# The Identification, Isolation and Partial Characterisation of VapA-encoding virulence plasmids in *Rhodococcus equi* found in New Zealand.

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**ABSTRACT**

This thesis describes the identification, isolation and partial characterisation of VapA-encoded virulence plasmids present in *Rhodococcus equi* (*R. equi*) found in foals in New Zealand. *R. equi* infection associated with the presence of virulence plasmids causes severe pyogranulomatous pneumonia in foals and, if left untreated, it can be lethal. Foals are thought to be infected when they ingest or breathe in bacteria present in soil, dust and faecal particles after which the bacteria multiply inside macrophages and cause pneumonia. Studies have shown that virulence is associated with the presence of the *vapA* gene in a plasmid that encodes a VapA protein. Twelve different types of VapA-encoding virulence plasmids have been described so far. Epidemiological studies of America, Australia, Korea, Japan and some parts of Europe have revealed that more than one subtype can exist in a farm or even a country. The aim of the study was to investigate if more than one subtype of VapA-encoded plasmids exists in a farm in New Zealand.

Nasal swabs for the analysis were obtained from the Auckland Veterinary Centre in Papakura. Based on colony morphology and 16S primers, successful identification of *R. equi* was possible, VapA primers assisted with distinguishing bacteria that carried VapA-encoded plasmids. Since success with plasmid isolations was modest, characterization of plasmids was conducted by sequence analysis of an approximately 1-kb variable region present in VapA-encoded plasmids. Sequence analysis and restriction digestion patterns revealed two distinct patterns of sequences from the variable region which were divided into two groups: PI and PII.

Results indicated that PI sequences were significantly similar to published 85-kb type I plasmids and must also be from 85-kb type I plasmids but more detailed analysis of other regions is required to confirm the results. PII sequences on the other hand were distinctly different from PI sequences and displayed poor matches with published plasmid sequences. This is the first attempt at characterising VapA-encoded virulence plasmids from *R. equi* isolates in New Zealand. This work will contribute towards increasing our knowledge regarding the unique characteristics of the VapA-encoding plasmids, which could define transmission characteristics, possible sources of infection in disease outbreaks and vaccine development.

## PREFACE

This thesis begins with a Literature Review (Chapter 1), which describes *R. equi* growth, morphology, biochemistry, infection, spread of infection, immunity against infection and treatment of infection. A review on *R. equi* species-specific molecular markers, *R. equi* virulence molecular markers and VapA genetics follows after which there is a brief description of the proposed methodology which includes the aims of the project and finally a conclusion that highlights the importance of the study. Chapter 2 is presented in the form of a research paper prepared for publication in a scientific journal. The main findings of this research project are presented here. Chapter 3 provides a conclusion, including a summary of results and some suggestions and recommendations for further research. The references are found in Chapter 4. Chapter 5, which is the Appendix section, includes additional protocols, recipes of solutions and results that are not presented in Chapter 2, including DNA extractions, PCR optimisation and post-PCR processing of DNA. The appendices also contain additional gel photos of all strains of *R. equi* tested with 16S, VapA and TraA primers. The appendices section concludes with a BLAST analysis of all PI and PII isolates of *R. equi* and electropherograms of a PI and PII isolate. The alignment of the four published plasmid sequences, table of ORFs in virulence plasmid p33701, amplification regions of primer 74 and 76 and the electropherogram data for all 21 PI and PII sequences can be found on the CD at the back of the thesis. Also, the area selected for amplification is highlighted in the plasmid alignments and the links to software required to view the electropherograms are available.

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# 1 Literature Review

## 1.1 Introduction

Members of the *Rhodococcus* genus which was first described by Zopf in 1891 are gram-positive, aerobic, non-motile, non-sporulating (Singer & Finnerty 1988) and metabolically diverse bacteria (Larkin *et al*. 2005). The genus *Rhodococcus* ('red pigmented cocci') belongs to the phylogenetic group described as nocardioform actinomycetes, on the basis of cell wall composition (Tkachuksaad & Prescott 1991; Finnerty 1992). The order being *Actinomycetales*, the suborder is *Corynebacterineae* and the family is *Nocardiaceae* (Magnusson 1923; Goodfellow & Alderson 1977). They can be found in various environments including plant surfaces, soils, insect guts, ground water, boreholes, marine sediments, rocks, animal faeces etc (Goodfellow 1989; Bell *et al*. 1998). Warhurst and Fewson (1994) have commented that *Rhodococci* can persist in soil even under starvation conditions thus emphasising their robustness. Virulent *Rhodococcus* have been isolated in countries such as Australia, Japan, Korea, America and in parts of Europe. Since the *Rhodococcus* cell wall consists of aliphatic chains of mycolic acids, thus making the cell hydrophobic, they may be able to degrade hydrophobic pollutants by allowing cells to adhere oil/water interphases (Bell *et al*. 1998). The *Rhodococcus* species include symbionts like *R. rhodnii* and other members like *R. globerulus, R. erythropolis, R. rhodochrous* which are known for their ability to degrade hazardous chemicals such as polychlorinated biphenyls (Bell *et al*. 1998) *R. percolatus, R. erythropolis* and *R. rubber* are known to produce surfactants which contain both strongly hydrophobic and hydrophilic groups and are thus able to migrate to interphases between oil and

aqueous phases, this allows the inexpensive purification of biosurfactants that in turn is used for biodegradation of pollutants (Bell *et al*. 1998). *Rhodococcus (R. equi)* - previously called *Corynebacterium equi* -was first described by Magnusson in 1923 based on an isolate from the lung of an infected foal (Takai 1997). The bacteria are surrounded by a complex thick and lamellar polysaccharide capsule (von Bargen & Haas 2009) that contains mycolic acid side chains (Hondalus 1994). *R. equi* strains are pathogenic (Anzai *et al*. 1997), affect foals less than four months of age (Tkachuksaad & Prescott 1991), have a circular chromosome that is *c*. 5 Mb (Sanger 2008) and can have different types of plasmids some of which are found to be linked with virulence (von Bargen & Haas 2009). The *R. equi* genome is 5,043,170 bp in length and has a G+C content of approximately 68.82%. Shotgun sequencing data reveals that there are 91,118 reads totalling 43.169 Mb and giving a theoretical coverage of 99.97% of the genome (Sanger 2008).

### 1.1.1 Media, Morphology and Biochemistry

A selective medium that prevented the growth of many competing organisms was developed; it consisted of a trypticase soy agar (TSA) base to which nalidixic acid (20 µg/ml), novobiocin (25 µg/ml), actidione (Cyclohexamide) (40 µg/ml) and potassium tellurite (0.005%) (Woolcock *et al*. 1979). This medium was named NANAT and has been used for *R. equi* selective growth for the past 30 years (Muscatello 2006). However, the base medium TSA and the modifications made to it produced unsatisfactory colony morphology and “did not allow” differentiation of bacteria, thus blood agar was substituted as the base medium (Woolcock *et al*. 1979). Comparisons of NANAT with other media such as CAZ-NB {Mueller-Hinton agar

base with the addition of ceftazidime (20 µg/ml) and novobiocin (25 µg/ml)} for *R. equi* recovery have been made but none are found to be significantly more effective than NANAT (Woolcock *et al*. 1979; Muscatello 2006). Although sheep blood agar has been recommended, many recent studies have successfully used Blood Heart Infusion (BHI) agar as the base medium and this is also the media chosen for use in this study. The addition of polymyxin B (30 µg/ml) to the NANAT medium has been suggested to resolve the problem of *P.aeruginosa* growth (Woolcock *et al*. 1979) and is also incorporated in the NANAT medium used for this study.

Depending on the growth conditions, *R. equi* morphology varies, after 4 h (hours) of growth in culture broth bacteria are rod shaped but become coccoid after a day of growth in liquid media or on blood agar (von Bargen & Haas 2009), viscous-mucoid coalescing colonies are predominant in fresh cultures (Prescott 1991). After 24 h, colonies are 1 to 2 mm in diameter but by 48 h they develop their characteristic appearance: irregularly round, smooth, mucoid, glistening, semitransparent, salmon pink to yellow coloured and coalescing colonies (Prescott 1991). They are not known to have flagellae but some strains have pili or appendages (von Bargen & Haas 2009). The optimal growth temperature for *R. equi* growth is between 30 and 37ºC and colonies develop into a shade of salmon pink to yellow after a few days of growth on (Prescott 1991).

Species within the *Rhodococcus* genus have an oxidative metabolism and are categorized by their capability to utilize unusual compounds such as alicyclics, polycyclic hydrocarbons, steroids and nitroaromatic compounds (DeLaPenaMoctezuma *et al*. 1996). The organism is biochemically un-reactive as it is

unable to oxidize or ferment any alcohols or carbohydrates (Prescott *et al*. 1991). Their nutritional requirements are simple; they can utilize carbon from many different sources and simple organic acids such as acetate, which can be obtained from herbivore manure (von Bargen & Haas 2009), as well as a variety of sugars (DeLaPenaMoctezuma *et al*. 1996). Nitrogen can be utilized from ammonium sulphate or potassium nitrate (Prescott 1991). *R.equi* produces equi factors that are known to interact with the beta-toxin of *S. aureus*, phospholipase D of *Corynebacterium psuedotuberculosis* and a partial hemolysin of *L. monocytogenes* thereby giving an area of complete hemolysis with cattle erythrocytes (Prescott *et al*. 1982). Although this characteristic is not unique to *R. equi*, no negative isolates of *R. equi* display this property, so it is useful for *R. equi* identification (Prescott *et al*. 1982).

### 1.1.2 *R. equi* Infection in Foals and other Species

For over 80 years *R. equi* has been recognized as a pulmonary pathogen of horses. It is known for zoonotic infections in foals that are between 1 and 4 months of age (Tkachuksaad & Prescott 1991; Yager *et al*. 1991; von Bargen & Haas 2009). It has been ranked as among the four most important disease problems in the horse industry especially because of its high prevalence and mortality rate, which is approximately 28% (Blood-Horse 2009; Muscatello 2009). Symptoms in foals include fever and respiratory distress; pulmonary lesions and chronic pus-filled lung abscesses, which develop in early stages of the disease and if left untreated can cause death by asphyxiation (Wichtel *et al*. 1991; Lavoie *et al*. 1994). Clinical signs include coughing, thick greenish-white nasal discharge, lethargy and an increased respiratory

rate (Blood-Horse 2009). The infection can spread from the lungs to other organs and joints when granulomatous foci in the lung open up (Prescott 1991). Infection of the gut lining causes diarrhoea with an ulcerative enteritis and mucosal invasion of *R. equi* which is frequently observed in chronic disease (Bell *et al*. 1998; Vazquez-Boland *et al*. 2009). Immune complex deposition in synovial membranes can cause polysynovitis which contribute to the development of uveitis, anemia or thrombocytopenia in infected foals (Giguere *et al*. 1999). Occasionally, osteomyelitis and arthritis are also observed (Giguere *et al*. 1999). Although infections can sometimes occur in healthy adult horses they are more common and severe in foals due to their compromised immunity (Hondalus 1994). However, both adult horses and some foals are able to develop protective immunity and quickly clear infection when re-exposed to virulent *R. equi* (Lopez *et al*. 2002). It has been found that only a small proportion of all *R. equi* in soil are able to cause the infection and only *R. equi* carrying virulence plasmids can cause disease in foals (Muscatello *et al*. 2006b). Foal to foal transmission of *R. equi* has not yet been documented (von Bargen & Haas 2009) but exhaled air from foals may be a significant source of virulent *R. equi* for other foals (Muscatello 2006). In some strains of *R. equi*, presence of a plasmid (as shown in Figure 1.1 below) encoding a 15-17kDa protein called VapA (Virulence Associated Protein A) is known to be responsible for virulence (Takai 1991b; Tkachuksaad & Prescott 1991).

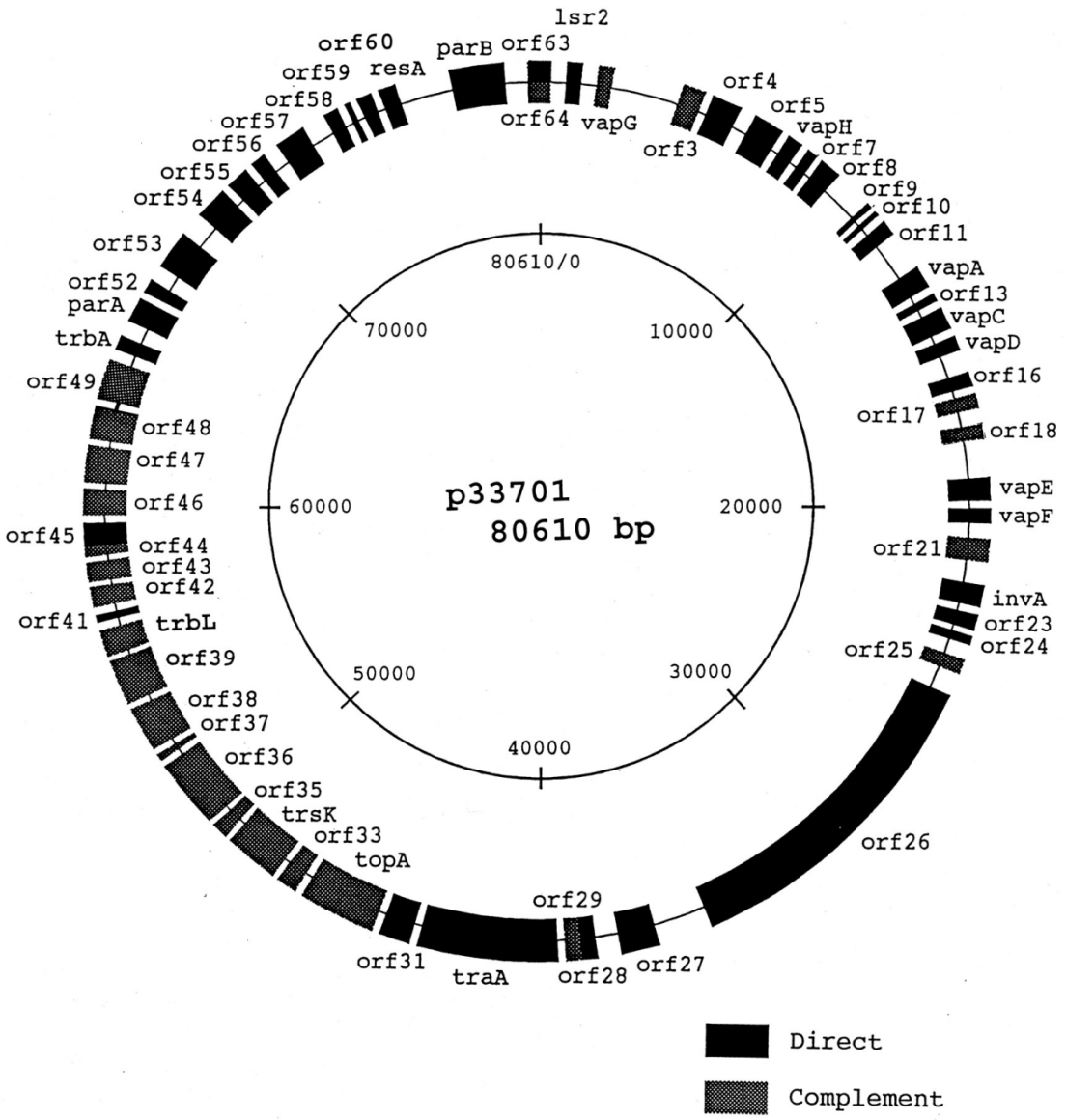


Figure 1.1**:** Map of the *R. equi* virulence plasmid p33701. The circle shows orientation of the ORFs, with the direction of reading denoted by shading. Genes with a known or suspected putative function based on homology with genes of a known function in DNA databases are named (Takai *et al*. 2000).

