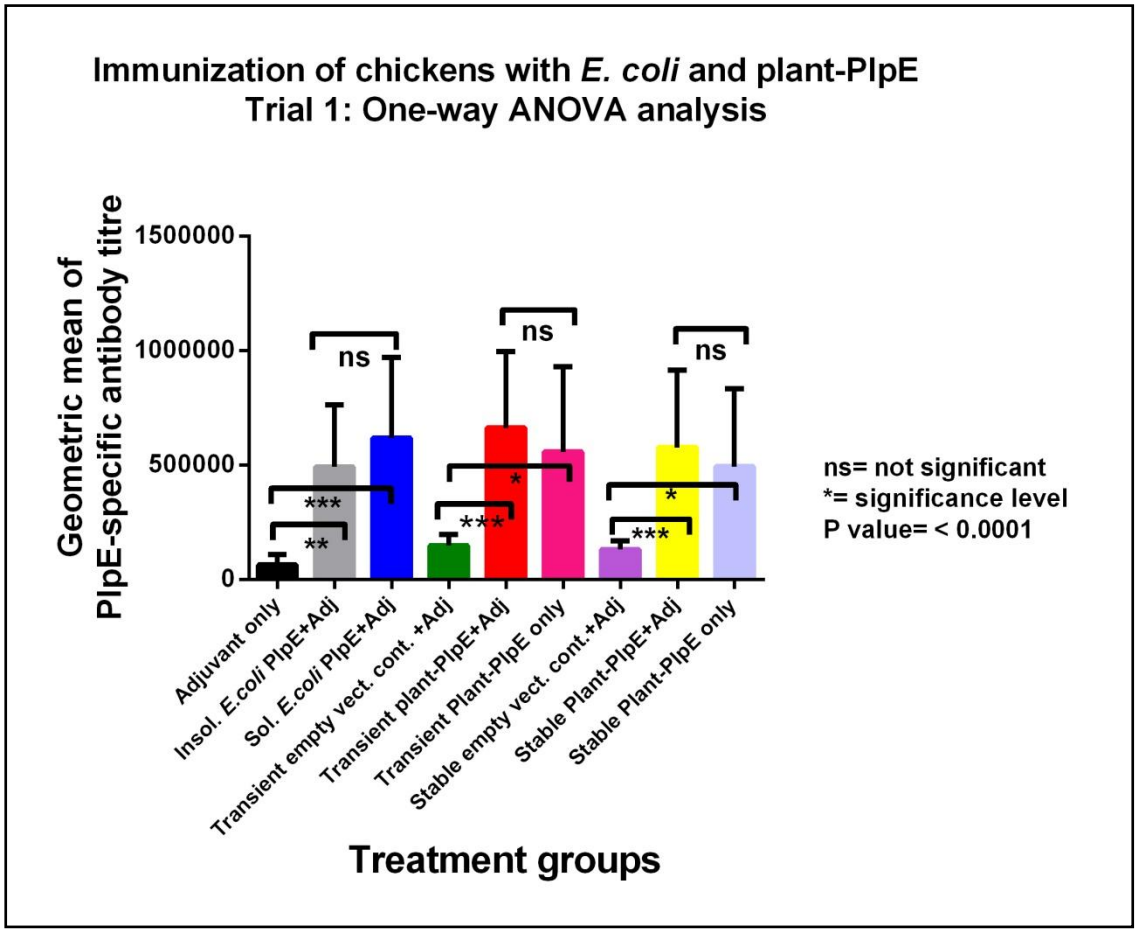

Figure 5.6 Detection of PlpE-specific antibody titres in chicken sera by ELISA (Trial 1). Comparison of antibody titres in *E. coli* and plant-PlpE injected chicken groups after the 3rd or final immunization. The data shows average values originated from 3 independent experiments. The comparisons were performed using one-way ANOVA followed by a Tukey's Multiple Comparison test. The bars represent mean values of the titre for chicken groups and the error bars represent the standard error of the mean (SEM).

Table 5.3 Results of the *P. multocida* challenge trial 1 in chickens

Treatment group	Challenge strain and dose	% survival	Average hours to death	Immune response on Western blot
Transient plant-PlpE with adjuvant	X-73 1.4×10^3 & VP161 1.3×10^5	**40 (4/10)**	**87**	**Strong**
Transient plant-PlpE without adjuvant	X-73 1.4×10^3 & VP161 1.3×10^5	**60 ^{ns} (6/10)**	**87**	**Moderate**
Transient empty vector control with adjuvant	X-73 1.4×10^3 & VP161 1.3×10^5	**60 ^{ns} (6/10)**	**87**	**None**
Stable plant- PlpE with adjuvant	X-73 1.4×10^3 & VP161 1.3×10^5	**70 ^{ns} (7/10)**	**89**	**Moderate**
Stable plant- PlpE without adjuvant	X-73 1.4×10^3 & VP161 1.3×10^5	**60 ^{ns} (6/10)**	**89**	**Western blot analysis not done**
Stable Empty vector control with adjuvant	X-73 1.4×10^3 & VP161 1.3×10^5	**50 ^{ns} (5/10)**	**87**	**None**
E. coli PlpE Insoluble with adjuvant	X-73 1.4×10^3 & VP161 1.3×10^5	**70 ^{ns} (7/10)**	**89**	**Strong**
E. coli PlpE Soluble with adjuvant	X-73 1.4×10^3 & VP161 1.3×10^5	**100* (10/10)**	**N/A**	**Strong**
Adjuvant only	X-73 1.4×10^3 & VP161 1.3×10^5	**53 (8/15)**	**87**	**None**

*=significant, ns= not significant, N/A=Not Applicable

5.3.2 Chicken immunization trial 2

All sera collected from groups of chickens injected with either soluble or insoluble *E. coli* PlpE reacted with recombinant *E. coli* PlpE in Western blotting (Figure 5.7 & 5.8). 70% of the sera collected from chickens immunized with transient plant-PlpE reacted with recombinant *E. coli* PlpE in Western blotting; the transient empty vector control group remained negative (Figure 5.9). The chickens immunized with soluble *E. coli* PlpE or transient plant-PlpE reacted more strongly than those immunized with insoluble *E. coli* PlpE. The Western blot results were confirmed by PlpE-specific ELISA. The PlpE-specific antibody titres were `significantly higher in sera from chickens immunized with 100 µg *E. coli* PlpE, soluble or insoluble, compared to that in chickens injected with adjuvant only (Figure 5.10). Similarly, significantly higher antibody titres were observed in chickens immunized with 100 µg transient plant-PlpE than those in chickens immunized with transient empty vector control. However, no survival of birds was observed in this group, whereas 80% and 50 % of survival was observed in birds immunized with soluble and insoluble *E. coli* PlpE (Figure 5.11). However, the average time to death was significantly delayed in chickens immunized with 100 µg transient plant-PlpE compared to birds injected with transient empty vector control. No significant difference was seen between the surviving and non-surviving chickens that received soluble or insoluble *E. coli* PlpE in terms of serum anti-PlpE antibody titres (Figure 5.12). The results of the challenge trial 2 are summarized in table 5.4.