*R. equi* infect immunocompromised hosts by multiplying within macrophages, seizing the normal pathway of phagosome maturation and this

eventually results in necrotic death of the infected cell (Hondalus 1994; Luhrmann *et al*. 2004). This causes severe pyogranulomatous pneumonia in foals which is characterized by inflammation and lung abscesses and, if left untreated, can be fatal (Takai *et al*. 1993). This disease (commonly called "rattles") is estimated to affect up to 17% of foals worldwide (Elissalde *et al.* 1980). In Australia 1-10% of foals are affected, although antibiotic treatment reduces mortality rates to less than 1%; yet, in some farms mortality rates are as high as 20% or greater (Muscatello *et al*. 2006b). As shown in Figure 1.2 which shows the distribution of VapA among species, in 85% of cases the presence of a VapA encoding virulence plasmid has been associated with *R. equi* infection in foals for the last couple of decades (Rodriguez-Lazaro *et al*. 2006). Experiments with the presence of the VapA-expressing plasmid in *R. equi* showed an increase in the percentage of killed macrophages in a standard assay using trypan blue by roughly 20 to 70% in comparison with its cured equivalent strain (Luhrmann *et al*. 2004).

*R. equi* infrequently infects other animals such as pigs, goats and cattle but is increasingly responsible for AIDS-associated pneumonia in HIV-infected individuals (Meijer & Prescott 2004; Wall *et al*. 2005). Infections in bovine and pig isolates have been associated with the presence of VapB-encoding plasmids (Ocampo-Sosa *et al*. 2007). Infections are also observed in patients with organ transplants, renal failure, alcoholism, lung cancer, leukaemia and other conditions related to immunodeficiency (Kedlaya *et al*. 2001). Human infections with *R. equi* are uncommon, affecting approximately 10-15% of immunocompetent patients (Gabriels *et al*. 2006). The best therapeutic approach is a two-step strategy; in the first step a

combination of bactericidal drugs such as vancomycin plus imipenem is used to kill extracellular organisms and, in the second step, a combination of drugs that penetrate the cell, such as erythromycin plus rifampicin, are administered for a period of at least two months (Gabriels *et al*. 2006). The pathogen in plants is known as *R. fascians* (von Bargen & Haas 2009). The infection causes growth abnormalities such as loss of apical dominance and the growth of lateral shoots, evidence suggests that the abnormalities are caused by the production of cytokines by *R. fascians* (Bell *et al*. 1998). Plasmids in *Rhodococcus* species other than *R. equi* have been linked to resistance to heavy metals and to chloramphenicol, disease processes in plants and the metabolism of extraordinary compounds (DeLaPenaMoctezuma *et al*. 1996). *R. fascians* infections in crops on farms can lead to large economic losses.

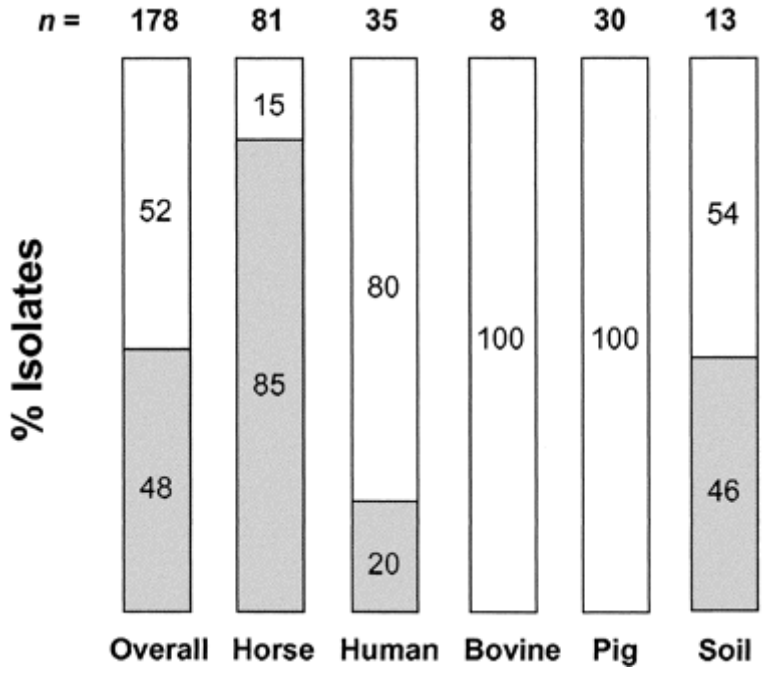


Figure 1.2**:** Distribution of the *vapA* allele according to isolate origin. The number of isolates within each category is indicated above the bars; within the bars are the percentages of *vapA*$^{+}$ (shaded region) and *vapA*$^{-}$ (blank region). (Rodriguez-Lazaro *et al*. 2006)

### 1.1.3 Spread of *R. equi* Infection in foals

Foals with clinical disease have been found to have considerably high concentrations of *R. equi* in their faeces, thus faeces from foals is thought to be the main source of soil contamination (Takai 1991a; Takai *et al.* 1994; Takai 1995; Muscatello *et al.* 2006a). *R. equi* multiplies exceptionally well during temperate spring and summer conditions in soil that in contaminated with the manure (Muscatello *et al*. 2006a). It has been observed that as the concentration of virulent organisms in the environmental *R. equi* population increase there is an increased risk of inhalation of *R. equi* from the soil (Takai 1997). Minimising the time foals spend in laneways and

holding pens where areas are covered with dry and acidic soils can help reduce their exposure to *R. equi* (Muscatello *et al*. 2006a). Environmental conditions favourable for *R. equi* multiplication in the soil and aerosolization of *R. equi* from the soil are considered high risk for foals (Muscatello 2009). Due to the problematic recovery of *R. equi* from tracheal lavage fluid samples with clinical *R. equi* pneumonia, the intracellular habitat of *R. equi* in the alveolar macrophages of infected foals and the production of abscesses in the lungs of infected foals, it is suggested that direct foal to foal transmission is unlikely (Martens *et al.* 1982; Barton & Embury 1987). Although the sensitivity of air sampling techniques is similar to tracheal lavage fluid samples, the specificity of the test is poor since most of the healthy foals' also exhale virulent *R. equi* thus proving that attempting to diagnose *R. equi* pneumonia is difficult, as healthy foals could also be a source of infection (Muscatello *et al.* 2006a)

### 1.1.4 Immunity against *R. equi* infections in Foals

There is evidence to supporting the idea that susceptibility to infection is determined by differences in immune functions in foals (Nerren *et al*. 2009b). A study by (Chaffin *et al*. 2003) has confirmed that the ratio of CD4+/CD8+ T cells in blood samples from 2-week-old foals was considerably lower in foals that later developed *R. equi* pneumonia than in foals that did not develop the disease. Protection against *R. equi* infection is provided by CD4+ and CD8+ lymphocytes, therefore clearance of virulent *R. equi* in mature horses is dependent on interferon gamma production by CD4+ and CD8+ lymphocytes (Nerren *et al*. 2009a) *R. equi* infection in adult horses is also known to be cleared by *R. equi*-specific cytotoxic T lymphocyte responses (Nerren *et al*. 2009b). Pulmonary clearance of *R. equi* is shown to be enhanced by

antibodies of the Vap proteins but these do not provide complete protection (Jacks *et al*. 2007). Although the mechanism is unclear, there is evidence that neutrophils play an important role in resistance to infection with *R. equi* and may also have a protective effect by forming a link between adaptive and immune responses (Nerren *et al*. 2009a). Neutrophils produce a number of cytokines and chemokines that activate other effector cells of the immune system thereby initiating an immune response (Nerren *et al*. 2009a). Analysis of the expression of 8 cytokines (IFNy, TFNα, IL-6, IL-8, IL-12p40, ILP-12p35 and IL-23p19) in response to *R. equi* infection from newborn foals and subsequently at 2-, 4- and 8 weeks of age revealed that on exposure to virulent *R. equi*, expression of these cytokines would increase with age (Nerren *et al*. 2009b). The *R. equi* specificity of the above responses was unable to be examined since other bacterial species were not included in the experiment (Nerren *et al*. 2009b). Furthermore, a generalized IFN-γ deficiency or inapt polarization of the immune response towards the Th2 phenotype cannot alone explain susceptibility to *R. equi* infection (Jacks *et al*. 2007). However, understanding the mechanism of innate and adaptive immunity and the reasons why some foals are more susceptible to *R. equi* infection than others will provide helpful information for vaccine development.

### 1.1.5 Treatment of *R. equi* infection

*R. equi* is usually resistant to beta-lactam antibiotics such as penicillin G, oxacillin, ampicillin, carbeicillin and cefazolin (Kedlaya *et al*. 2001). Current treatment involves the usage of a combination of erythromycin estolate and rifampin

(Jacks 2003). Before the advent of the above combined antimicrobial therapy, fatality rates were as high as 80% but studies conducted around 2006 showed that these rates could be reduced to between 12.5 and 42% after administration of the antibiotic cocktail (Heidmann *et al*. 2006). This combination allows the drugs to penetrate the lung abscesses, macrophages or neutrophils where the bacteria multiply (Prescott 1991). Normally treatment of 30 days is prescribed but prolonged treatment of 6-8 weeks can often be required. Radiographs and fibrinogen levels are used to determine when to stop treatment (Prescott 1991). However, the side-effects of antibiotic usage include hyperthermia, fatal diarrhoea and are normally self-limiting. Erythromycin has variable absorption and is associated with effects such as colitis and tachypnea in hot weather (Heidmann *et al*. 2006). In the last decade the minimal inhibitory concentrations of erythromycin and rifampin for *R. equi* isolates have been rising and there have been reports of strains becoming resistant to these antibiotics (von Bargen & Haas 2009). In one study, prophylaxis with azithromycin tested on foals for a period of 28 days proved to be insufficient for reducing the incidence of pulmonary abscesses (Venner 2007). This highlights the need for non-antibiotic strategies to combat *R.equi* infection. Other methods for treatment rely on reducing faecal contamination and dust on studs but the effects of such methods have been variable and little is known about how much these factors account for higher rates of mortality and infection on some studs (Muscatello 2006). Another monitoring tool in farms with endemic *R. equi* infection is the periodic analysis of white blood cell concentrations (von Bargen & Haas 2009), the cultivation of bacteria from tracheal washes and identifying *R. equi* by PCR (as carried out in this study) is also

recommended (Heidmann *et al*. 2006). Another study also implies that laneways and holding pens are major infection areas, if the time spent by the foals in these environments is reduced and if these areas were regularly irrigated then there may be a decline in airborne concentration of virulent *R. equi* (Muscatello *et al*. 2006a).

The only useful precautionary medical strategy against *R.equi* is the intravenous administration of hyperimmune plasma (HIP) (Blood-Horse 2009). This contains high levels of antibodies against the bacteria. HIP can provide passive immunity to treated foals and also reduces the incidence of pneumonia (Blood-Horse 2009). Nonetheless, it is expensive, labour intensive to administer and not universally effective (Blood-Horse 2009). Despite these limitations, HIP is commercially available and still being used on many farms (von Bargen & Haas 2009). There is currently no effective vaccine or a rapid, inexpensive method of early diagnosis and prevention of *R. equi* infection in foals (Rodriguez-Lazaro *et al*. 2006). Many attempts for vaccine developments have been made over the years but none have proven to be very successful. The development of a GroEL/VapA epitope chimeric (combination) protein/DNA vaccine was possible however there is still a need for a non-equine model of *R. equi* infection (Heuzenroeder *et al*. 2005).

### 1.1.6 Research on *R. equi* Virulence Determinants

*R. equi* grown on acetate- or lactate-containing minimal media revealed its high activities of isocitrate lyase (Kelly *et al*. 2002). Isocitrate lyase is the first enzyme of the glyoxylate bypass, which allows assimilation of acetate via the

glyoxylate bypass (Wall *et al*. 2005). Isocitrate lyase is encoded by the *aceA* gene, thus by constructing an aceA mutant of strain *R. equi* 103 (*R. equi* lacking isocitrate lyase activity) (Wall *et al*. 2005) were able to understand the role of isocitrate lyase in virulence of *R. equi*. On the surface of *R. equi*, a Haemagglutinin has been described as a potential virulence marker of this species (Soedarmanto *et al*. 1998). A relationship between haemaglutinin's adherence of this bacteria to HeLa cells (an immortal carcinoma cell line) or to murine macrophages has been found (Soedarmanto *et al*. 1998).

Bacterial capsules have been shown to adhere to biotic and abiotic surfaces, are resistant to complement-mediated killing, suppress adapted immune responses and are resistant to desiccation (Sydor *et al*. 2008). In a study, the authors created a transposome mutation in the gene for mycolyl transferase FbpA which resulted in the apparent loss of capsule structure in *R. equi* (Sydor *et al. 2008).* These mutants with disturbed capsule structures were shown to release factors for virulence more successfully and therefore had stronger virulence characteristics (Sydor *et al*. 2008). Since the mutation inflicted by the transposome affected capsule adhesion, mycolic acid compound synthesis and possibly other phenotypes it was difficult to understand the role of each of these effects (Sydor *et al*. 2008). However the role of the capsule in virulence remains negligible due to the fact that the overall role of virulence in mice was unaltered (Sydor *et al*. 2008). Although the capsule showed little relevance to virulence it might be crucial for *R. equi* survival in dry soil (von Bargen & Haas 2009). HeLa cell association with the presence of virulence plasmids in *R. equi* was examined in eight *R. equi* isolates but no relationship between them was found (De la

Pena-Moctezuma & Prescott 1995). A determinant that influenced the association was the colony type, the dry colonies showed a higher HeLa cell-presence of plasmid association possibly because they are more hydrophobic (play a role in permitting adhesion) than the mucoid colonies; however there was no relation between hydrophobicity and possession of the VapA plasmid (De la Pena-Moctezuma & Prescott 1995). The HeLa cell was not a useful model in assessment of virulence plasmids thus suggesting that macrophages may be a better cell model for *in vitro* assessment of virulence. Pei *et al*. (2006) examined the application of a system they developed to target gene mutations and to assess their importance in resistance to immune defences. Mutations in a gene *htr*A (a stress-induced serine protease) required for maturation and folding of secreted proteins which is known to be involved in virulence of many gram negative bacteria has shown to be required for *R. equi* virulence (Pei *et al*. 2006). The gene *nar*G (encodes nitrate reductase G) in the absence of oxygen is known to play a role in respiration, the indication of its requirement for virulence in *R. equi* was observed when the narG mutant of *R. equi* in mice was fully attenuated (Pei *et al*. 2006). The limitation of this study is that using healthy adult mice to assess virulence of mutant strains will fail to show attenuation that might only be observed in activated macrophages (Pei *et al*. 2006). It seemed that in pathogenic bacteria there are examples of virulence factors that act cooperatively to cause membrane damage (Navas *et al*. 2001).

## 1.2 Species-Specific Molecular Markers of *R. equi*

### 1.2.1 16S Ribosomal RNA

16S rRNA sequencing has been generally accepted as a means for species differentiation. The gene sequence for 16S rRNA is largely phylogenetically conserved therefore variable regions in its sequence can be characteristic of particular organisms (Bell *et al*. 1996). Comparative analysis of 16S rRNA sequences can be carried out with universal PCR primers due to the presence of highly conserved primer binding sites and hypervariable regions that provide species specific signatures (Coenye & Vandamme 2003). The primers allow amplification of a 441-bp segment of DNA from all *R. equi* strain (Sellon *et al*. 2001) and have been used in this study and others to confirm that the visually identified isolates belong to *R. equi*.

### 1.2.2 Colony Hybridisation using an *rrnA* probe

In a study by Muscatello and Browning (2004), a *rrnA* probe was used to hybridize to and identify *R. equi* colonies; however, of the 14 colonies that hybridized to the *rrnA* probe, six of them had colony morphology atypical of *R. equi*. On increasing the hybridization temperature and the stringency of the wash buffer, the binding of the *rrnA* probe to these atypical *R. equi* isolates was eliminated, leaving genuine positive colonies (Muscatello & Browning 2004). Thus specific identification of *R. equi* colonies was possible using the *rrnA* probe.

### 1.2.3 Cholesterol oxidase gene (*choE*) gene

Studies have suggested a high degree of conservation of the *choE* gene in *R. equi* (Ladron *et al*. 2003). DNA sequencing and Restriction Fragment Length

Polymorphism (RFLP) analysis have confirmed the above suggestion i.e. RFLP analyses of the 959-bp choE amplicons from 20 previously analyzed *R. equi* isolates showed identical patterns for each of the seven restriction enzymes that were used (Ladron *et al*. 2003). Thus choE was a good target for species specific identification of the nocardioform actinomycete, *R. equi*. In another study, a molecular analysis of *R. equi* virulence was conducted by investigating cholesterol oxidase using *choE* mutants (Navas *et al*. 2001). Inactivation of *choE* annulled the rhodococcal cohemolytic activity but was restored on complementation of choE (Navas *et al*. 2001). The expression of choE in a strain of *E. coli* conferred the same membrane damaging activity as in the wild-type strain of *R. equi* (Navas *et al*. 2001). This supports the current belief that cholesterol oxidase is a major cytotoxic factor that is possibly involved in the macrophage and leukocyte destruction that characterizes infections in humans and animals (Prescott 1991; Linder & Bernheimer 1997). There is evidence that *R. equi* also produces phospholipase activity that, together with choE, forms a system that efficiently alters host membranes and causes cytotoxicity and tissue destruction (Linder & Bernheimer 1997; Navas *et al*. 2001).

## 1.3 Molecular Markers of Virulence in *R. equi*

### 1.3.1 VapA

A number of studies have shown that the possession of a *vapA* gene encoding VapA protein has been associated with virulence in *R. equi* (Takai 1991b, a; Tkachuksaad & Prescott 1991; Takai *et al.* 1993). Although many phenotypic markers such as congo

red binding, resistance to antibiotics, conventional clinical microbiological tests, utilization of different carbon sources have been tested as methods for detection of virulence, VapA seems to be a more reliable indicator of virulence (DeLaPenaMoctezuma *et al*. 1996). A plasmid–typing scheme described by (Ocampo-Sosa *et al*. 2007) suggests that the presence or absence of the plasmid genes *traA* (959-bp)[A conserved gene for plasmid transfer shown to be functional in other Rhodococcus species (Yang *et al*. 2007)] and the genes *vapA* (286-bp) and *vapB* (477-bp) determine virulence in Rhodococcus species. Strains that have the characteristic $traA^{+}$ $vapA^{+}$ $vapB^{-}$ are found in isolates from sick foals, $traA^{+}$ $vapA^{-}$ $vapB^{+}$ prevail in those from predominantly asymptomatic pigs, the $traA^{+}$ $vapAB^{-}$ is characteristic of virulence plasmids frequently found in diseased cattle and $traA^{-}$ $vapAB^{-}$ (no plasmid) is typical nonpathological isolates (Ocampo-Sosa *et al*. 2007; von Bargen & Haas 2009). The distribution of the above mentioned TRAVAP categories is shown below in Figure 1.3. Thus the presence or absence of the *vapA* gene can be used as a marker to show presence or absence of the VapA-encoding virulence plasmid. Virulence plasmid negative strains of *R. equi* do not express *vapA* and are therefore incapable of causing disease (Jain 2003).

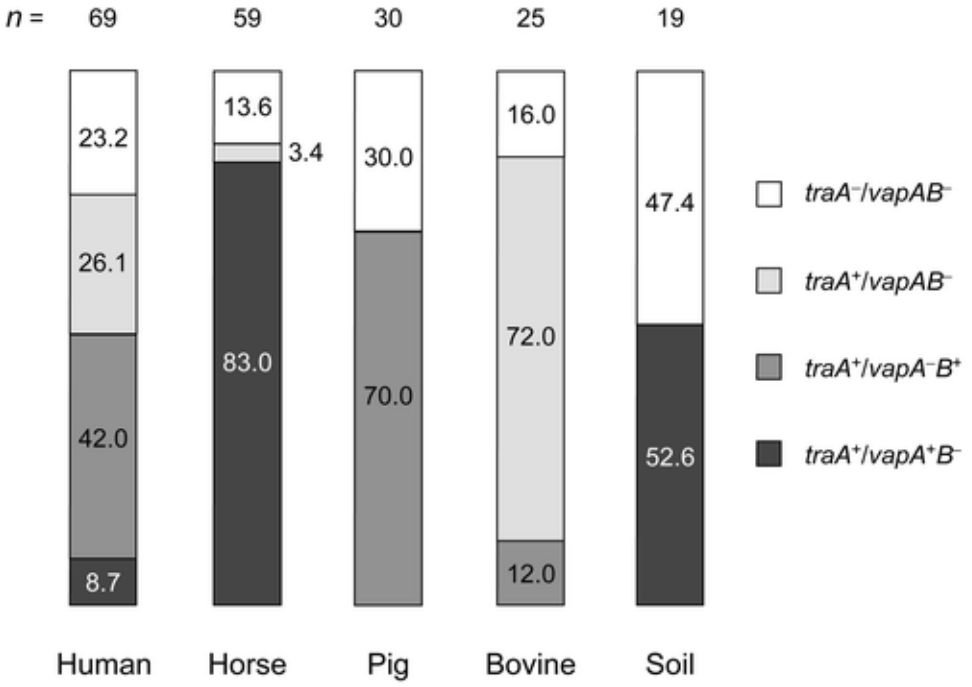


Figure 1.3**:** Distribution of the 4 *traA* - *vapA* - *vapB* (TRAVAP) categories as a function of the origin of the *R. equi* isolates. The no. of isolates within each group is indicated above the bars, and the percentages of the different plasmid categories are indicated inside the bars (Ocampo-Sosa *et al*. 2007).

When the *vapA* gene was expressed in a plasmid-cured isogenic strain of *R. equi* it was not sufficient enough to restore the capacity to proliferate in macrophages and to colonize the lungs of experimentally infected foals (Navas *et al*. 2001). Thus the role of vapA in virulence has been questioned. Since the involvement of vapA and the presence of the virulence plasmid alone do not appear to be essential in pathogenesis in non-horse hosts, such as clinical isolates from humans and other animal species, it has been suggested that chromosomally determined factors are involved in *R. equi* pathogenicity (Navas *et al*. 2001). Candidates for such chromosomal virulence factors include; the capsular polysaccharide (which might

interfere with phagocytosis), mycolic acid-containing glycolipids (which might be involved in granuloma formation), cholesterol oxidase (an enzyme that might be involved in cytoxicity and macrophage destruction) (Navas *et al*. 2001). However, there is no direct proof of any of the above factors involvement in pathogenesis (Navas *et al*. 2001). VapA is a greatly immunodominant protein and is abundantly expressed on the bacterial surface (Takai *et al*. 2001). It is thought that VapA is linked to the cell wall through an abnormal lipid modification that occurs at the N-terminal end so that at least the conserved C terminal region of VapA is presented on the surface of the organism (Tan *et al*. 1995; Takai *et al*. 2001).

### 1.3.2 VapB

VapB is identified in plasmids from 'nonequine' *R. equi* isolates (Lazaro *et al*., 2006). A 20 kDa virulence protein seems to be related to 79 to 95 kb plasmids that are generally found among *R. equi* isolates from pigs and HIV infected persons (Soedarmanto *et al*. 1998). These 20 kDa proteins are suggested to represent intermediately virulent *R. equi* (Soedarmanto *et al*. 1998) and in order for them to impose lethality in mice there needs to be bacterial numbers 10 times higher than for VapA-expressing strains (Takai 1995; Takai *et al*. 2000). VapA and VapB sequences are strongly related to each other and have a sequence identity of 83.6% (von Bargen & Haas 2009), clinical isolates can express VapA or VapB but not both (Monego *et al*. 2009). The VapB-encoding plasmid codes for 4 proteins along with VapB which are different to those found in VapA they are: VapJ, VapL, VapM and two copies of VapK (Letek *et al*. 2008).

## 1.4 VapA Genetics

### 1.4.1 VapA Regulation and Expression

VapA expression is thermoregulated and pH regulated, occurring at temperatures greater than 32ºC and at a pH of 5.0 (Takai 1991b, 1999; Benoit *et al.* 2001, 2002). Studies have shown that increased temperatures have the strongest impact on induction of VapA expression (Ren 2004). Broth composition has also shown to be involved in regulating the expression of VapA: when additional amounts of yeast extract is added to brain heart infusion broth (BHI) VapA is synthesised even at 30ºC (Byrne *et al*. 2007). Oxidative stress and the concentration of calcium and magnesium also control expression of VapA gene and other genes in the pathogenicity island (Wall *et al*. 2005)

Two genes encoding transcriptional regulators are located in a five-cistron operon (the virR operon) and are found to play an important role in controlling the expression of pathogenicity island genes (Byrne *et al*. 2007). The *virR* operon contains ORF4 and ORF8 genes that are found on virulence plasmids and are involved in regulation of VapA (Russell *et al*. 2004). ORF4 codes for a LysR-type transcription regulator (VirR), and ORF8 is homologous to response regulators of two-component regulatory systems (VirS), both are identified in the pathogenicity island (Takai *et al*. 2000; Russell *et al*. 2004). When *virR* or *virS* were knocked out an upregulation of mRNA levels of all *vap* genes, including *vapA*, was observed (Ren 2004). This led to the assumption that regulation of genes encoded in the pathogenicity island is mainly based on a negative transcription regulatory network (Ren 2004). Even while *vapA* mRNA increased when *virS* was deleted, VapA protein

was not expressed and the explanation for this remained to be investigated (Russell *et al*. 2004). Although *virR* is transcribed at a constitutive low level its transcription is driven by the $P_{virR}$ promoter which has a start site 53 bp upstream of the virR initiation codon (Byrne *et al*. 2007) . Deletion analysis of the *virR* operon revealed that transcription of the 4 genes downstream of virR is driven by a second promoter ($P_{orf5}$) which is also located within the virR gene (Byrne *et al*. 2007). Investigation of virulence plasmid-free *R. equi* strains showed that the activity of $P_{orf5}$ was significantly reduced than that in the presence of the virulence plasmid, suggesting that for full $P_{orf5}$ activity one or more components of the virulence plasmid was required including the *virR* gene (Byrne *et al*. 2007). Although the role of Orf5 is unclear, inducing growth conditions to upregulate $P_{orf5}$ activity will result in the increased expression of the response regulator Orf8 which would in turn lead to full induction of the virulence genes (Byrne *et al*. 2007).

### 1.4.2 VapA-encoding Plasmids

Plasmids encoding VapA that are around 85-90 kb have been analysed intensely due to their central role in virulence in foals (Jain 2003). These plasmids belong to the CURV (conjugation-unknown function-replication/partition-variable region) family of plasmids, the variable region contains the 'pathogenicity islands' and hence is a region of interest (von Bargen & Haas 2009). A distinct feature of the CURV plasmid family is the presence of a highly conserved syntenic gene cluster which includes conserved conjugal transfer genes and spans part of the conjugation and unknown-function modules (Letek *et al*. 2008). A family of four other *vap* genes (*vap*C, *vap*D, *vap*E and *vap*F) that comprise the pathogenicity island are also known

to be involved in virulence with the exception of two other individual genes *vap*G and *vap*H (Jain 2003). Proteins encoded by *vap* genes exhibit significant sequence conservation and no proteins homologous to the Vap are found in other organisms (Jain 2003). The plasmid is essential for multiplication in macrophages and by enhancing cytotoxicity is able to allow prolonged inhibition of phagosome maturation (Hondalus 1994; Giguere *et al.* 1999; Luhrmann et al. 2004). Twelve different (85-kb types I to IV, 87-kb types I to III, and 90-kb types I to V) but remarkably conserved VapA-encoding plasmids have been described based on their endonuclease restriction patterns and some seem to be accumulated geographically (Takai *et al.* 2001; Takai *et al.* 2003; Ribeiro 2005; Venner *et al.* 2007). A second type of plasmid, the VapB-expressing plasmid contains the VapB gene which is structurally homologous to the VapA gene, this VapB plasmid has been isolated from pigs and AIDS (Acquired Immune Deficiency Syndrome) infected patients (Luhrmann *et al*. 2004). Along with some evolutionary reasons for the presence of different forms of the virulence-associated proteins which is documented further below, the involvement of lipid modifications has been suggested (von Bargen & Haas 2009).

Plasmids can be divided into four major portions: one is involved in replication, one in conjugation, a third section serves unknown functions and the fourth is a likely pathogenicity island.

Replication region:

ORF41 to ORF61 of the plasmid appears to be involved in replication and partition (Takai *et al*. 2000). This region was found to contain the putative *ori* (origin of

replication) during studies conducted on the development of an *R. equi-E. coli* plasmid shuttle vector (Singer & Finnerty 1988). The origin of replication of *R. equi* virulence plasmid has no homology with genes involved in replication; the only exception is a gene (ORF50) which is most homologous with the *trbA* gene (Singer & Finnerty 1988). The *trbA* gene produces a protein that represses both plasmid replication and conjugative transfer (Takai *et al*. 2000). ORFs (Open Reading Frames) that have homology to *parA* and *parB* are found in this replication and partition region, while *parB* is unexpectedly present inside the pathogenicity island (Takai *et al*. 2000). Protein domain analysis identified ORF56 as a helix-turn-helix regulatory protein (Takai *et al*. 2000). It seems likely that the other unknown ORFs in this large area are also concerned with replication and partition functions, but further work is required to confirm the supposition (Takai *et al*. 2000).

Conjugation region:

This region appears to be concerned with conjugation functions, complex functions that involve many genes (Takai *et al*., 2000). It was observed from sequence analysis of plasmid p33701 and p103 that the conjugation region includes ORF23 to 40, thus including ORF39 which has homology to a *Streptomyces* integral membrane protein which is probably involved in conjugation and ORF40 which has homology to a gene in the transfer gene complex of plasmid RP4 (Takai *et al.*, 2000). Protein domain analysis of ORF26, which is the largest ORF of the plasmid, identified a domain involved in transcriptional regulation, DNA repair or chromatin unwinding and a domain characteristic of the C-terminal domain of helicases which are compatible with conjugation activities (Takai *et al.,* 2000). ORF30 was found to be homologous

to a gene called *traA* that encodes conjugal transfer proteins (Takai *et al.,* 2000). ORF32 is homologous to a gene *topA* that encodes a putative DNA type 1 topoisomerase that controls the topology of DNA and is known to have a role in DNA transfer during conjugation (Takai *et al.,* 2000). ORF36 was identified to be required for its lysozyme-like activity in hydrolysing the polyglycine interpeptide bridge of peptidoglycan to assist the conjugation process (Takai *et al.,* 2000).

Pathogenicity Island:

A 27,536-bp region between ORF61 and ORF22 has the characteristics of a pathogenicity island since it is bounded by insertion elements, contains a block of foreign genes (60.8% GC content), contains a tRNA gene (a lysyl-tRNA synthetase [*lsr2*] gene), contains *vapA* and six other related virulence-associated protein genes that are also known as the *vap* gene family (VapC, VapD, VapE, VapF, VapG, VapH) (Takai *et al*. 2000). Following phagocytosis of *R. equi* by macrophages the genes in the variable region (VR) of the virulence plasmid are highly expressed (Ren and Prescott, 2003). Studies that showed deletion of *vapA* gene, which lies within the variable region, renders the resulting strain avirulent (with no virulence plasmid), it is therefore believed that the VR is a pathogenicity island that contains genes essential for virulence (Jain *et al*., 2003).

### 1.4.3 Virulence Plasmids Isolation Difficulties

1) Plasmids may exist in multiple copies and are lost after repeated passages of bacteria in broth culture (von Bargen & Haas 2009). Therefore even when

strains are found to be plasmid positive initially and are stored at -80ºC, they might lose plasmid when thawed and grown on agar or in broth.