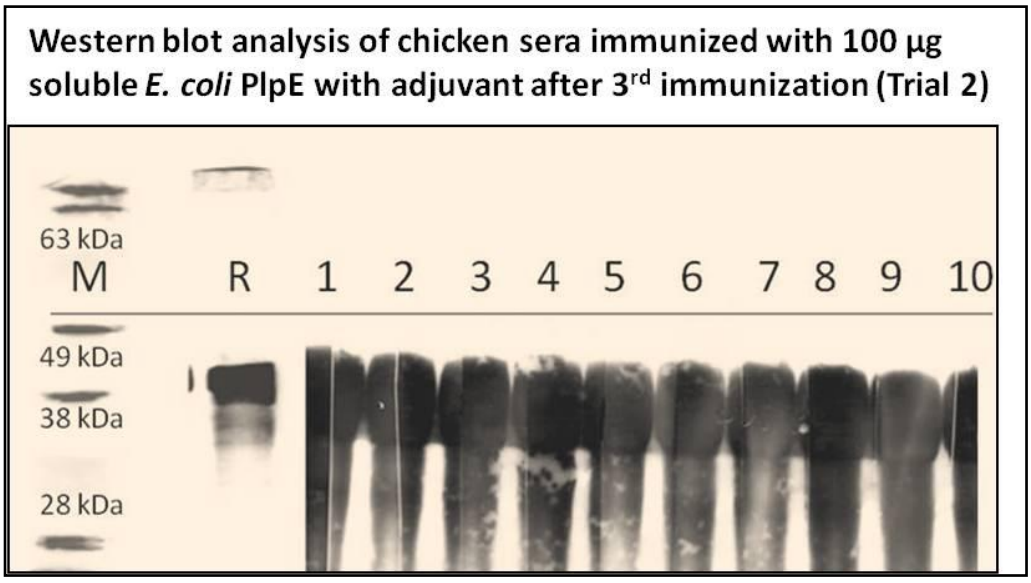

Figure 5.7 Western blot analyses showing the antibody responses in chicken sera after the 3rd immunization (Trial 2). *E. coli* insoluble PlpE (600 ng/lane) was probed with chicken sera collected after 3rd immunization (1:200) and with HRP conjugated polyclonal IgY donkey secondary antibody (1:1000). Lane M: molecular mass standards (SeeBlue® Plus2, invitogen™). Lanes 1 to 10: show sera from 10 chickens immunized with 100 µg soluble *E. coli* PlpE with adjuvant.

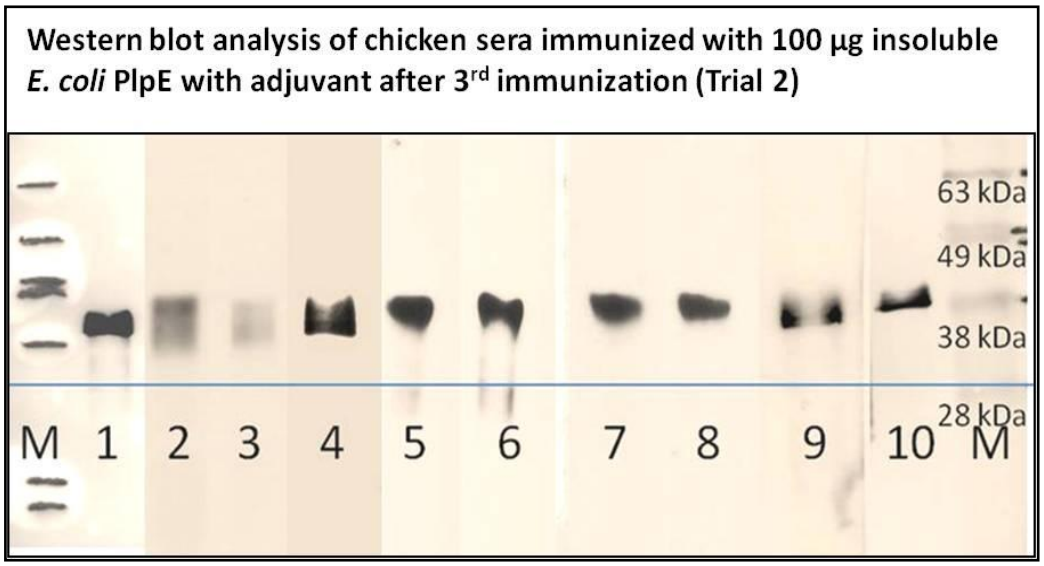

Figure 5.8 Western blot analyses showing the antibody responses in chicken sera after the 3rd immunization (Trial 2). *E. coli* insoluble PlpE (600 ng/lane) was probed with chicken sera collected after 3rd immunization (1:200) and with HRP conjugated polyclonal IgY donkey secondary antibody (1:1000). Lane M: molecular mass standards

(SeeBlue® Plus2, invitogen™). Lanes 1 to 10: show sera from 10 chickens immunized with 100 µg insoluble *E. coli* PlpE with adjuvant.

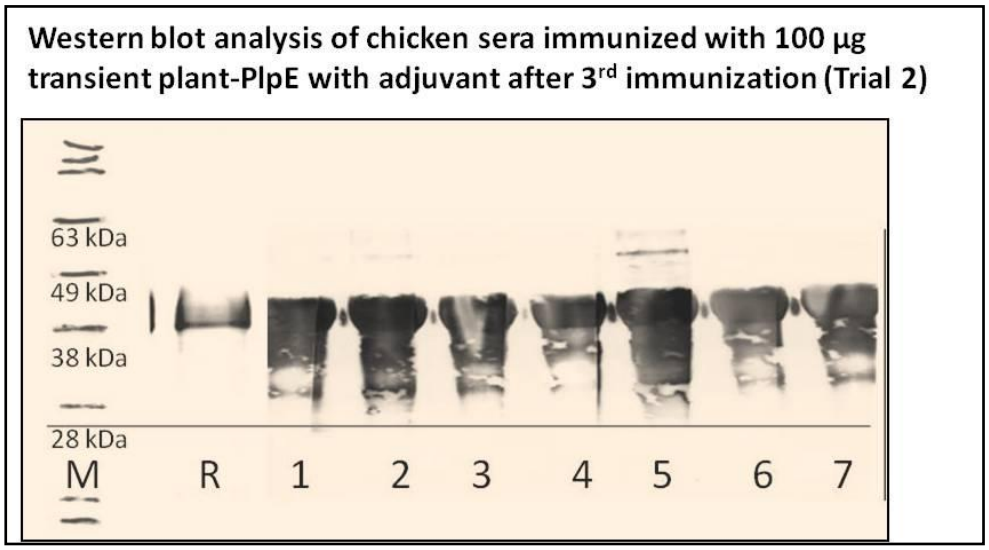

Figure 5.9 Western blot analyses showing the antibody responses in chicken sera after the 3rd immunization (Trial 2). *E. coli* insoluble PlpE (600 ng/lane) was probed with chicken sera collected after 3rd immunization (1:200) and with HRP conjugated polyclonal IgY donkey secondary antibody (1:1000). Lane M: molecular mass standards (SeeBlue® Plus2, invitogen™), R shows anti-PlpE serum from rabbit (positive control), Lanes 1 to 7: show sera from 10 chickens immunized with 100 µg transient plant-PlpE with adjuvant.