2) Isolating the 80-90kb plasmids from *R. equi* has been difficult due to their low copy numbers owing to their large size, thus repeated attempts have been necessary (Tkachuksaad & Prescott 1991). Because of a low copy number, it is possible that some strains classified as plasmid negative by identification techniques might actually contain plasmids.
3) *Rhodococcus* bacteria are surrounded by thick and lamellar polysaccharide capsules that are antigenically variable (Prescott 1991). This makes it difficult to lyse the cells during plasmid isolations which in turn results in fewer or none isolated plasmids.

### 1.4.4 Vap Evolution

As mentioned earlier the VapB and the VapA-encoded plasmids have found to contain 6 vap family genes (*vapJ, vapK1, vapK2, vapM, vapL* and *vapB*) and (*vapC, vapD, vapE, vapG, vapH, vapF* and *vapA*) respectively (Letek *et al*. 2008; Monego *et al*. 2009). Figure 1.4 shows a phylogenetic tree for the mature Vap proteins using the amino acid p-distance model (Letek *et al*. 2008) thereby pointing out the evolutionary relationships between *vap* genes in VapA and those in VapB plasmids. Comparing the genetic structures and linkage results of the pathogenicity island from pVAPB1593 and pVAPA1037 (previously published plasmid sequences obtained from GenBank) the authors were able to reconstruct a model for evolutionary dynamics of the vap multigene family (Figure 1.5) which showed that VapA and VapB seem to be divergent allelic variants of the same ancestral gene (Letek *et al*.

2008). It has been suggested that the *vap* Pathogenicity island which was acquired via lateral gene transfer by an ancestral plasmid, later evolved by *vap* gene duplication and sequence diversification and adapted to specific hosts after plasmid acquisition (Letek *et al*. 2008). In order to analyse host specificity, VapB deletion strains can be changed to VapA and similarly virulence in mice can be altered from intermediate to high by interchanging VapB with VapA (von Bargen & Haas 2009). Evolutionary comparisons of VapA and VapB will help gain understanding regarding Vap protein functions, offer insights into *R. equi* virulence mechanisms and in turn aid vaccine development.

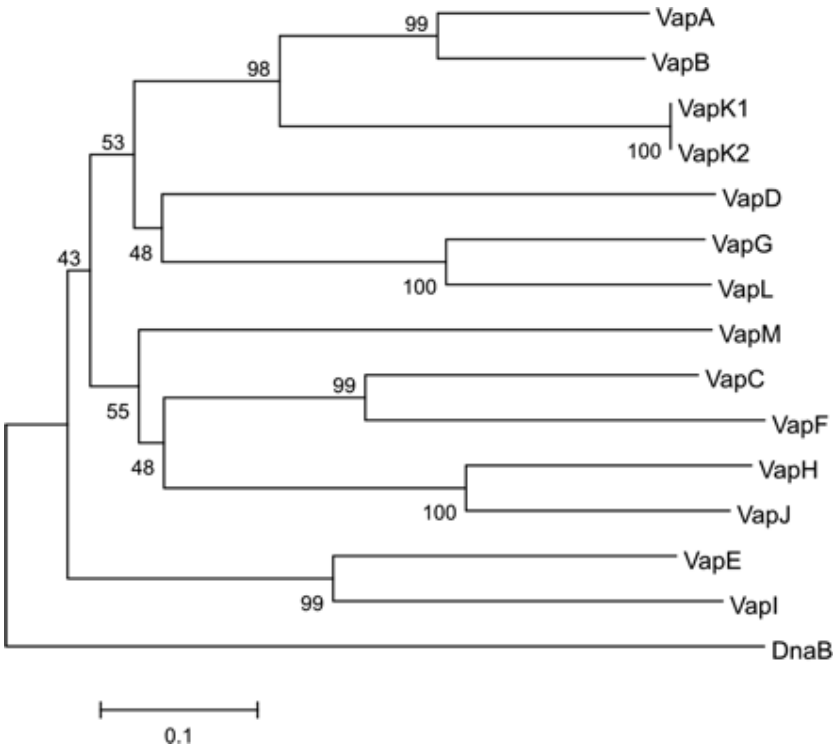


Figure 1.4: Phylogenetic tree of *vap* multigene family. It was constructed from a ClustalW alignment of the mature Vap proteins encoded by pVAPB1593 and

pVAPA1037. For the analyses, a full-length VapF protein was reconstructed by correcting the two frameshift mutations in the 3' region of the gene. Bootstrap values (10,000 replicates) are shown at the nodes and the bar indicates genetic distance (Letek *et al*. 2008).

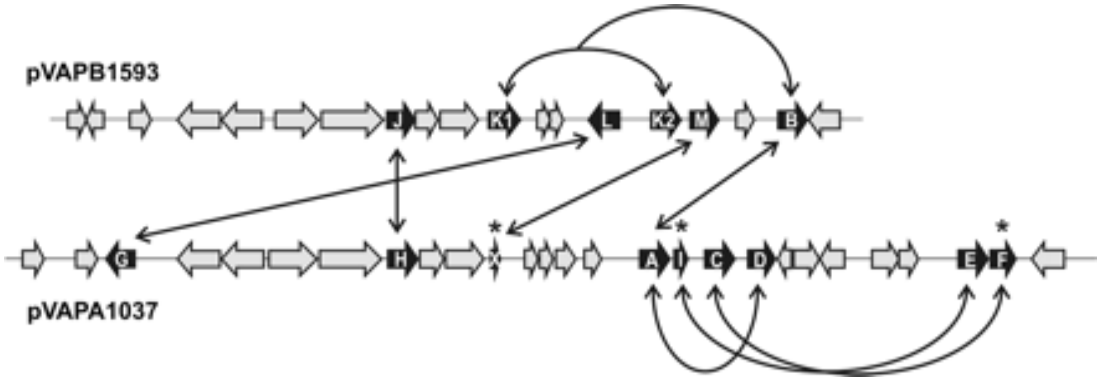


Figure 1.5: Map of the evolutionary dynamics of the *vap* multigene family. The straight arrows connect the *vap* genes that are presumably derived from vertical evolution of a common ancestor. The genes connected by cured arrows were probably originated from gene duplication. The asterisks denote degenerate *vap* genes i.e. one gene of each of the duplicated *vap* IE and *vap* CF is undergoing decay (Letek *et al*. 2008).

### 1.4.5 Heterogeneity in *R. equi* strains

In a study by Soedarmanto *et al*. (1998) heterogeneity among bacteria was found when they were subjected to DNA-fingerprinting. It was performed by macrorestriction analysis of chromosomal DNA of bacteria by Pulsed-field gel electrophoresis (PFGE) (Soedarmanto *et al*. 1998). DNA-fragments revealed DNA-

restriction patterns that were different for most isolates investigated, indicating that different farms seemed to be contaminated with various *R. equi* clones (Soedarmanto *et al*. 1998). In only one of six farms studied did all *R. equi* isolates have identical DNA-fingerprints; however most isolates did not contain plasmids (Soedarmanto *et al*. 1998). It would be worthwhile using the same techniques for strains that have virulent plasmids. In another study, genotypic variation among virulence plasmids was investigated in 543 foal isolates from five different countries (Takai 1999). Restriction Fragment Length Polymorphisms (RFLP) revealed that isolates from Canada and Argentina contained either an 85-kb type I or an 87-kb type I plasmid; isolates from Australia contained mostly an 85-kb type I plasmid; isolates from France contained either an 85-kb type I, an 87-kb type I or an 85kb-type II plasmid; Japanese isolates did not contain any of the three types instead they revealed 87-kb type II or a 90-kb plasmid, thus there seemed to be differences in geographical distribution of the 5 virulence plasmids obtained (Takai 1999). A global surveillance of virulent *R. equi* might reveal more about the evolution of horses and possibly the transmission of virulent *R. equi* using plasmid profiles.

### 1.4.6 Virulent *R. equi* Epidemiology

**Brazil:** The prevalence of virulent *Rhodococcus* in clinical isolates was investigated in foals obtained from seven horse breeding farms in the south of Brazil (Ribeiro 2005). Three types of plasmids (85-kb type I, 87-kb type II and 87-kb type III) were described based on the restriction enzyme digestion patterns of the isolated plasmids in order to gain more information about the *vap* gene family and the antigens

associated with *R. equi* (Ribeiro 2005). This has importance in exemplifying the epidemiological and pathogenic nature of the disease in equines.

**Japan:** Virulent *R. equi* in Japan were found to contain five distinct plasmid types based on EcoRI restriction digestion fragments of plasmids (87-kb type II, 90-kb type I, 90-kb type II, 90-kb type III, 90-kb type IV) which were in agreement with the dispersal of the native horses over a period of time thus providing insight into the origin, ancestry and dispersal of *R. equi* (Takai *et al.* 2001b).

**Korea:** Restriction enzyme digestion patterns of Plasmid DNA extracted from foal isolates obtained from four Jeju farms in Korea showed the presence of two closely related plasmid types (Takai *et al*. 2003). These profiles, compared with those found in Japan, suggested a common origin and ancestry for isolates from both countries and this is supported by the fact that native horses both countries originated from the Mongolia horse (Takai *et al*. 2003).

**Germany:** Four different restriction patterns were observed in VapA positive isolates from a horse breeding farm in Germany, with the majority of the infected foals displaying three subtypes of the VapA positive *R. equi*. This characterisation was carried out in the hope of gaining more information regarding the route and source of infection.

**Texas, U.S.A:** Virulent *R. equi* isolated from three farms in Texas revealed the presence of at least 4 different virulence associated plasmids based on RFLPs: 85-kb type I, 87-kb type I, 85-kb type III and 85-kb type IV (Takai *et al*. 2001).

The majority of the VapA positive *R. equi* that infect foals seems have the 85-kb type I plasmids (Venner *et al*. 2007). The additional DNA present in the 87- and 90-kb types is thought to be the result of insertion of mobile genetic elements (Takai *et al*. 2000). The characterisation of VapA virulence plasmids from *R. equi* in New Zealand will provide an understanding regarding the ancestry and origin of *R. equi* isolates, the pathogenicity of virulent factors, the genetics of *R. equi* and eventually some information on vaccine development. Since the first horses brought to New Zealand were from Australia, it is likely that the *R. equi* plasmids are of similar origin but that remains to be investigated. Most Australian *R. equi* isolates carry 85-kb type I and 87-kb type I plasmids and majority of virulent *R. equi* that infect foals in general carry 85-kb type I plasmids (Venner *et al*. 2007) and since there also seems to be a geographical correlation for accumulation of certain plasmid types (von Bargen & Haas 2009) it can be predicted that *R.equi* isolates in New Zealand would carry plasmids similar to the Australian type. Although plasmids have successfully been isolated in a number of studies, it has been a somewhat painstaking process involving testing of a variety of modifications to existing methods and this is discussed below.

## 1.5 Methodology

The methods of plasmid isolation used generated a large amount of chromosomal DNA and, on some occasions, small amounts of plasmid DNA. Because of the low relative amounts of plasmid DNA, banding patterns produced with restriction enzymes such as EcoR1 appeared to be very faint and the lane in the gel had smears of DNA thus showing that the plasmid product was not clean. If the plasmid band was cut out from the gel, purified and sequenced there was still a high probability of

not being able to repeat the obtained result, even after sequencing. It would be futile to use restriction enzyme digests of purified plasmid samples to characterise them since all isolations would have different levels of purity and that could obscure banding patterns of digests thereby showing RFLPs in plasmids without there being a true difference between the isolated plasmids.

The primary benefit of this research is to the horse breeding industry of New Zealand. Our main aim was to find out if based on *R. equi* plasmid differences there can be multiple subtypes of *R. equi* in one farm. It not only provides a head start in successful growth of *R. equi* from foal nasal swabs but also in the recognition of virulent *R. equi* and an attempt at using a variety of methods to isolate virulence plasmids, some of which proved successful but owing to the nature of the plasmids were unreliable, even when repeated under exactly the same conditions. Successful isolations were required for the characterization of the plasmids which could help find differences in plasmids thus improve our understanding about plasmid function and its virulence. However, a more user-friendly and reliable way of plasmid characterisation was necessary. It aimed to characterise plasmids by looking at approximately 1kb highly variable regions within the plasmid. Four published plasmid sequences obtained from GenBank (accession numbers: AM947676, AP001204, AF116907 and AM947677) were aligned and a 1000bp area of high variation amongst the sequences was selected. Forward and Reverse primers were designed for a products that would be roughly 920bps. Comparison of sequences from PCR amplification of Plasmid isolations as well as those from DNA isolations showed the same sequence results. Therefore sequencing was continued with PCR

amplified product from isolated DNA not only because it is quicker and easier but it is also more reliable than plasmid isolations.

## 1.6 Conclusion

Although *R. equi* is a disease of major economic importance to the horse breeding industries around the world the exact figures of financial losses in New Zealand are unknown. However, estimates from an Australian study can give an idea regarding the costs involved in diagnosis and treatment of infection. An RIRDC project on disease surveillance estimated the cost to be roughly AU$370 per case thus 1% of foals being infected with *R. equi* can pose an annual cost of about AU$3-4 million (Muscatello *et al*. 2006a). The breeding and racing of thoroughbreds in New Zealand is as much a part of its history and culture as is the development of dairy herds that produce export wealth to the community. Thoroughbred horses raised in New Zealand are amongst the most sought after in the world as they have been known to consistently outperform their competition in every country they race. Studies have shown that foals affected with *R. equi* perform poorer as race-horses than unaffected one's thus posing economic losses to New Zealand's horse breeding industry. The suggested methodology of comparing RFLP patterns of variable regions will not only give more insight into epidemiology of *R. equi* infections here in New Zealand but also allow the industry to devise management strategies to minimise exposure to the bacteria.

# 2 Research Paper

**The identification, isolation and partial characterisation of VapA-encoding virulence plasmids in *Rhodococcus equi* found in New Zealand.**

## 2.1 Abstract

*Rhodococcus equi* (*R. equi*) is a facultative intracellular bacterium that survives and multiplies within macrophages by establishing a niche inside host cells and causing severe pyogranulomatous pneumonia in foals. In foals, virulence is associated with the presence of 85-90kb plasmids carrying the *vapA* gene that encodes VapA protein. VapA primers have been used as genetic markers to identify these virulence plasmids. To date, twelve different types of VapA-encoding virulence plasmids have been described. Epidemiological studies of America, Australia, Korea, Japan and some other countries in Europe have revealed that more than one subtype can exist within a particular country or even within a particular farm. However the genetic diversity amongst VapA-encoding virulence plasmids in *R. equi* found in foals in New Zealand is not known. This study investigates a highly variable region found in virulence plasmids in *R. equi* from a farm in New Zealand using restriction digestion and sequence analysis techniques. Results from the analyses of 21 VapA-encoding virulence plasmids suggest the existence of two significantly distinct sequences PI and PII. Owing to geographical accumulation of plasmid types and the fact that PI sequences were found to be almost identical to previously published 85-kb type I plasmid sequences from GenBank there seems to be evidence for the presence of both the 85-kb type I plasmids and another type in populations of VapA-encoding virulent *R. equi* in foals in New Zealand.

## 2.2 Introduction

*Rhodococcus equi (R. equi),* previously called *Corynebacterium equi* was first described by Magnusson in 1923 based on an isolate from the lung of an infected foal (Takai 1997). *R. equi* cause infections in foals less than 4 months old due to their compromised immunity (Tkachuksaad & Prescott 1991). It has been ranked as among the four most important disease problems in the horse industry (Muscatello 2009) causing fever, respiratory distress, pulmonary lesions and chronic pus-filled lung abscesses, which are characteristic of severe pyogranulomatous pneumonia (Lavoie *et al*. 1994). *R. equi* are characterised by the presence of 85-90-kb virulence-associated plasmids that encode 15- to 17-kDa antigens (VapA) (Venner *et al*. 2007). Twelve different VapA-encoding plasmid types (85-kb types I to IV, 87-kb types I to III, and 90-kb types I to V) have been described based on Restriction Fragment Length Polymorphisms (RFLPs) from foals around the world (Venner *et al*. 2007; von Bargen & Haas 2009). Pyogranulomatous pneumonia caused by *R. equi* infections is estimated to affect up to 17% of foals worldwide (Elissalde *et al*. 1980). In Australia 1-10% of foals are affected and has mortality rates usually less than 1% due to antibiotic treatment; yet, in some farms mortality rates as high as 20% or more can be found (Muscatello *et al*. 2006b). *R. equi* infrequently infects other animals such as pigs, goats and cattle, and these infections are caused by *R. equi* isolates with the presence of VapB-encoding plasmids (Ocampo-Sosa *et al*. 2007). *R. equi* is increasingly responsible for AIDS-associated pneumonia in HIV-infected individuals, affecting approximately 10-15% of immunocompetent patients (Gabriels *et al*. 2006). Foals with clinical disease are found to have high concentrations of *R. equi* in their

faeces thus faeces from foals is thought to be the main source of soil contamination (Muscatello *et al*. 2006a). It was observed that when the population of virulent *R. equi* increases there is also an increase in inhalation of *R. equi* from the soil (Takai 1997). *R. equi* multiples exceptionally well during the temperate spring and summer months therefore minimising the time foals spend in laneways and holding pens - where areas are covered with dry and acidic soils - can help reduce exposure of foals to *R. equi* (Muscatello *et al*. 2006a). It is suggested that direct foal-to-foal transmission is unlikely. CD4+ and CD8+ lymphocytes provide protection against *R. equi* infection thus clearance of infection in adult horses is due to production of these lymphocytes (Nerren *et al*. 2009a). Neutrophils play an important role in resistance to infection with *R. equi* and may also have a protective effect by forming a link between adaptive and immune responses (Nerren *et al*. 2009a). However, understanding the mechanism of innate and adaptive immunity and the reasons why some foals are more susceptible to *R. equi* infection than others will provide helpful information for vaccine development. *R. equi* is usually resistant to Beta-lactam antibiotics and current treatment involves the usage of a combination of erythromycin estolate and rifampin (Kedlaya *et al*. 2001; Jacks 2003). The intravenous administration of hyperimmune plasma (HIP) can provide passive immunity to treated foals and also reduce the incidence of pneumonia. Nonetheless, it is expensive and labour intensive to administer and not universally effective.

*R. equi* have a characteristic appearance after 48 h in culture and develop irregularly round, smooth, mucoid, glistening, semitransparent, salmon pink to yellow coloured and coalescing colonies (Prescott 1991). Along with the phenotypic

identification of *R. equi* 16S rRNA sequencing has been generally accepted as a means for species differentiation. The gene sequence for 16S rRNA is largely phylogenetically conserved therefore variable regions in its sequence can be characteristic of particular organisms (Bell *et al*. 1996). They amplify a 441-bp segment of DNA from all *R. equi* strains (Sellon *et al*. 2001) and have been used in this study and others to confirm that the visually identified isolates belong to *R. equi*. A plasmid –typing scheme described by (Ocampo-Sosa *et al*. 2007) suggests that the presence or absence of the plasmid genes *traA* (959-bp)[A conserved gene for plasmid transfer shown to be functional in other Rhodococcus species(Yang *et al*. 2007)] and the genes *vapA* (286-bp) and *vapB* (477-bp) determine virulence in Rhodococcus species (Ocampo-Sosa *et al*. 2007). Strains that have the characteristic $traA^{+}$ $vapA^{+}$ $vapB^{-}$ are found in isolates from sick foals (Ocampo-Sosa *et al*. 2007; von Bargen & Haas 2009). The expression of VapA is thermoregulated and pH regulated, occurring at temperatures over 32 ºC and pH of 5.0 (Takai 1991b, 1999; Benoit *et al.* 2001, 2002). Oxidative stress and the concentration of calcium and magnesium are also known to control expression of VapA gene and other genes in the pathogenicity island (Wall *et al*. 2005). VapA-encoding plasmids are 85-90 kb in size and have been studied intensely due to their central role in virulence (Jain 2003). These plasmids belong to the CURV (conjugation-unknown function-replication/partition-variable region) family of plasmids, the variable region contains the ‘pathogenicity islands’ and hence is a region of interest (von Bargen & Haas 2009). The plasmid is known to essential for multiplication in macrophages and by enhancing cytotoxicity is able to allow prolonged inhibition of phagosome maturation

(Hondalus 1994; Giguere *et al*. 1999; Luhrmann *et al*. 2004)The vap gene family of VapA-encoding plasmids includes genes *vapC, vapD, vapE, vapG, vapH, vapF* and *vapA* (Letek *et al*. 2008; Monego *et al*. 2009). A second type of plasmid, the VapB-encoding plasmid contains the *vapB* gene which is structurally homologous to the *vapA gene,* this VapB plasmid has been isolated from pigs and AIDS infected patients (Luhrmann *et al*. 2004).Along with some evolutionary reasons for the presence of different forms of the virulence-associated proteins, an observation suggests the involvement of lipid modifications. It has been suggested that the *vap* pathogenicity island which was acquired via lateral gene transfer by an ancestral plasmid, later evolved by *vap* gene duplication and sequence diversification and adapted to specific hosts after plasmid acquisition (Letek *et al*. 2008). Evolutionary comparisons of VapA and VapB will help gain an understanding regarding Vap protein functions, offer insights into *R. equi* virulence mechanisms and in turn supply information required for vaccine development.

A macrorestriction DNA analysis of *R.equi* by Pulsed-field gel electrophoresis (PFGE) indicated that different farms seemed to be contaminated with various *R. equi* clones. In fact, of the six farms studied only one farm showed identical *R. equi* isolate DNA fingerprints; however, most isolates did not contain plasmids (Soedarmanto *et al*. 1998). In another study genotypic variation among virulence plasmids was investigated in 543 foal isolates from five different countries (Takai 1999). Restriction Fragment Length Polymorphisms (RFLP) revealed that isolates from Canada and Argentina contained either an 85-kb type I or an 87-kb type I plasmid; isolates from Australia contained mostly an 85-kb type I plasmid; isolates

from France contained either an 85-kb type I, an 87-kb type I or an 85kb-type II plasmid; Japanese isolates did not contain any of the three types instead they revealed 87-kb type II or a 90-kb plasmid, thus there seemed to be differences in geographical distribution of the 5 virulence plasmids obtained (Takai 1999). A global surveillance of virulent *R. equi* might reveal more information regarding the evolution of horses and possibly the transmission of virulent *R. equi* using plasmid profiles.

The purpose of the study was to find out if multiple subtypes of *R. equi* can be found within one farm in New Zealand. Isolating the 85-90kb plasmids from *R. equi* has been difficult due to their low copy numbers owing to their large size, existence in multiple copies which are lost after repeated passages of bacteria in broth culture and being surrounded by thick and lamellar polysaccharide capsules which are difficult to lyse (Prescott 1991; Tkachuksaad & Prescott 1991; von Bargen & Haas 2009). Based on *R. equi* plasmid RFLPs and sequence analysis of a variable region from 21 VapA-encoded plasmids two distinct types of sequences were found suggesting polymorphisms in population of *R. equi* in New Zealand.

## 2.3 Materials and Methods

Collection, growth and isolation of Bacteria

Nasal swabs of 40 foals with clinical *R. equi* infection obtained by clinical vets were used in this study. Swabs were stored at -80ºC, defrosted at room temperature when required and individual swabs were streaked on NANAT selective media and grown at 37 ºC for approximately 4-5 days. *R. equi* colonies were identified based on their colony morphology and subsequent confirmation via PCR. Three *R. equi* colonies from each plate were numbered and streaked onto BHI (Blood Heart Infusion) agar and grown at 37 ºC for 48 h (hours).

PCR and Gel Electrophoresis to identify virulence plasmid positive bacteria

Each single colony was inoculated in 100 µl of 20% PEG (Polyethylene glycol) lysis solution for quick crude DNA extraction and heated at 95 ºC for 10 min. 2 µl of each DNA sample (400-600 ng/µl) was added to a 20 µl reaction of PCR (Polymerase Chain Reaction) in order to amplify DNA. PCR was carried out on a Peltier Thermal Cycler DNA engine® (BIO-RAD) or on a Peltier Thermal Cycler PTC-200 (BIO-RAD). A 16S primer that corresponded to its gene was used as genotypic confirmation for the phenotypic selection of *R. equi* isolates. VapA (specific marker for the virulence plasmid *vapA* gene) and TraA (specific marker for all *R. equi* plasmids) primers were used for identifying the presence of virulence plasmids in

samples. Primers used in this study were purchased from Invitrogen and designed using Primer3 Input-version 0.4.0 (Whitehead Institute for Biomedical Research; http://frodo.wi.mit.edu/primer3/). Platinum Taq DNA Polymerase (Invitrogen, New Zealand) or i-star Taq (INtRON Biotechnology) was used for DNA cycling. Cycling conditions with VapA primer were as follows: 95 ºC for 2 min, 39 cycles of 95 ºC for 20 s, 55 ºC for 20 s, 72 ºC for 30 s and a final step at 68 ºC for 5 min. For TraA the temperature in the third step was increased to 60 ºC and the time was increased from 20 s to 1 min. 10 µl of each sample was subjected to electrophoresis in a 1% Agarose gel (containing 0.5 µg of Ethidium Bromide) at 110V using 1 X SB Buffer (Sodium Borate) and visualised under a UV transilluminator (AIRPRO Scientific Limited).

DNA Extraction

Tubes were labelled and 350 µl of Lysis Solution A (0.1M Tris, 0.5 SDS, 40mM EDTA, 100 mM NaCl) was pipetted into each tube (1.7 mL 'Eppendorf' tubes). Single colonies were scraped from the BHI plates that were incubated at 37ºC using a loop and were put into their respective tubes. Samples were placed in a thermomixer (Eppendorf) at 95ºC at 650rpm for about 10-15 min. 350 µl of Lysis Solution B (5M LiCl) was added into each tube after which they were inverted a number of times. 750 µl of Chloroform was added to each tube which was then vortexed for a few seconds before being put on a rotator for 10 min. The tubes were centrifuged at 16k for 15 min which created the division of the DNA and cell-debris pellet. The top aqueous layers of each tube containing the DNA were pipetted out into fresh tubes. Roughly

equal amounts of 100% isopropanol were added to each of the tubes and were inverted about 5 times. The tubes were left on the bench at room temperature for 20 min to allow DNA precipitation. They were centrifuged at 16k for 15 min and the isopropanol was poured out from the tubes. 1ml of 70% ethanol was added to each tube and then spun again in the centrifuge at 16k for 5 min. The ethanol was poured out; the tubes were spun as before in the centrifuge for a couple of minutes after which the excess ethanol was pipetted out. 50 µl of TE (100mM Tris-Cl, 10mM EDTA) was added to each tube and left on the bench for about 30 min. They were then put in a 37ºC shaker for 30 min, vortexed to allow mixing then the DNA concentration was measured using a NanoDrop (ND-1000 Version 3.5.2 produced by Coleman Technologies Inc for NanoDrop Technologies).

Plasmid Isolation

Of all the plasmid Isolation methods tried the following seemed to be the most successful, it was called Method E. A few µL of broth stored at -80ºC was streaked onto plates and colonies grew overnight. Loops full of bacteria were transferred to 10 mL of BHI broth which were grown overnight at 37ºC in a shaking incubator. Tubes were labelled, 90 µl of TE and 10 µl of lysozyme (70 mg/mL) was added to each tube. The contents of the tubes were mixed well by running them along PCR trays. They were put in a thermomixer at 37ºC for an hour. 600 µl of Tens Buffer (0.1M NaOH, 0.2% SDS, 1mM EDTA, 10mM Tris pH 7.5) was added to each tube, which were then put on a rotator for about 30mins. 300 µl of 3M NaOAc was added; the tubes were mixed by inverting a few times and then spun in the centrifuge at 16k for

5 min. The supernatant was put in a fresh tube without disturbing the pellet. 600 µl of phenol: chloroform (1:1) was added to each tube and then put on a rotator for 10 min. They were spun in a centrifuge at 16k for 10 min after which the top layers of the contents were put into fresh tubes. Equal amounts of 100% Isopropanol or 20% PEG (20g PEG 8000, 14.8g NaCl/100 mL $H_2O$) was added to each tube, they were inverted a few times then left on the bench for about 10 min to allow precipitation. As before, they were spun in a centrifuge at 16k for 10 min. The Isopropanol was tipped out of the tubes without disturbing the pellet. 350 µl of Lysis Buffer, 350 µl of 5M LiCl and 80 µl of CTAB (10g CTAB, 4.1g NaCl/100 mL $H_2O$) was added to each tube and then heated at 65ºC for 10 min. The tubes were vigorously shaken followed by addition of equal volumes of Chloroform. They were spun in the centrifuge at 16k for 14 min. The top layer was removed and put into separate tubes as before. Equal volumes of Isopropanol was added, the tubes were left on the bench for 10 min and then spun at 16k for 10 min. The isopropanol was poured out leaving behind the pellet which was washed twice with 1 mL of 70% Ethanol. TE was added to each tube depending on the size of pellet obtained.

PCR and Restriction Digestion of highly variable regions

Four plasmid sequences available from NCBI GenBank (Accession numbers: AM947676, AP001204, AF116907, AM947677) were aligned using software called ClustalX (EMBL-EBI). Regions displaying high variation were sought out in order to select an approximately 1Kb region showing maximum differences in base pairs. One particular highly variable region was found and primers were designed. Two primers:

74 and 76 (74 being 50 bp larger than 76) were designed and used in PCR to amplify the region of interest. PCR conditions were as follows: 95 ºC for 2 min, 35 cycles of 95 ºC for 20 s, 60 ºC for 20 s, 72 ºC for 30 s and a final step at 68 ºC for 5 min. Samples were digested with restriction enzyme HindII (Boehringer Mannheim) or HincII (New England BioLabs) and an 11µl volume of digested DNA was electrophoresed in TAE buffer at 70V for 1.5 h in 2% agarose gels.

Sequencing Data Analysis

The 20 DNA samples that were sent out for sequencing to the Waikato DNA Sequencing Facility were extracted from gels using the Zymoclean Gel DNA Recovery Kit™. The facility offers a DNA sequencing service based on the Amersham Biosciences MegaBACE DNA Analysis System that utilizes dye terminator chemistry. Electropherograms data obtained were aligned using program ClustalX from EBI web server (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Comparisons of sequences were done using BLAST (Basic Local Alignment Search tool) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) at the server of the National Centre for Biotechnology information.

| | | |
|---|---|---|
| VapAF | GACTCTTCACAAGACGGT | (Ocampo-Sosa et al. 2007) |
| VapAR | TAGGCGTTGTGCCAGCTA | (Ocampo-Sosa et al. 2007) |
| TraAF | AGAGTTCATGCGTGACAACG | (Ocampo-Sosa et al. 2007) |
| TraAR | GTCCACAGGTCACCGTTCTT | (Ocampo-Sosa et al. 2007) |
| 16SF | TCGTCCGTGAAAACTTGGG | (Sellon et al. 2001) |
| 16SR | CGACCACAAGGGGGCCGT | (Sellon et al. 2001) |
| 74F | CCGGTGTGTACGTGTACCTG | |
| 74R | ATCCGTAGTCGAGCATCACC | |
| 76F | CCCTAGACCACGCAATCG | |
| 76R | ATCCGTAGTCGAGCATCACC | |

Table 1: Primer sequences used in PCR amplifications and sequencing. F- Forward

R- Reverse

## 2.4 Results and Discussions

Bacteria Recovery and Storage

The repeated transfer of bacteria from one media to another or storage of bacteria on agar in a refrigerator over a period of time (von Bargen & Haas 2009) was responsible for the large VapA plasmids getting expelled from the bacterial cell. Thus when bacteria grown in NANAT media were transferred to BHI plates and finally to BHI broth plasmids were lost from the bacterial cells. Thus a more efficient way to store positive samples was to use half the single colony to do a quick PEG Lysis, PCR and confirm the presence of plasmids then use the other half of the virulence plasmid (VP) positive isolate to grow VP positive isolates in broth and freeze them at -80 ºC until required. 10 mL of bacteria was frozen in approximately 1 mL of glycerol which allows preservation of bacteria and prevents damage that can be encountered during freezing or thawing of samples. Freezing the VP positive isolate in the initial steps was essential so that once the positives were found only those samples were used for DNA /Plasmid isolation. Since plasmids were getting lost from the samples, storing plates was not an option thus one had to grow only a few samples at a time so that they could be dealt with on the same day as any plates left for another day or two could potentially be void of plasmid. This was very time consuming but there was a better chance of preserving plasmids in isolates for use in further experiments.