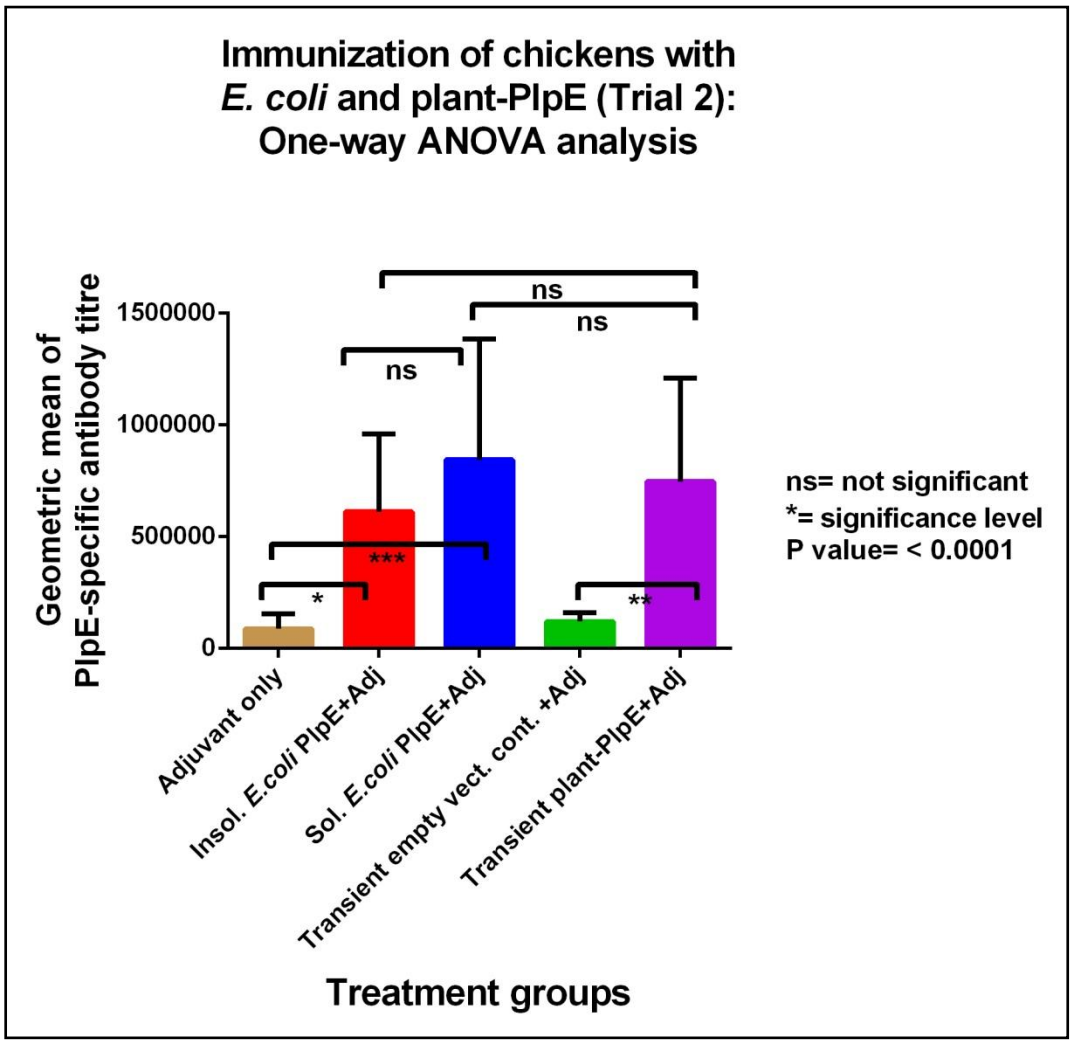

Figure 5.10 Detection of PlpE-specific antibody titres in chicken sera by ELISA (Trial 2). Comparison of antibody titres in *E. coli* and plant-PlpE injected chicken groups after the 3rd or final immunization. The data shows average values originated from 3 independent experiments. The comparisons were performed using one-way ANOVA followed by a Tukey's Multiple Comparison test. The bars represent mean values of the titre for chicken groups and the error bars represent the standard error of the mean (SEM).

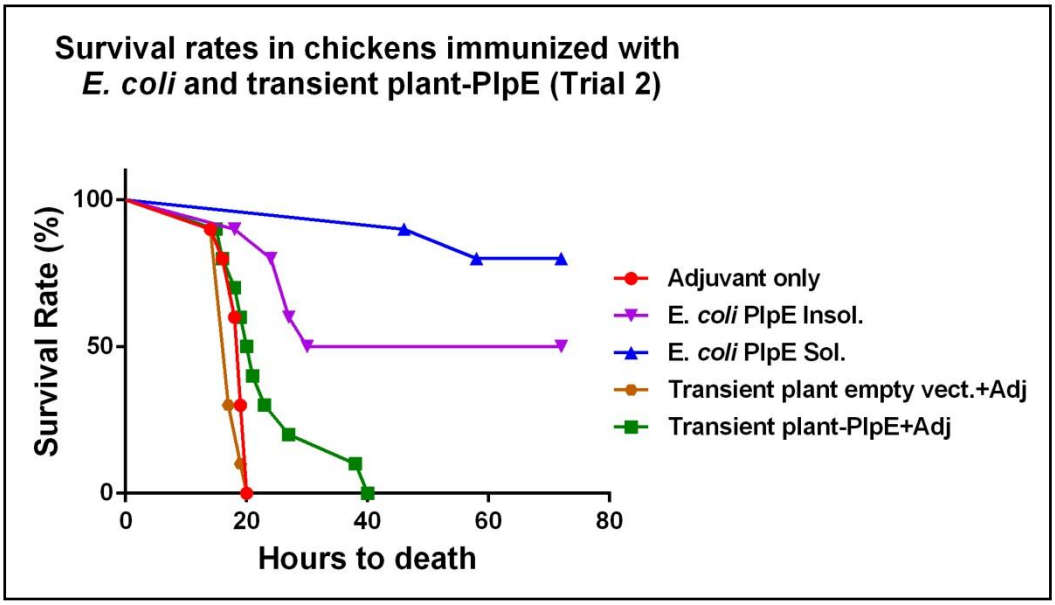

Figure 5.11 Percentage of Survival showing the effect of PlpE vaccines on chicken after a *P. multocida* infection (Trial 2). Statistical analysis was done following Log-rank (Mantel-Cox) Test.