Identifying *Rhodococcus* Bacteria

The growth of nasal swabs on NANAT selective media plates allowed the visual identification of *R. equi* based on the salmon pink to yellow coloured mucoid morphology of the colonies as described by (Prescott 1991). An occasional absence of *R. equi* growth on plates was observed due to the strength of the antibiotics used or their higher concentration in certain plates due to the media not being mixed properly. This was ensured by increasing the amount of media while maintaining the antibiotic concentration and doubling the time used for mixing it. Use of NANAT selective media successfully eliminated the growth of undesirable bacteria since the antibiotics used have the following activity. Polymyxin B is used against resistant gram negative as it binds to cell membrane and alters its structure by making it more permeable, Naladixic acid is known to be effective against gram positive and gram negative bacteria but if present in high concentrations it kills bacteria instead of inhibiting its growth (Monique *et al*. 2003), Novobiocin is a potent inhibitor of DNA gyrase and is an effective antistaphylococcus agent (Walsh *et al*. 1983) and Cyclohexamide is effective against eukaryotes such as yeast. The 16S rRNA gene is highly conserved between different species (Coenye & Vandamme 2003) and thus 16S primers which amplify a 441-bp segment of DNA from all strains of *R. equi* (Sellon *et al*. 2001) were used in this study to confirm the visual identification of *R. equi* as seen below in Figure 2.1.

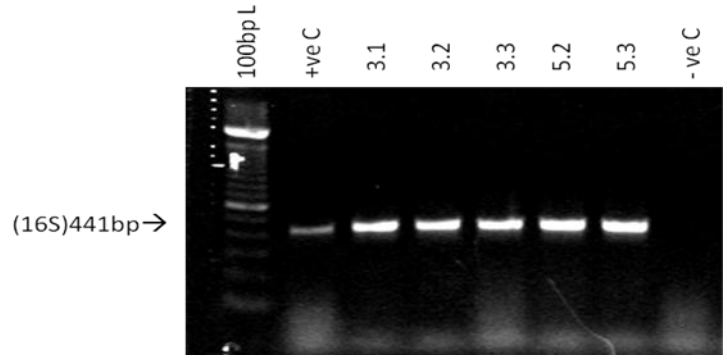


Figure 2.1: *R. equi* identification of 5 isolates. 16S primers were used in polymerase chain reaction.

Identifying Virulent Plasmid (VP) Positive Bacteria based on traA or vapA genes

The results obtained from the PEG Lysis of bacterial cells and Polymerase Chain Reaction (PCR) that was carried out with the VapA (Forward and Reverse) or TraA (Forward and Reverse) primers to find bacteria carrying the *vapA* or *traA* genes respectively (as shown in Figure 2.2 and Figure 2.3). VapA is indicative of the presence of *R. equi* virulence plasmids whereas TraA is indicative of the presence of large plasmids (Ocampo-Sosa *et al*. 2007). 50 out of 105 isolates tested by VapA primers were found to be VP positive as shown in Table 1 below.

| Foal tested for vapA/traA | Result | | |
|---|---|---|---|
| | 1 | 2 | 3 |
| B1 Victor Supreme 08’ | - | - | - |
| B3 Lislea Lass 08’ | - | - | - |
| B4 Invogorate 08’ | - | - | - |
| B5 Girl of your Dreams 08’ | - | + | + |
| B7 Angel of Tarcoola 08’ | + | + | + |
| B8 Got to be September 08’ | - | - | - |
| B9 Shy Angel 08’ | - | + | + |
| B11 Jaquie N 08’ | + | + | + |
| B12 Immortality 08’ | - | - | - |
| B13 Charmed life 08’ | - | - | - |
| B14 Susie Sands 08’ | + | + | + |
| B15 We Love to Party 08’ | - | + | - |
| B16 Cyclone Vance 08’ | - | - | - |
| B17 Idle Eve 08’ | - | + | - |
| B18 Culleys Asset 08’ | + | + | - |
| B19 Tania Bromac 08’ | - | + | - |
| B20 Foxy Mama 08’ | + | + | - |
| B22 Parisian Pearl 08’ | - | - | - |
| B23 Splendid Dreams 08’ | + | + | + |
| B25 Ally 08’ | + | - | + |
| B26 Speedy Star 08’ | - | - | + |
| B30 Socky Sweet Heart 08’ | + | + | + |
| B31 Twilight Chaser 08’ | + | + | + |
| B32 Dainty Falcon 08’ | + | + | - |
| B33 Perfecta Lobell 08’ | + | + | - |
| B34 Trumphant France 08’ | - | + | + |
| B35 Geld Bromac 08’ | - | + | - |
| B36 Poppy 08’ | + | + | + |
| B37 Classic Ace 08’ | - | - | - |
| B38 Darsia 08’ | + | - | + |
| B39 Rosemary Grace 08’ | + | - | - |
| B40 Explosive Atom 08’ | + | + | - |
| B41 Party Baby 08’ | - | - | - |
| B42 A little more magic 08’ | + | + | - |
| B43 In Hollywood 08’ | - | - | - |

Table 2: *R. equi* isolates tested for presence and absence of the virulence plasmid.

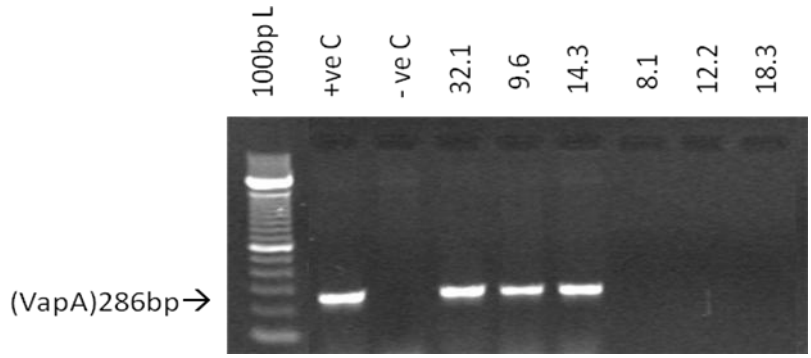


Figure 2.2: *R. equi* virulence plasmid detection of isolates. This was based on polymerase chain reaction with VapA primers.

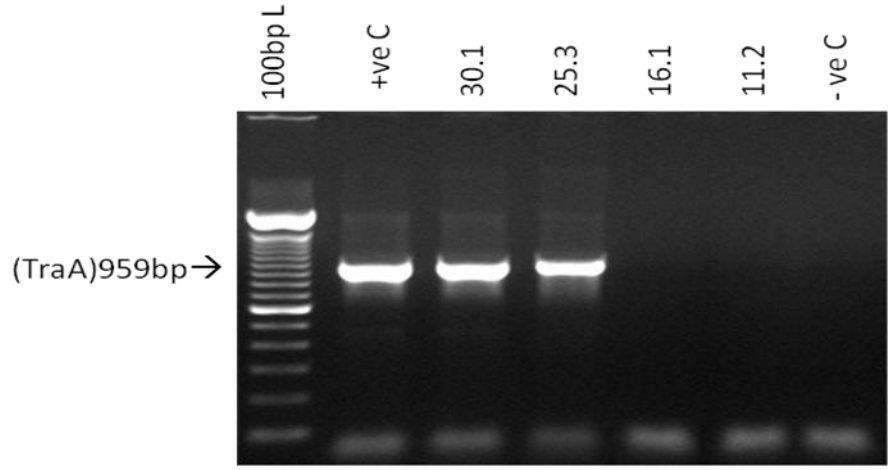


Figure 2.3: *R. equi* virulence plasmid detection of isolates. This was based on polymerase chain reaction with TraA primers.

The VapA amplification of 105 *R. equi* isolates is located in the Appendix 5.3: Gel Photos section of this thesis.

Plasmid Isolation

Large plasmids are difficult to see due to the obscuring by tailing of the chromosomal DNA, they are very thin and will run behind the chromosomal DNA band in an agarose gel (Walker 1984). As shown in figure, Method E was able to yield prominent plasmid DNA but unfortunately repeating the obtained result was very difficult. Since large plasmids are present in low copy numbers and nocardioform actinomycetes are relatively resistant to lysis, low yields of plasmid DNA are often found (De la Pena-Moctezuma & Prescott 1995). It was realised that the problem did not lie entirely with the methods being used but it was the nature of the plasmid that was making the isolations and their visualisations on gels so difficult. Plasmid visualisation on electrophoresis gels was quite cumbersome but was achieved for some plasmid isolations as observed in Figure 2.4. The supercoiled DNA migrates the fastest in the gel due to its tight conformation, followed by nicked DNA and then its linear form (Schleef 2001). Restriction digestion with EcoRI/NcoI of isolated plasmid showed extremely faint banding patterns and a fair amount of smearing emphasising the poor quality of the isolated plasmid, the gel photo is available in the Appendix Additional results section. It would be very difficult and time consuming to recognise different RFLPs in different plasmids and the difference in the quality of isolated plasmids would affect the banding pattern thus the RFLPs would be due to quality differences and not actual differences in plasmids. There was a need to find an easier way to characterise plasmids based on RFLPs.

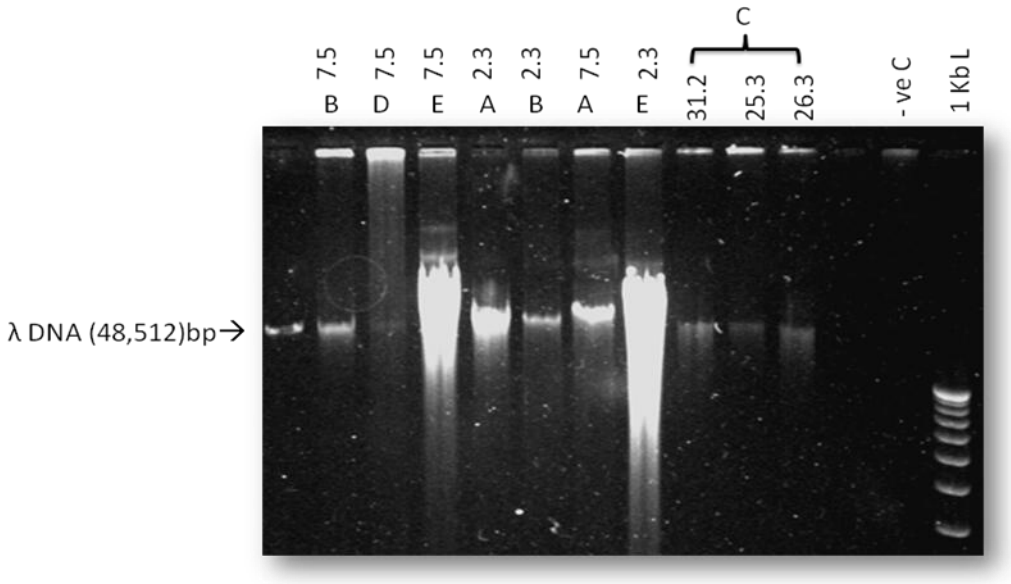


Figure 2.4: Agarose gel separations of plasmid DNA. These were isolated from various isolates of *R. equi* using a variety of methods and run for 10 hours in an electrophoresis gel.

Sequence Alignment

By aligning sequences of four available plasmids from GenBank (Accession numbers: AM947676, AP001204, AF116907, AM947677) a highly variable region was observed from which a region that was approximately 1 Kb in size was selected. With the exception of AM947676 which encoded VapB the three other plasmids encoded VapA. This highly variable region would be used to amplify part of the same variable region from DNA extractions/Plasmid isolations from VP positive isolates which would yield possible RFLPs when digested with restriction enzymes. Based on these assumption 21 VP positive strains were trialled. The sequence alignment of the

four plasmids is approximately 200 pages due to the large plasmid sizes and is available in section 4 on the CD attached to the back of the thesis. The highly variable region selected from the four plasmid alignments for this analysis is highlighted. Sequences of the two designed primers based on the variable region of plasmid accession number AF116907 which was named p103: 74 and 76 are available in section 2 on the CD. The only difference between the primers is that 74 consists of an extra 59 bp at the start of the sequence. This region was found to be void of any genes and is probably an intergenic region which is less conserved than the other areas but had the potential in characterising virulence plasmids if any differences did occur.

It was necessary to see if there was any cross reactivity between genomic DNA isolations and plasmid isolations of VP positive isolates for amplification with either primers 74 or 76. Figure 2.5 showed no difference in using plasmid isolations or DNA extractions for amplification of the variable region with primer 76. Because DNA extractions are relatively rapid and more efficient than plasmid isolations they were thus the method of choice for the analysis.

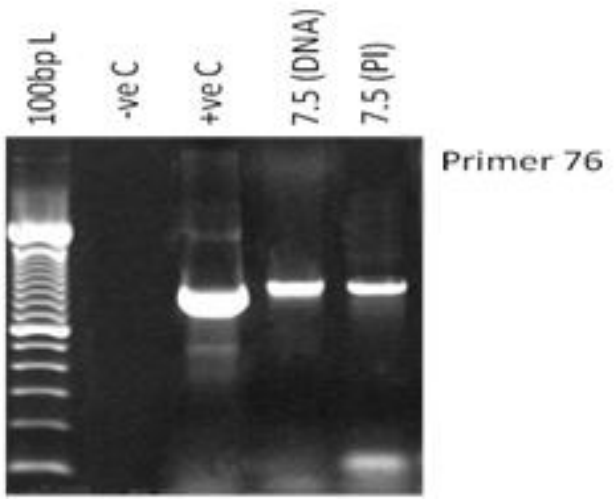


Figure 2.5: A comparison of plasmid isolation (PI) and DNA extraction (DNA) of VP positive isolate 7.5 with primer 76.

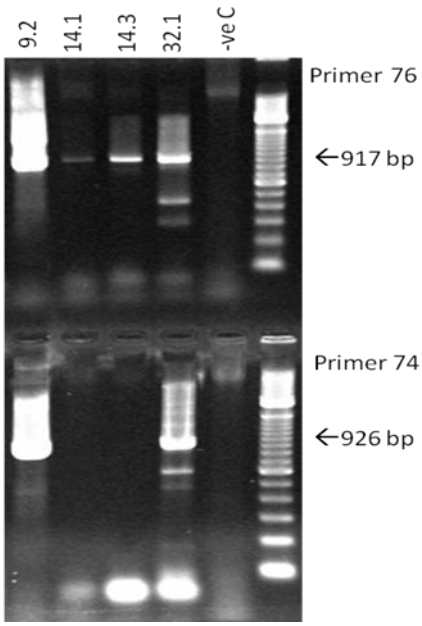


Figure 2.6: Comparison of 4 VP positive isolates. They were amplified with primers 74 and 76.

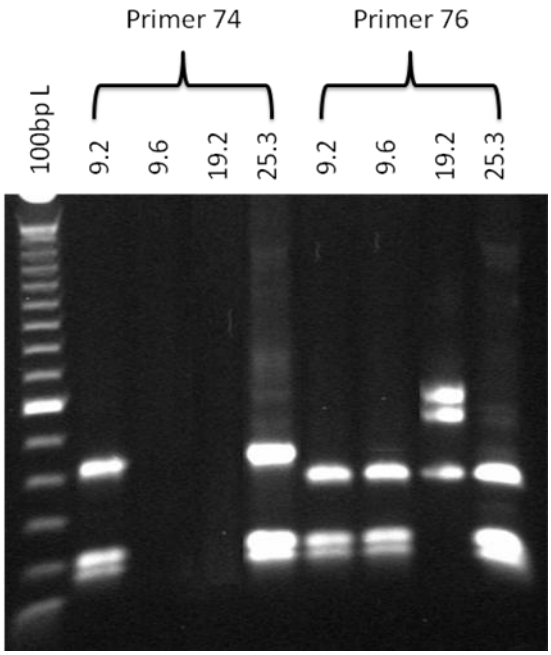


Figure 2.7: Comparison of restriction digestion of 4 VP positive isolates. They were amplified with primers 74 and 76 using restriction enzyme HindII.

Restriction Digestion

The region amplified by primers 74 and 76 generated amplicons of sizes 926-bp and 917-bp respectively. Primers 74 often failed to amplify the region of interest in positive samples (as shown in Figure 2.6) and even when the region was amplified it wasn't always sensitive to the restriction digestion by enzyme HindII , Primers 76 were a more reliable primer-set in generating amplicons and being digested with the restriction enzyme HindII/HincII and thus the preferred primer for the amplification of the selected variable region in plasmids (as shown in Figure 2.7). It was noticed from Figure 2.7 that *R. equi* isolate 19.2 had a different restriction digestion pattern compared to the three other isolates. This suggested the possibility of RFLPs in the isolates. 21 VP positive *R. equi* isolates were selected and subjected to restriction

digestion with HindII/HincII, as shown in the Figure 2.8. Distinct patterns were observed, the pattern with DNA bands of lower molecular weight was called PI and those with DNA of higher molecular weight was called PII. 13 VP positive *R. equi* isolates belonged to PI and 8 belonged to PII. All *R. equi* isolates from the same nasal swab had the same RFLP patterns and similar sequences. To confirm the observed result all 21 isolates were subjected to sequence analysis. Sequencing of many samples had to be repeated due to contamination with smaller products generated from non-specific binding, excising bands from the gel instead of purifying the PCR product solved the problem.

| **PI** | **PII** |
|---|---|
| **7.5** | **31.3** |
| **7.6** | **19.2** |
| **9.2** | **36.2** |
| **9.6** | **36.3** |
| **14.3** | **40.1** |
| **14.1** | **40.2** |
| **32.1** | **42.1** |
| **30.1** | **42.2** |
| **30.2** | |
| **30.3** | |
| **39.1** | |
| **18.2** | |
| **25.3** | |

Table 3: The distribution of PI and PII patterns among 21 VP positive *R. equi* isolates. The numbers refer to the foal number from Table 1.and the decimal points refer to strains of bacteria that were found to be positive.

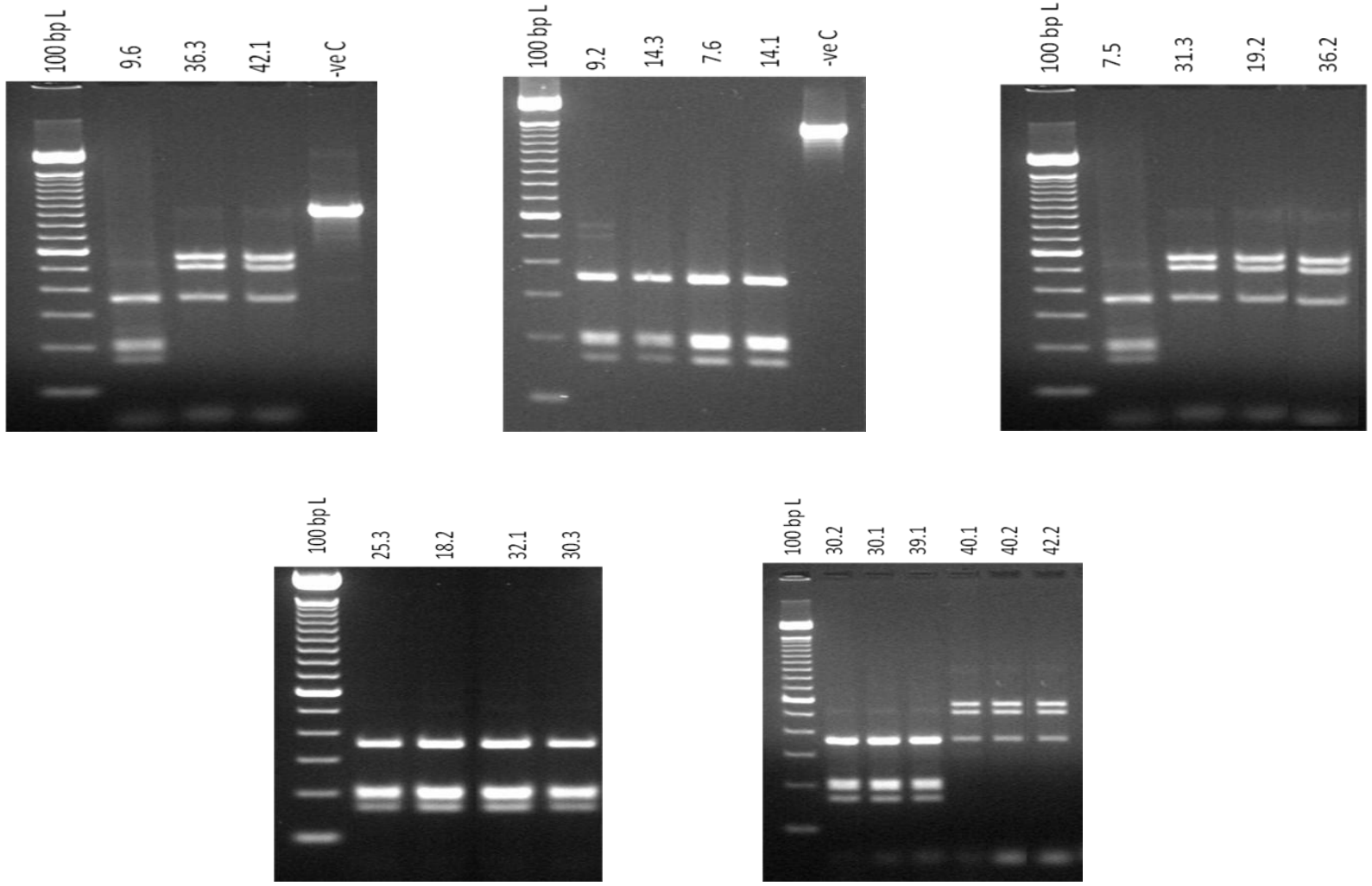


Figure 2.8: Restriction digests of 21 VP positive *R. equi* isolates using enzyme HindII .

PI Sequences

| Accession | Description | Max score | Total score | Query coverage | E value | Max ident |
|---|---|---|---|---|---|---|
| AM947677.1 | *Rhodococcus* vapA-type virulence plasmid (pVAPA1037), strain 103S | 1620 | 1620 | 100% | 0 | 100% |
| AP001204.1 | *Rhodococcus* plasmid pREAT701 DNA, complete sequence | 1620 | 1620 | 100% | 0 | 100% |
| AF116907.2 | *Rhodococcus* virulence plasmid, complete sequence | 1620 | 1620 | 100% | 0 | 100% |
| AM947676.1 | *Rhodococcus* vapB-type virulence plasmid (pVAPB1593), strain PAM1593 | 252 | 252 | 24% | 5.00E-68 | 88% |

Table 4: An example of a PI sequence producing alignments with published sequences. These were generated by the BLAST program.

All PI sequences had query coverage of 100% with a 98 to100% identity with plasmids of accession numbers: AM947677.1, AP001204.1, and AF116907.2 that all belonged to VapA virulence plasmids of 85-kb type I as shown in Table 4. Since query coverage of 24 to 25% was observed with accession number AM947676.1 of virulence plasmid VapB as seen in table it is certain that the query sequences are not from VapB-encoding plasmids. This data suggested that all PI sequences are a very

close match with the 80, 610 nt VapA plasmids, whose genome sequence are available in GenBank and are of 85-kb type I plasmids (Takai *et al*. 2000). However, if published genome sequences of other plasmid types were available to make comparisons, our results would be more conclusive.

PII Sequences

| **Accession** | **Description** | Max score | Total score | Query coverage | E value | Max ident |
|---|---|---|---|---|---|---|
| AM947676.1 | *Rhodococcus* vapB-type virulence plasmid (pVAPB1593), strain PAM1593 | 394 | 394 | 30% | 8.00E-111 | 88% |
| AM947677.1 | *Rhodococcus* vapA-type virulence plasmid (pVAPA1037), strain 103S | 224 | 503 | 31% | 1.00E-59 | 98% |
| AP001204.1 | *Rhodococcus* plasmid pREAT701 DNA, complete sequence | 224 | 503 | 31% | 1.00E-59 | 98% |
| AF116907.2 | *Rhodococcus* virulence plasmid, complete sequence | 224 | 503 | 31% | 1.00E-59 | 98% |

Table 5: An example of a PII sequence producing alignments with published sequences. These were generated by the BLAST program.

PII sequences unlike PI sequences had low query coverage of 29-37% with plasmids of accession numbers AM947677, AP001204 and AF116907 which are VapA type plasmids with 98-100% identity. Query coverage of 29-30% and identity of 88-90% was observed with plasmid of accession number AM947676 which is a VapB type plasmid. There is a possibility that PII sequences are from a different type of plasmid but more analysis of other sequences from variable regions of the plasmid and comparisons with published plasmid genomes of different types is required to confirm this.

Sequencing Data Analysis

Minor single nucleotide polymorphisms (SNPs) were observed in the sequences but they were acceptable since the region is highly variable thus less conserved and therefore more prone to SNPs. Poor quality in the first 15-20 bases and the deteriorating quality of sequencing traces after 700-900 bases was observed in the electropherograms of sequences as expected. Hence the first 30 and the last 50 bases from each of the sequences were not included in the analysis. Since the region being studied was GC-rich i.e. 64% and due to non-specific binding in some samples repeated attempts at sequencing were required.

PI sequences were found to have significant matches with three plasmid genome sequences, two of which i.e. plasmid p103 (accession number: AF116907), which is 80,609 nt in size and plasmid ATCC3370 pREAT701 (accession number:

AP001204), which is 80,610 nt have been found to be virtually identical and both are of the 85-kb type I (Takai *et al*. 2000). The third published plasmid 103S (accession number: AM947677) is 100% identical to the two above mentioned plasmids and thus must also belong to the 85-kb type I plasmid but comparisons with 87-kb type I plasmids is necessary. Due to the fact that most clinical isolates from Australia, America and Europe are found to contain either the 85-kb type I or 87-kb type I plasmids (Venner *et al*. 2007), the first horses were brought to New Zealand from Australia and that VapA-encoding plasmids of virulent *R. equi* infecting foals are mostly from 85-kb type I plasmids it is quite certain that the PI plasmids are 85-kb type I plasmids whereas PII could possibly correlate to 87-kb type I plasmids of any of the other types of 85-kb plasmids but more PII sequences need to be compared to those of 87-kb type I sequences to reach a valid conclusion but 87-kb type I sequences are unavailable. However sequences of PII could have matches with 85 Kb or 90 Kb types of plasmids but since the sequences of those too are unavailable in GenBank a comparison cannot be made. Selecting other variable regions from plasmid alignments and sequencing them can help confirm the observation made in this study. From our results it is likely that more than one subtype can exist from isolates on a single farm. Characterisation of these plasmids will be a useful tool in epidemiological studies. Since the sequence studied did not contain any known genes that determine proteins it could be an intergenic region, a regulatory sequence which is involved in regulating processes such as transcription or may even contain non-coding RNAs some of which are involved in maintaining cellular processes such as translation.

# 3 Conclusions

## 3.1 Summary

The results of the research presented in this thesis suggest that subtypes of *R. equi* isolates may exist in a single farm in New Zealand. Based on the fact that two of the published plasmid sequences that had significant matches with PI are 85-kb type I plasmids, that Australian *R. equi* isolates are mostly either 85-kb type I or 87-kb type I plasmids which were the first horses brought to New Zealand and that most *R. equi* isolates in the world are 85-kb type I it is speculated that a variable region from plasmids with PI patterns are 85-kb type I plasmids. This is the first attempt at characterisation of the New Zealand *R. equi* VapA-encoded plasmids. An in depth knowledge of the unique characteristics of plasmids and their relatedness of strains within the species could define transmission characteristics and possible sources of infection in disease outbreaks.

Use of a 16S primer provided rapid and accurate species identification whereas TraA and VapA primers were good indicators of the presence of virulence plasmids and of the presence of VapA-encoded plasmids respectively in *R. equi* found in foal nasal swabs. Studies have provided evidence of geographic diversity among isolates of *R. equi* by discovering twelve different but closely related virulence plasmid types from throughout the world.

Plasmid isolations were rather tedious thus selecting a variable region for analysis as used in this study to uncover plasmid characteristics is an efficient

alternative method. Associations between chromosomal differences, virulence and plasmid profiles will further evaluate the molecular diversity of *R. equi*. Analysis of the sequences deposited in GenBank provides a starting point for further analysis. However, more extensive sequencing of plasmid genomes, their mobile elements and sequence ontologies is necessary to address fundamental questions regarding its evolution and critical problems such as the spread of antibiotic resistance (Williams *et al*. 2006).

## 3.2 Future Work and Recommendations

Results in this study are based on sequence analysis of approximately 1-kb variable regions of VapA-encoding plasmids from 21 VP positive isolates of a farm in New Zealand. To lend support to the results presented in this thesis, it would be beneficial to investigate multiple regions of variability from plasmid alignments. The nasal swabs used in this investigation are all from foals in one farm; it would be interesting to get swabs from multiple farms to see if there is more variation in *R. equi* VapA-encoding plasmids or if the same variation as in this study exists in other farms in New Zealand. Factors that influenced the robustness of DNA sequence analysis studies included PCR contamination, DNA quality and quantity, GC content of the sample, resolution of the genetic markers and non-specific primer binding.

Contamination is a common problem when working with PCR especially on a daily basis as in this study. Because contamination is potentially expensive and

time-consuming it is advisable to aliquot all reagents used, such as primers. Contamination can be difficult to ferret out, thus cleaning pipettes, work area and regularly changing lab coats and gloves was an essential part of the daily routine to keep contamination away. PCR produces results quickly, is very useful for diagnostic purposes and it also has the advantage of ruling out *R. equi* pneumonia in culture-negative foals that have failed to improve with anti microbial therapy (Sellon *et al*. 2001). It may also be useful in monitoring response to therapy and deciding when to stop therapy in foals (Sellon *et al*. 2001). However, at the start of a PCR reaction, reagents are in excess whereas template and product are at low enough concentrations therefore product renaturation does not compete with primer binding and amplification proceeds at a constant rate. When the reaction enters linear phase, amplification is variable even among replicate samples this is mainly due to product renaturation competing with primer binding. In order to get precision and accuracy, when the sample is in constant rate of amplification it is necessary to collect quantitative data at that point. This is possible with RT-PCR (Real-Time Polymerase Chain Reaction) where analysis of reactions during exponential phase at a given cycle number provides several orders of magnitude of dynamic range. Thus RT-PCR can be used as quantitative analysis of sample DNA from the variable regions of plasmids alignments. Capillary gel electrophoresis to view large plasmids is available which allows separation of plasmid structures (supercoiled, linear or nicked DNA) in thin capillaries that are filled with liquid polymer solution that have high resolution, high sensitivity even when low DNA concentrations are used (Walker 1984). It has been suggested that using mCAZ-NB {ceftazidime (20 µg/mL), novobiocin (25 µg/mL)

and cycloheximide (40 µg/mL)} media might be more useful than NANAT media, due to higher rates of recovery of virulent *R. equi* from samples (Muscatello *et al*. 2006a).