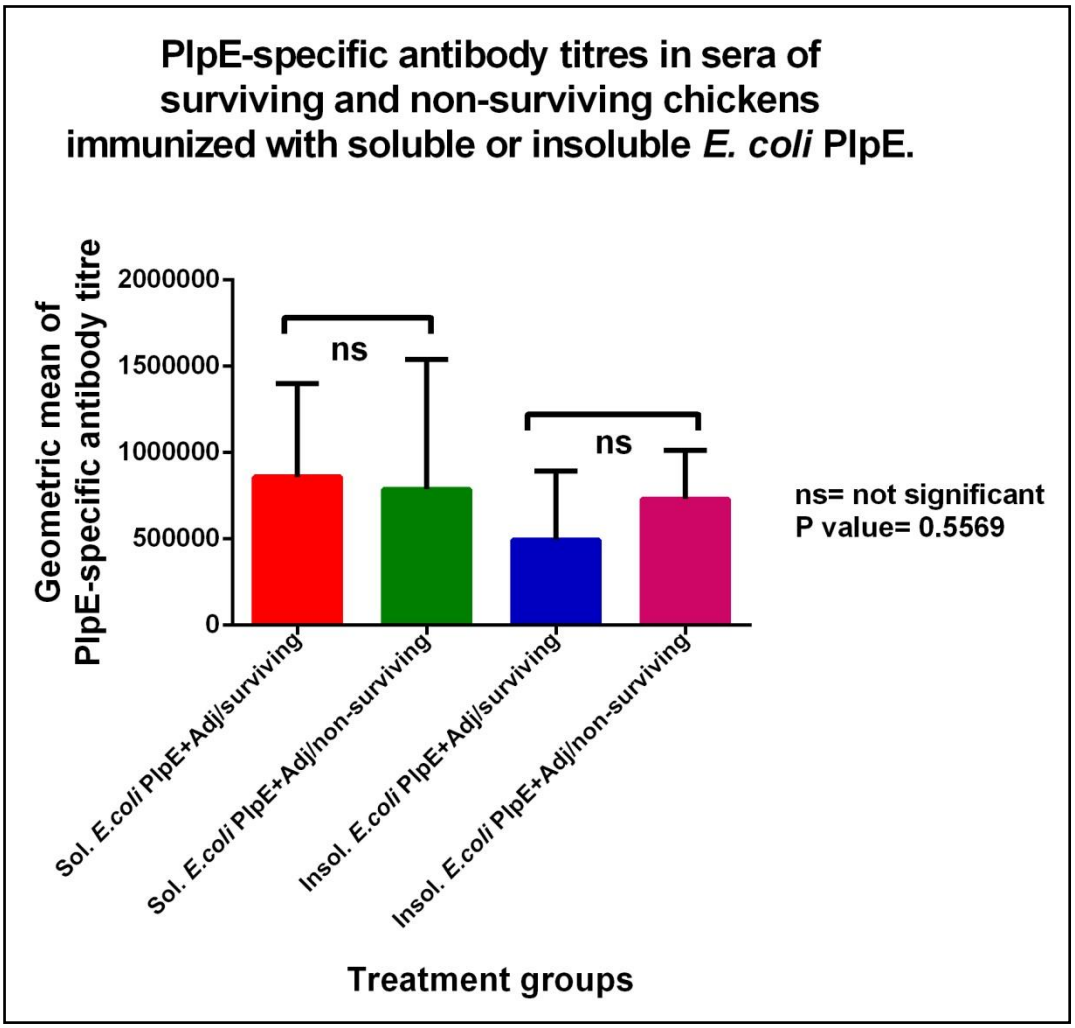

Figure 5.12 PlpE-specific antibody titres in sera of surviving and non-surviving chickens immunized with insoluble or soluble *E. coli* PlpE. The data show average values from 3 independent experiments. The comparisons were performed using one-way ANOVA followed by a Tukey's Multiple Comparison test. The solid bars represent the average titre of each group of birds and the error bars represent the standard error of the mean (SEM).

Table 5.4 Results of *P. multocida* challenge trial 2

Treatment group	Challenge strain and dose	% survival	Average hours to death	Immune response on Western blot
Transient Plant-PlpE 100 µg	**VP161 3 x 10^3**	**0 (0/10)**	**20.5**	**Strong**
Transient empty vector (control)	**VP161 3 x 10^3**	**0 (0/10)**	**17**	**None**
E. coli PlpE Insoluble 100 µg	**VP161 3 x 10^3**	**50* (5/10)**	**51**	**Moderate**
E. coli PlpE Soluble 100 µg	**VP161 3 x 10^3**	**80* (8/10)**	**52**	**Very Strong**
Adjuvant only control (Al Hydrogel®)	**VP161 3 x 10^3**	**0 (0/10)**	**19**	**None**

*=significant, ns= not significant

5.4 Discussion

Recombinant PlpE (100 µg) produced in *E. coli* was able to elicit 100% protection in chickens when injected in a soluble form, while 150 µg of the same antigen in insoluble form elicited 70% protection in birds (Wu et al., 2007b & Hatfaludi et al., 2012). Two different doses (50 or 100 µg) of soluble and insoluble *E. coli* PlpE were administered to chickens during these two chicken trials. It was observed that an increase from 50 to 100 µg did not significantly increase the PlpE-specific antibody titres in chicken sera. The result from the first pathogen trial was not conclusive, as the challenge strain X-73 had lost its virulence such that there were no signs of disease or any mortality the negative control groups. Accordingly,

the birds were re-infected with the highly virulent VP161 strain. However, this challenge did not produce the normally observed mortality. It is therefore likely that an innate and/or adaptive immune response mounted within 48 hours of the X-73 challenge compromised the subsequent VP161 challenge, thus limiting the conclusions that could be drawn from this experiment. Nevertheless, the birds immunized with 50 µg soluble PlpE were 100% protected, which was significantly higher compared to that in negative control adjuvant only group. The 70% survival of the birds immunized with insoluble PlpE was not significant.

During the second trial both soluble and insoluble *E. coli* PlpE were delivered in 100 µg doses and elicited 80% and 50% protection in birds, respectively. However, the use of a heterologous challenge strain may have reduced the levels of protection achieved. The PlpE protein was produced from strain X-73 (A:1), whereas VP161 (A:1) was used for challenge. This finding was similar to that by Wu et al. (2007). They found that chickens were 100% protected when the challenge strain was X-73 (A:1), but it reduced to 63% when the infection experiment was done with a different strain P-1662 (A:4).

No significant differences were observed in PlpE-specific antibody titres between surviving and non-surviving birds (Figure 5.12), similar to the results obtained from the mouse trials (Chapter 3). Therefore, it is clear that the protection in chickens conferred by PlpE had not originated from antibody-meadited immune response solely and there might be a role of cell-mediated immunity. Baba (1984) reported a role for cell-mediated immunity in the protection of chickens against *P. multocida*, possibly involving mediators such as macrophage activating factor from T lymphocytes. Similar type of findings were reported by Tsuji & Matsumoto (1990), who suggested that the phagocytes in chicken liver played a crucial role in the intravascular defence against *P. multocida* in the presence of specific antibodies. The findings from the current study and available literature suggest that the mechanisms behind the protection of chickens by PlpE antigen against *P. multocida* infection might require the induction of both humoral and cell-mediated immunity. It is possible that antibodies produced against PlpE help to inhibit the rapid spread of *P. multocida* through the host body and at the same time PlpE might also stimulate agents like neutrophils and macrophages, which interact with T lymphocytes for the later development of cell-mediated immunity. Another possibility is that the protection conferred by PlpE might be mediated by specific isotype or sub-class of immunoglobulins G (IgG) (Marandi & Mittal, 1997; Goh et al., 2011). Further research is required to test these hypotheses.

Plant-produced PlpE vaccines, both transient and stable, elicited high antibody titres in chicken sera when delivered by sub-cutaneous injection (Figure 5.6). These antigens were injected into birds with or without adjuvant to test if the plant cells could act as adjuvants. No significant difference was observed in PlpE-specific titre in sera from birds that received plant-PlpE antigens with or without adjuvant. After the second infection with *P. multocida* strain VP161 the survival rates were 40 and 60 percent in chickens immunized with transient plant-PlpE with and without adjuvant respectively. The rates were 70 and 60 percent in birds immunized with stable plant-PlpE with and without adjuvant respectively. These survival rates were not significantly higher than the survival rates observed in birds in negative control groups of transient and stable plant empty vector (Table 5.3).

During the second chicken trial, only transient plant-PlpE was tested in birds as no significant protection was conferred to mice by the stable plant-PlpE vaccine in an earlier mouse vaccination trial (Chapter 3). Transient plant-PlpE delivered with adjuvant in 100 µg doses did not protect chickens (Figure 5.11), although a high PlpE-specific antibody titre was found in those birds' sera, which was similar to that elicited by the soluble and insoluble *E. coli* PlpE (Figure 5.10). However, the mean time to death was delayed significantly in chickens injected with plant-PlpE compared to that in birds injected with adjuvant only or plant empty vector with adjuvant (Figure 5.11).

It was observed that both transient and stable plant-PlpE were *N*-glycosylated (Chapter 2). It is thus possible that the difference in protection level elicited by transient plant-PlpE antigen in mice (70%) and chickens (0%) may be explained partially by the glycosylated nature of this plant-made antigen being perceived differently by the different immune systems. However, it was not determined if this N-glycosylation helped to induce and improve the protective immunity in mice as the deglycosylated plant-PlpE was not tested in animals. The core structures of *N*-glycans are common in mammals, birds, insects, yeast and plants (Varki et al., 2009). The mature complex-type *N*-glycans differ to some extent in plants as the complex-type *N*-glycans contain β1,2-xylose and/or core α1,3-fucose residues (Faye et al., 2005), which are not present in mammals. However, no literature could be found on these structures with regards to chickens. Parenteral immunization of rabbits and rats with plant-derived glycoproteins elicited the production of xylose- and core α1,3-fucose-specific antibodies (Prenner et al., 1992; Faye et al., 1988; Bardor et al., 2003; Jin et al., 2006), whereas immunization of BALB/c mice with the same plant glycoproteins did not seem to elicit any humoral immune response (Chargelegue et al., 2000; Bardor et al., 2003; Jin et al.,

2006; Petruccelli et al., 2006). Therefore, immunogenicity of these plant-specific *N*-glycans may depend on the species being immunized (Fischer et al., 2004; Ma et al., 2005). The effect of these plant-derived *N*-glycans on antibody responses of chickens is not known yet. Perhaps that the chicken immune system reacted differently than mice to these plant-specific *N*-glycans present on the plant PlpE antigen due to difference in avian and mammal immune architecture. It is likely that those *N*-glycans were recognized by the chicken's immune system resulting in high PlpE-specific total IgG antibody titres observed in chicken sera but it may be that a specific IgG isotype and/or a cell-mediated immune response is required to stimulate protection, and this response is weakened by glycosylated PlpE. To test this hypothesis, further immunization experiments need to be performed in chickens with both glycosylated and deglycosylated forms of plant-based PlpE.

Another important issue to be considered in this chicken trial is the use of a different *P. multocida* strain VP161 as the challenge strain. Though both X-73 and VP161 belong to the same serogroup and serotype A:1, there are 10 amino acids differences in their PlpE proteins (Appendix 2.1). When amino acid sequences of X-73 and VP161 were analyzed with N-glycosylation site prediction software it was found that one potential N-glycosylation site at amino acid 186 was absent in PlpE of VP161 (Figure 5.13) and the positions of four other potential sites were slightly different between these two strains. Though the *N*-glycosylated plant-PlpE could produce PlpE-specific antibody in chickens it is probably that this antibody could not recognize and bind to PlpE protein of VP161.

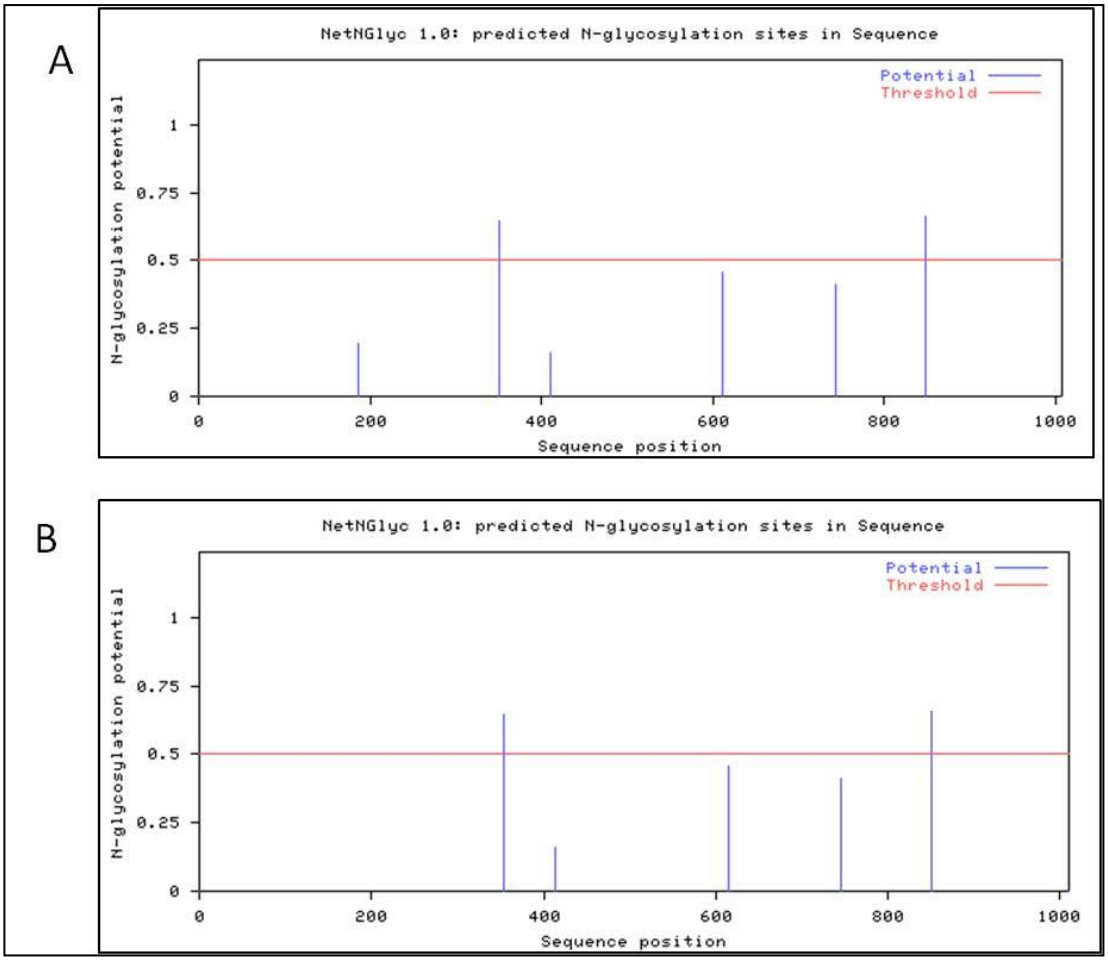

Figure 5.13 Potential N-glycosylation sites (http://www.cbs.dtu.dk/services/NetNGlyc/) **in amino acid sequences of PlpE proteins from *P. multocida* strain X-73 (A) and strain VP161 (B).** (http://www.vicbioinformatics.com/webtools.shtml)

5.5 Conclusions and future directions

E. coli and plant recombinant PlpE antigens were tested in chickens in two different vaccination trials. During the first trial both *E. coli* (soluble or insoluble) and plant-based PlpE (transient and stable) were able to elicit high PlpE-specific antibody titres in chicken sera when administered subcutaneously in 50 µg doses. However, the challenge experiment was not conclusive as two different *P. multocida* strains (X-73 and VP161) were used, due to loss of virulence in the first strain X-73. Only soluble *E. coli* was found to induce 100% protection in birds. Consequently, an increased dose of vaccine was used and a different *P. multocida* strain was used for challenge. Both *E. coli* and transient plant-PlpE induced strong PlpE-specific antibody responses in chicken sera. Both soluble and insoluble *E. coli*

PlpE induced significant protection, with the plant-PlpE failing to elicit any protection. However, the mean time to death was significantly delayed in chickens immunized with plant-PlpE. The non-protective nature of plant-PlpE in chickens might be partially explained by the difference in avian and mammal immune system that reacted differently to the *N*-glycans attached to plant-PlpE antigens. Plant-PlpE was able to induce a high PlpE-specific total IgG titre in chicken sera but it may be that a specific IgG isotype and/or a cell-mediated immune response was necessary to stimulate protection and that was moderated due to the presence of the *N*-glycans on plant-PlpE antigen Additionally, the use of VP161 as the challenge strain might reduce the efficacy of plant-based PlpE as a vaccine. Though plant-PlpE elicited antibody titre in chickens specific to PlpE it might be possible that this antibody could not recognize and bind to PlpE protein of VP161 strain. Therefore, in future experiments, both glycosylated and deglycosylated plant-PlpE antigens should be tested in chickens through systemic administration and a virulent X-73 strain should be used for challenge. To the author's knowledge this is the first report of a plant-based candidate vaccine against fowl cholera as well as any bacterial pathogen of poultry, which has been tested in chickens and shown to be immunogenic.

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Chapter 6

Conclusions and future directions

6.1 Conclusions

The outer membrane lipoprotein, PlpE of *Pasteurella multocida* has been previously demonstrated immunogenic and cross-protective in mice and chickens (Wu et al., 2007, and Hatfaludi et al., 2012). This thesis describes production of PlpE antigen in plants, transiently in *Nicotiana benthamiana* leaves and stably in *N. tabacum* cell lines, with the aim to develop a plant-based recombinant vaccine preferably orally delivered against fowl cholera. It also describes the immunization trials with these plant-based PlpE vaccines in mice and chickens as well as the pathogen challenge trials with *Pasteurella multocida* strains X-73 and VP161.

The N-terminal signal peptide of the LTA protein was fused to the 5'end of the *plpE* gene to direct the plant-expressed PlpE protein to the apoplast of the plant cells. It was predicted that the PlpE would undergo post-translational modification or glycosylation along its route through the plant ER and Golgi complex to the apoplast. This was observed to be the case with both transient and stable plant-PlpE being *N*-glycosylated but not *O*-glycosylated. Western blot analysis of transiently expressed PlpE demonstrated four bands of 44, 42, 40 and 38 kDa. Upon deglycosylation, these bands resolved to 40 and 38kDa. Therefore transient expression resulted in two glycosylation states (44 and 42 kDa) that aren't found in the native host, a product the same size as the native protein (40kDa) and a specific cleavage product (38kDa). Western blot analysis of PlpE derived from stable transformation demonstrated one band running at 42kDa. Upon deglycosylation, this band shifted to 40kDa. Therefore stable expression resulted in one glycosylation state that isn't found in the native host. This different banding pattern due to difference in glycosylation states between stable and transiently produced PlpE was thought due to different species and/or tissues resulting in different environments. Since we did not know the bioactivity of the glycosylated forms or the cleavage product, when the concentration of the recombinant protein was determined in transiently produced plant materials, to be conservative only the 42 and 40 kDa band was utilised (Western blot analysis) or the yield was halved (ELISA). When the concentration of the recombinant protein was determined in stably produced plant materials, the only band at

42 kDa was quantified. The yield of the transient plant-PlpE and stable plant-PlpE were estimated to be 400 mg/kg of freeze-dried leaf powder and 100 mg/kg of freeze-dried cell suspension powder, respectively (Chapter 2). Therefore, plant production systems were able to express PlpE that was recognised by antibodies raised against a recombinant bacterial form of PlpE and in good quantity.

The alkaloid content was found to significantly differ according to the method of transient transformation used. The alkaloid content was significantly higher is plant extracts that had been transformed using hand-injected infiltration as opposed to levels found in vacuum infiltrated materials. The alkaloid levels reached in hand-injected infiltration proved toxic when subcutaneously delivered to mice and deterred consumption. Since this method was also labour and time expensive, the vacuum infiltration method was used in further experiments.

The efficacy of transiently and stably expressed plant-PlpE was tested in mice through subcutaneous delivery (Chapter 3). In the first mouse immunization trial, a subcutaneous dose of 8-10 µg of transient plant-PlpE adjuvanted with 20% (v/v) Al hydrogel® protected 64% of mice after pathogen challenge with *P. multocida* strain X-73. The PlpE dose was increased in the second mouse trial to 100 µg per dose. The transient plant-PlpE delivered subcutaneously to mice with adjuvant provided 70% protection. However, the same antigen could not provide any protection when delivered without adjuvant. Interestingly the PlpE-specific total antibody titre in the animals did not correlate with the protection level. A plant-PlpE derived from stable transformation was also delivered to mice during the second immunization trial with or without the adjuvant Al hydrogel®. 100 µg of stable plant-PlpE delivered subcutaneously without the adjuvant could protect only 10% of the animals while the same antigen with the adjuvant could not provide any protection. It was concluded the difference in protection level observed between the transiently and stably produced plant-based PlpE was due to difference in glycosylation state affecting the bioactivity. Both soluble and insoluble *E. coli* PlpE were delivered to mice with 20% (v/v) Al hydrogel® as positive controls. 10 µg of insoluble *E. coli* PlpE protected only 10% of the animals, whereas 50 µg of the same antigen protected 20% of the animals. Meanwhile 100 µg of soluble *E. coli* PlpE protected 50% of mice whereas, 60% animals immunized with 100 µg insoluble *E. coli* PlpE were protected. This was not significantly different. Additionally, a whole cell killed vaccine prepared from *P. multocida* strain X-73 was delivered to a group of mice with Al hydrogel®

subcutaneously as positive control. This vaccine could not protect the animals at all, likely due to an insufficient dose.

Only transient plant-PlpE was tested orally in mice since not only was the quantity of stable plant-PlpE estimated to be insufficient but it had proved less protective when subcutaneously delivered. In the first oral immunization trial, 7 mice were fed with *N. benthamiana* leaf powder containing 50 µg of transient PlpE and 7 mice were fed with control leaf powder four times. No significant PlpE-specific antibody titre was observed to be elicited in mice sera fed plant-PlpE material compared to that in mice sera fed control plant material. In addition, no animal survived challenge with *P. multocida* strain X-73 ($1.3X10^3$ CFU).

During the second oral immunization trial, a group of 10 mice were fed with *N. benthamiana* leaf powder containing 100 µg of transient plant-PlpE and 10 mice were fed with control leaf powder three times. This time a crude saponin powder was added to the vaccine diets as an adjuvant. Additionally, one positive control group of 10 mice were fed three times with a whole cell killed vaccine prepared from *P. multocida* strain X-73 ($4X10^7$ CFU) and saponin. Another positive control of 10 mice received a subcutaneous dose of 100 µg of insoluble *E. coli* PlpE adjuvanted with Al hydrogel® three times. After challenge with *P. multocida* strain X-73 ($1.4X10^3$ CFU) no mice survived in the fed groups whereas, 70% mice survived in *E. coli* PlpE injected group. However, a significantly high level of PlpE-specific antibody titre was observed in sera of the mice fed with transient plant-PlpE material compared to that in mice fed with control plant material and mean hours to death was also significantly delayed as compared to the control group. The lack of protection afforded by the transient plant PlpE was thought likely due to insufficient dose, inappropriate delivery route and/or glycosylation state. Mice fed with whole cell killed vaccine did not produce a significant level of PlpE-specific antibody titre in their sera compared to that in mice fed with plant-PlpE or in mice injected with *E. coli* PlpE and it also did not protect, most likely due to degradation/digestion. However, the oral route of delivery for this vaccine candidate has not been previously tried (Ian Wilkie, 2011, Personal Communication).

During the first chicken immunization trial (Chapter 5) both transient and stable plant-PlpE antigens were tested with or without the adjuvant Al hydrogel® via subcutaneous injections. 50 µg of transient or stable plant-PlpE were delivered with or without adjuvant three times to groups of 10 chickens. Both insoluble and soluble *E. coli* PlpE were delivered to chickens in 50 µg dose with adjuvant three times as positive controls. Both plant and *E. coli* antigens

were able to elicit immune responses in vaccinated chicken sera. The pathogen challenge trial with *P. multocida* strain X-73 did not work as the strain lost its virulence. A second infection of the birds was performed with *P. multocida* strain VP161. The results from this pathogen challenge trial were not conclusive as more than 50% of the birds in the negative control groups were still alive even after the second infection. This was most likely due to the first failed challenge acting as an inactivated vaccine.

During the second immunization trial chickens were only immunized with 100 µg transient plant-PlpE with the adjuvant Al hydrogel® three times. Both insoluble and soluble *E. coli* PlpE were delivered to chickens in 100 µg dose with adjuvant as positive controls. After the pathogen challenge trial with *P. multocida* strain VP161 ($3X10^3$ CFU), 50% and 80% of the birds were protected in the groups immunized with insoluble and soluble *E. coli* PlpE, respectively. Despite having a strong PlpE-specific antibody titre and the mean time to death being significantly delayed in chickens injected with plant-PlpE compared to that in birds injected with negative controls, no birds were protected in the group immunized with transient plant-PlpE. In addition, no significant differences were observed in PlpE-specific antibody titres between surviving and non-surviving birds immunized with soluble or insoluble *E. coli* PlpE. Therefore, it is clear that the protection in chickens conferred by bacterial PlpE does not originate from antibody-mediated immune response alone. Instead there might be a role for specific IgG isotypes and/or of cell-mediated immunity. It is possible that antibodies produced against PlpE help to inhibit the rapid spread of *P. multocida* through the host body and at the same time PlpE might also stimulate agents like neutrophils and macrophages, which interact with T lymphocytes for the later development of cell-mediated immunity. Further research is required to test these hypotheses.

It was observed that both transient and stable plant-PlpE were *N*-glycosylated (Chapter 2). It is thus possible that the difference in protection level elicited by transient plant-PlpE antigen in mice (70%) and chickens (0%) may be explained partially by the glycosylated nature of this plant-made antigen being perceived differently by the different immune systems. However, it was not determined if this N-glycosylation helped to induce and improve the protective immunity in mice as the deglycosylated plant-PlpE was not tested in animals. Another possible reason could be the use of a different *P. multocida* strain VP161 as the challenging pathogen. Although PlpE is a cross-protective antigen, the protection level still varies with challenge of different strains (Wu et al, 2007).

The aims of this project as mentioned in the Introduction (Chapter 1) have been met except that plant-based PlpE vaccine was not tested orally in chickens since it was thought protection should first be achieved through injection or perhaps intranasal delivery before contemplating the more difficult route of oral delivery. The hypotheses that plants could express PlpE antigen and plant-based PlpE antigen could elicit immune response in mice and chickens have been proved to be correct while the hypothesis that plant-based PlpE vaccine could protect mice and chickens against *P. multocida* infection has been proved to be partially correct as protection was demonstrated in mice but not in chickens. Further research is needed to improve the efficacy of plant-based PlpE vaccine in chickens against fowl cholera.

6.2 Future directions

The over-all outcomes made from this PhD project are promising. This is the first time that an antigen from *P. multocida* has been expressed in plants, found immunogenic in mice and the host and protective when subcutaneously delivered to mice. This thesis also provides evidence that protection against *P. multocida* challenge is not provided by humoral immune response alone.

To improve the efficacy of plant-based PlpE several strategies can be taken. Intranasal delivery of plant-PlpE should be tested as an alternate vaccination route since nasal immunization results in a strong immune response at respiratory mucosa, the site of *P. multocida* entry and colonization (Wilkie et al, 2000). In addition intranasal delivery induces a more broad-spectrum mucosal response than oral immunization (Ogra et al, 2001). However, intranasal delivery of a non-replicating subunit vaccine is unlikely to be able to be performed in timely and non-labour intensive manner. Therefore, due to the ease of oral delivery, increased dose of orally delivered antigen should also be investigated to determine if a protective immune response can be induced. To assist in improving the induced immune response a prime-boost vaccination strategy should be investigated with parenteral delivery of purified plant-PlpE followed by an oral boosting with the same plant-PlpE (Judge et al., 2004; Lauterslager et al., 2001). This may also guard against oral tolerance (Rogalska et al., 2011). Also, the immunogenicity of plant-based PlpE antigen should be compared in animals in its glycosylated and deglycosylated forms. Glycosylation can be avoided by deleting the glycosylation site, targeting the accumulation of PlpE to the cytoplasm or to plant organelles such as the chloroplast where glycosylation doesn't result (Daniell et al 2001). In addition

alternative adjuvants could be tried such as co-delivery with LTB or CTB. These adjuvants could improve mucosal immunity (Arakawa et al., 2001; Mason et al., 1998).

In this study we observed variation in protection afforded against different strains within the same serotype (A1). It would not be unexpected to see this continue with different serotypes. To improve the cross-protectiveness of a plant-based fowl cholera vaccine a multicomponent subunit vaccine (Yu & Langridge, 2001) should be investigated that combines the protective antigens of *P. multocida*, PlpE, OmpH and FhaB2 (Wu et al., 2007; Lee at al., 2007; Tatum et al., 2009). It would be interesting to also investigate the success of cross-protection should these antigens be derived from different serotypes (A1, A3). As highlighted by this thesis, the TH1 response induced by plant-made PlpE vaccines should also be investigated as it appears more than a humoral response is required for protection against *P. multocida*. The ultimate goal should be to test mucosal delivery of the vaccine in chickens, the natural host of fowl cholera.

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Appendix 1

Nucleotide Sequence for *plpE* gene

atgaaacaaatcgttttaaaaacaagcttattgatgaccctctcttcattattagttgcatgtagcggcggtggcggtagcgctggaaatcg
tgctgaccgtgtagaggaaaaagcacaaccggttcaatcaaatagtgagccttcttccgctccaatcaaaaatcctactaataccgctac
gaatgattctcttcatgacaaactttcaatgtcttctcatgacacatccaaagaaaatagtcaacaatcctcctttaaagcccctctagaaca
agaaaaaaaccaacctgcacaagaaaatctcacttggacaggttatcatgtttcagaagtgggaaatgcgagtaataatgtagataaag
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attagttgacggaaaaaatgagaataaagaggattattataactttacgttaaaagacgctttattttattatggaagttatggacaaccttca
gcagattacaaaaaagtagaaaaaaattatatttatgcaattaaaccagatgcaataaataatgagaacctcaatgcactaactgcaactt
attatcaagaagatggttttatatattccgtattaagtgatgtaaatcgagttggttcagaatatattcctcagtatggcaatgtgactcttactt
tccgaaatggcaagatttatggtgaaatctacagatataatagaggacgtgatgatttgtttcagctctcaggagaaggacaaaacttaac
tataacaccacacaaggacaatccccataaactatcccctacaggacccgacaacatggcaatggagctgaattttatcaacgcagaa
aaaactgataaaaaatacgttgttggtgtaggaaaagctgaaaaatattatgggttattatttgctgaaaaaagtcaccaagcacaataa

Appendix 2

2.1 Amino Acid sequence for PlpE protein

2.1.1 Pasteurella multocida strain X-73(A:1)

ATGAAACAAATCGTTTTAAAAACAAGCTTATTGATGACCCTCTCTTCATTATTAGTTGCATGTAGCGG
TGGTGGCGGTAGCGCTGGAAATCGTGCTGACCGTGTAGAGCAAAAAGCACAACCGGTTCAATCAAA
TAGTGAGCCTTCTTCCGCTCCAATCAAAAATCCTACTAATACCGCCACGAATGATTCTCTTCATGACA
AACTTTCAATGTCTTCTCATGACACATCCAAAGAAAATAGTCAACAATCCTCCTTTCAAGCCCCTCTAG
AACAAGAAAAAAACCAACCTGCACAAGAAAATCTTACTTGGACAGGTTATCATGTTTCAGAATGGG
GAAATGCGAGTAATAATGTAGATAAAGATAATGTTACGGTATTCACTTTCGTAAAATATAATTCTCAA
TACAATGATGATCCAGTTTTTGATAAAACAAAAACACAAAGTAAAACAATATCATTAGTTGACGGCA
AAAACGAAAGTAAAGAAGATTATTATCACTTTACGCTAAAAGACGCTTTATTTTATTATGGCAGTTAT
GGACAACCTTCAGCAGATTACAAAAAAGTAGAAAAAAATTATATTTACGCGATTAAACCAGATGCAA
TAAATAATGAGAACCTCAATGCACTAACTGCAACTTACCATCAAGAAGATGGTTTTATATATTCCGTA
TTAAGTGATGTAAATCGAGTTGGTTCAGAATATATTGCTCAGTATGGCAATGTGAGTCTTACTATACA
AAATGGTAAAATTCATGGTGAGATTTATAGGCATAACCGAGGGTACGATGATCTATTTAAGCTCTCT
GGAGAAGGCCGGAATTTAATATTAACGCCACATAAAAATAACCCTCATGATCTTTCCCCAACAGGAC
CCGACAACATGACAATGGAGCTGAATTTTATCAACGCAGAAAAGACTGATAAAAAATACGTTGTTG
GTGTAGGAAAAGCTGAAAAATATTATGGGTTATTATTTGCTGAAAAAAGTCACCAAGCACAATAA

2.1.2 Pasteurella multocida strain VP161 (A:1)

ATGAAAAAAATCGTTTTAAAAACAAGCTTATTGATGACCCTCTCTTCATTATTAGTGGCATGTAGCGGTGGTGG
CGGTAGCGCTGGAAATCGTGCTGACCGTGTAGAGGAAAAAGCACAACCGGTTCAATCAAATAGTGAGCCTTC
TTCCACTCCAATCAAACATCCTATGACTAATAGTGCTACGAATACTTCTCTTCATGACAAACTTTCAATGTCTTCT
CATGACACATCCAAAGAAAATAGTCAACAATCCTCCTTTCAAGCCCCTCTAGAACAAGAAAAAAACCAACCTG
CACAAGAAAATCTTACTTGGACAGGTTATCATGTTTCAGAATGGGGAAATGCGAGTAATAATGTAGATAAAG
ATAATGTTACGGTATTCACTTTCGTAAAATATAATTCTCAATATAATGATGATCCAGTTTTTGATAAAACAAAAA
CACAAAGTAAAACGATATCATTAGTTGACGGAAAAAATGAAAATAAAGAGCATTATTATCACTTTACGCTAAA
AGACGATTTATTTTATTATGGCAGTTATGGACAACCTTCATCAGATTATAAAAAAATAGAAGAAAACTATATTT
ATGCAATCAAACCAGATGCAATAAATAATGAGAACATCAATGCACTAACTGCAACTTACCATCAAGAAGATGG
TTTTATATATTCCGTATTAAGTGATGTAAATCGAGTTGGTTCAGAATATATTCCTCAGTATGGCAATGTGAGTC
TTACTATACAAAATGGTAAAATTTATGGTGAGATTTATAGGCATAACCGAGGGTACGATGATCTATTTAAGCTC
TCTGGAGAAGGTCGGAATTTAATATTAACACCACATAAAAATAACCCTTATGATCTTTCCCCTACAGGACCCGA
CAACATGACAATGGAGCTGAATTTTATCAACGCAGAAAAGACTGATAAAAAATACGTTGTTGGTGTAGGAAA
AGCTGAAAAATATTATGGGTTATTATTTGCTGAAAAAAGTCACCAAGCACAATAA

Appendix 3

3.1 LB Medium: Component Quantity per liter

Bacto-tryptone (Sigma T-9410) 10.00 g

Yeast extracts (Difco 212750) 5.00 g

Sodium chloride (Sigma S-6191) 10.00 g

Bacto Agar (Difco) 15.00 g

3.2 YM Medium: Component Quantity per liter

Yeast extracts (Difco 212750) 400 mg

Mannitol (Sigma M-9546) 10.00 g

Sodium chloride (Sigma S-6191) 100 mg

Magnesium sulfate heptahydrate (Sigma M-7774) 200 mg

Potassium phosphate, monobasic (Sigma P-9791) 500 mg

3.3 NT-1 Liquid Medium: Component Quantity per liter

MS salts (PhytoTech M524) 4.30 g

MES stock (20x) (as below) 50.00 mL

B1 inositol stock (100x) (as below) 10.00 mL

Miller's 1 stock (as below) 3.00 mL

2,4-D (1 mg/mL stock) (Sigma D-7299) 2.21 mL

Sucrose (PhytoTech S391) 30.00 g

pH was adjusted to 5.7 +/- 0.03

Miller's 1 (1 liter total)

Potassium phosphate, monobasic (Sigma P-9791) 60.00 grams

B1 Inositol Stock (100x) (1 liter)

Thiamine HCl (Vitamin B1) (Sigma T-3902) 0.10 g

Myo inositol (PhytoTech I703) 10.00 g

MES Stock (1 liter total)

2-(N-morpholino) ethanesulfonic acid (MES) (PhytoTech M825) 10.00 grams