When performing plasmid isolations, taking the number of bacterial cells in to consideration might give more control over the plasmid yield. Since chromosomal DNA reduces plasmid visibility in electrophoresis gels, methods that reduce the content of chromosomal DNA in plasmids isolation might be beneficial. A better understanding of the cell wall structure of *R. equi* will improve our understanding in its complexity, its contribution in virulence and possibly improve plasmid isolations. Some aspects that can be studies in the future include; designing a reliable vaccine for foals that are younger than three months of age, understanding phagosome maturation by *R. equi* (von Bargen & Haas 2009) and infecting non-virulent strains with virulent plasmids to understand direct foal to foal transmission and its epidemiological relevance. Further research on *R. equi* virulence determinants discussed in Chapter 1 is required to fully understand the *R. equi* virulence mechanism.

The murine model of *R. equi* infection is not a good one, because the route of infection in mice does not mimic that in foals. In foals, the respiratory route is the route of infection caused by the smaller number of organisms, whereas the route in mouse models is intravenous and a large number of organisms are administered (Heuzenroeder *et al*. 2005). A more efficient inexpensive and accurate approach to obtain plasmid DNA of sufficient amount and quality to generate libraries remains to be found (Heuzenroeder *et al*. 2005). An important research area for the future will be

the analysis of chromosomally encoded proteins and their associations with *R. equi* virulence plasmid factors (von Bargen & Haas 2009). Techniques of gene manipulations of *R. equi* will help discover factors involved in phagosome establishment and host cell cytotoxicity. An enormous amount of surface-associated proteins have been revealed in the *R. equi* genome and their possible interaction with host cells in the course of infection requires investigation (von Bargen & Haas 2009).

Studies in recent years have highlighted the importance of airborne *R. equi* population and ecological factors that facilitate aerosolization of virulent *R. equi* (i.e. soil, moisture, sandy soils and poor pasture cover). Furthermore the growing resistance of *R. equi* to antibiotics emphasizes the importance of vaccine development. A better understanding of *R. equi* virulence plasmid epidemiology will lead to improved strategies for prevention and control of the disease.

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# 5 Appendix

## 5.1 Appendix: Additional Protocols

### 5.1.1 Media

Making NANAT selective media to isolate *R. equi*

1. To make 400 mL of the selective media, 6g of agar and 14.8g of BHI were added to 400 mL of $H_2O$.
2. After autoclaving the media and cooling it to 50ºC, 1 mL of Polymyxin B (10mg/mL), 1 mL of Naladixic Acid (10mg/ml), 1 mL of Novobiocin (10mg/ml) and 2 mL of Cyclohexamide (10mg/mL) were added and the media was mixed by inverting it ten times.
3. Approximately 15 mL of the media was poured into each of the pentri dishes which were cooled overnight and then refrigerated for future use.

Making BHI (Blood Heart Infusion) agar to grow *R. equi*

1. 7.5g of Agar and 18.5g of BHI was added to 500 mL of distilled water.
2. The medium was autoclaved in a pressure cooker for 20 -40 min, cooled in a water bath at 50ºC for about 30 min and then approximately 15 mL was poured into each pentri dish after which they were cooled overnight.

Storage of nasal swabs and broth containing positive bacteria samples

1. Nasal swabs were stored in a -80 ºC refrigerator.
2. BHI broth was made in the above mentioned way excluding the addition of agar, 10 mL of broth was poured into 15 mL universals.
3. Broth was inoculated with a loop full of virulent positive bacteria and grown in a shaking incubator at a speed of 200 rpm at 30-37 ºC for 24 or 48 h.
4. 1 mL of Sterile Glycerol was added to each of the universals, they were inverted a few times and approximately 1 mL of the broth was put into long storage tubes.
5. These tubes were stored in a -80 ºC refrigerator.

### 5.1.2 Colony Hybridization

1. Nylon Membranes (Hybond) were cut into circles roughly the same size as the plates.
2. Single *R. equi* colonies were grown on plates overnight.
3. The Nylon membranes were placed on the plates for about a minute and markings were drawn on the edges of the membrane as well as on the plates so that the orientation is known.
4. Using tweezers the membranes were lifted off the plates and put on a small tray (Bacteria facing up) containing Lysis Solution (10 mM Tris-HCl, 1 mM EDTA pH 8.0, 80mg/mL lysozyme) and then heated at 80ºC for 10 min.

5. The membranes were put on a filter paper for a couple of minutes then were transferred onto another filter paper that was immersed in Denaturing Solution (0.5M NaOH, 1.5M NaCl) for about 3 min.
6. The membranes were transferred twice for 3 min each onto separate trays with filter papers (Whatman 3MM) immersed in 3 X SSC (20 X SSC is 3M NaCl and 0.3M sodium citrate) Solution.
7. Small lids were filled with 3 X SSC just about 1cm high and with the bacteria facing up membranes were placed in the lids.
8. They were put on an Orbital shaker (Bellco Glass, INC.) set at speed 3.5 for about 10 min.
9. The 3 X SSC was drained out from the lids and fresh 3 X SSC was added to them and again shaken on the Orbital shaker for 10 min.
10. The membranes were then dried on filter paper at 80ºC for a couple of hours, the crosslinker (BLX-Z54, Life Technologies) was set at 0.120 and the membranes were exposed to UV light.
11. The membranes were put into tubes and then pre-hybridised with 5 mL of Church and Gilbert (0.5M $Na_2HPO_4$ pH 7.2, 1% BSA, 1 mM EDTA, 7% SDS) for about 30 min rotating at 55ºC.
12. The Church and Gilbert solution was drained out of the tubes and 5 mL of the radioactively labelled VapA probe (10 pmol) {purified VapA PCR product was used to construct 32P labelled probes using a Primed DNA Labelling Kit (Roche Diagnostics) according to the manufacturers recommendations} tagged with the primer was added to the tubes.

13. The tubes were set onto a rotator such that they were balanced and allowed to rotate at 55ºC overnight after which the probe was poured back into its falcon tube and stored in the fridge for future use.
14. The membranes were washed twice with 3 X SSC while shaken at 37ºC for 20-30 min each time.
15. The membranes were dried completely by placing them on a filter paper and heating at 37ºC for two hours.
16. The membranes were taped onto a filter paper and put in a cassette, a photographic paper was placed on the membranes in the dark room; the cassette was shut, wrapped in a black bag and put in a cupboard overnight.
17. The next day, the photographic paper was developed and fixed for 90 sec.

### 5.1.3 Staining

DAPI Staining

1. About 15 µl of Milli-Q $H_2O$ was used to form a drop on a slide.
2. The drop was emulsified with a single streak of *R. equi* bacteria, heat-fixed and dried at room temperature.
3. The cells were fixed with 2-3 drops of cold methanol and then dried at room temperature.
4. The above step was repeated three times after which the slide was washed with large amounts of tap water about 4-6 times and dried as before at room temperature.

5. 10 µl of poly-L-lysine (5 µg $ml^{-1}$) was smeared over the fixed and washed cells and they were left to dry at room temperature.
6. 10 µl of DAPI (0.2 µg $ml^{-1}$ in 40% glycerol) was used to stain the cells after which a cover clip was placed on the slide then viewed on a microscope.

Gelstar Staining

Exactly the same procedure as for DAPI staining was followed with the exception of a 1/5000 dilution of Gelstar used instead of DAPI.

Stains were used to allow better visualisation of plasmids

Ethidium Bromide (EtBr)

1. As opposed to adding EtBr while making the agarose gel, EtBr was used to stain the gel by adding it to the buffer in which it was soaked once the DNA had already been electrophoresed.
2. Approximately 6 µl of EtBr (10 mg/mL) was added to 150 mL of buffer.

SYBR Green

1. A couple of µl of SYBR Green (10,000 X) was directly added to the DNA before gel electrophoresis.
2. 2.5 µl of SYBR Green was added to 10 µl of plasmid DNA.

The two stains were compared to see which one was more useful in plasmid DNA visualisation.

### 5.1.4 DNA Extraction

PEG Lysis

1. Single colonies were inoculated in 1.7 mL Eppendorf tubes containing 100 µl of 20% PEG Lysis Solution (20g PEG 8000, 14.8g NaCl/100 mL $H_2O$) each.
2. Tubes were heated at 95°C in an Eppendorf Thermomixer for 10 min.

### 5.1.5 PCR Conditions

1. PCR tubes were labelled for each DNA sample as well as a negative control.
2. About 20 µl of Master Mix (MM) was required for each tube.
3. 1 µl of primer was used per 50 µl of MM whereas 1 µl of Taq per 200 µl of MM was used.
4. According to the above concentration and the number of DNA samples a mix of the MM, primer and Taq was made, vortexed then 20 µl of the mix was put into each PCR tube in the PCR cabinet.
5. 1 to 2 µl of DNA was added to each tube depending on the achieved DNA concentration.
6. A PCR (Peltier Thermal Cycler PTC-200, BIO-RAD) machine was used for the amplification.

PCR Master Mix

10mM Tris (pH8.3)

2.5mM MgCl2

250mM deoxynucleotide triphosphates (dNTP)

Deoxynucleotide triphosphates (dNTP) stock solution:
dATP, dCTP, dGTP and dTTP (Eppendorf) dissolved to a final concentration of 250µM in TE buffer.

Temperature Gradient PCR

A different annealing temperature is programmed into the thermocycler for each tube column across the thermocycler.

### 5.1.6 PCR Cleanup Methods

Purifying PCR Product for sequencing

1. 10 µl of PCR product was put into a 1.7 mL tube.
2. An equal volume of 20% PEG/NaCl was added to the tube.
3. It was left on the bench for about 15 min to allow precipitation.
4. The tube was spun in the centrifuge for 10 min at 16k.
5. 1ml of 100% Ethanol was added to the tube; once it was mixed well by inverting it a few times it was spun in the centrifuge for 15 min.
6. All the ethanol was pipetted out without disturbing the pellet.
7. 1 mL of 70% Ethanol was added to the tube; it was inverted a few times, spun in the centrifuge for 10 min at 16k and again discarded while making sure the pellet is not disturbed.

8. 15 mL of sterile water was added to the tube and then the concentration of DNA was measured using a nanodrop.

Reprecipitation to cleanup PCR products

1. Add 5 µL of NaOAc and 50 µL of nuclease-free $H_2O$ to 10 µL of DNA.
2. 2 volumes of 100% Ethanol was added to the above and precipitated on ice for 20-30 min.
3. Centrifuged at 16k for 15 min.
4. Supernatant discarded and pellet was resuspended in 7-10 µL of nuclease-free $H_2O$.
5. Nanodrop was used to check the concentration.

### 5.1.7 DNA and PCR Quality and Quantity Indicators

NanoDrop

1. Place 2 µl Milli-Q $H_2O$ on the lens and close. Click OK to BLANK.
2. Wipe lens using KimWipe tissue.
3. Place 2 µl Milli-Q $H_2O$/elution buffer (TE) on lens, close and click REBLANK.
4. Wipe using KimWipe.
5. Place 2 µl of the sample on lens and close.
6. Enter sample name and click MEASURE.

7. After all samples have been measured click SHOW REPORT, adjust as necessary.

Electrophoresis

- 1% Agarose and 1 X SB buffer for DNA under 1200bp run at about 110 V.
- 0.7% Agarose and 1 X TBE buffer for large plasmids and DNA over 1000bp run at about 40 V.
- 2% Agarose and 1 X TBE buffer for digested DNA under 500bp with multiple bands that are of similar length run at about 60 V.

➢ 10 µL DNA + 2 µL gel loading buffer (BPB).

➢ 1 µL of Ethidium Bromide (10mg/mL) stain per 10 mL of Agarose

➢ UV Alpha Imager used to capture image of gel.

1% Agarose gel:

35 mL 1 X SB buffer

0.35 g Agarose

1.5 µl Ethidium Bromide solution (10mg/mL)

0.7% Agarose gel:

35 mL 1 X SB buffer

0.24 g Agarose

1.5 µl Ethidium Bromide solution (10mg/mL)

2% Agarose gel:

35 mL 1X SB buffer

0.7 g Agarose

1.5 µl Ethidium Bromide solution (10mg/mL)

10 X SB (Sodium Borate) buffer (pH 8.5) (Stock Solution 2 L):

56 g Boric acid

9 g NaOH

1 X SB buffer:

200 mL 10 X SB buffer

1800 mL deionised $H_2O$

5 X TBE (Tris Borate EDTA) buffer (pH 8.0) (Stock Solution 1 L):

54g Tris Base

27.5g Boric Acid

20ml 0.5 EDTA

1 X TBE buffer:

100 mL 5 X TBE

1900 mL deionised $H_2O$

50 X TAE (Tris-acetate-EDTA) buffer (pH 8.3) (Stock Solution 1 L):

242g Tris

57.1 mL Acetic Acid

100 mL 0.5 EDTA

1 X TAE buffer:

20 mL 50 X TAE buffer

980 mL deionised $H_2O$

Gel loading buffer:

10 mL Glycerol

4 mL EDTA

2 mL 10% SDS

1.5 mL $H_2O$

### 5.1.8 Gel Extractions

Conventional Band Stabs

1. Run 1% Agarose gel with 1 X TAE buffer
2. Place on UV box
3. Cut tip of 100 µl pipette tip.
4. Turn on UV and stab target band with pipette tip.
5. Turn off light and remove tip from gel.
6. Expel gel from tip into 1.7 mL Eppendorf tube.

DNA excision

1. Place gel on a glass above the UV base (Safe Imager, Invitrogen), put on protective headgear, and the lights were switched off.
2. Using a scalpel, razor blade or cover slip splice band in gel (Keep UV light turned on for a minimum amount of time to prevent DNA damage).
3. Lift slice out of the gel and place in an 1.7 mL Eppendorf tube.

Freeze and Squeeze method for gel band purification

1. A hole was made at the bottom of a 0.7 mL Eppendorf tube using a sterilised 25mm needle.
2. A glass bead was placed inside the tube and the cut out band from the agarose gel was placed just on top of the glass bead in the tube.
3. The 0.7 mL tube was put inside a 1.5 mL Eppendorf tube and frozen at -80ºC for 30 min.
4. It was then centrifuged at 16k for 15 min. For the rest of the extraction the same protocol as the one above from PCR product purification was followed.

Gel Band Purification using the Illustra™ Extraction Kit

1. Following PCR to amplify the desired areas of the plasmid, bands of the PCR products that were run on the gel were cut out using a razor blade.
2. These were purified using the manufacturer's protocol in Illustra™ GFX PCR DNA and gel Band Purification Kit (This was done by adding capture buffer

to the gel sample, incubating it at 60ºC to dissolve the agarose, transferring it to a GFX column placed in a collection tube and washing it with wash buffer).

3. They were sent out to the Waikato DNA sequencing facility to ensure matches of the sequences with those available from GenBank.

Gel Band Purification using the Zymoclean™ Gel DNA recovery Kit.

1. DNA fragments from the agarose gel were removed by band stabbing and transferred to 1.5 mL Eppendorf tube.
2. 3 volumes of ADB Buffer™ was added to each volume of agarose excised from the gel.
3. Samples were incubated at 55 ºC for 5-10 min until the gel slices were completely dissolved.
4. The melted agarose solution was transferred to a Zymo-Spin I ™ Column in a Collection tube.
5. They were centrifuged at 10,000 rpm for 45 sec and the flow-through was discarded
6. 200 µl of Wash buffer was added to the column and centrifuged at 10,000 rpm for 45 sec, flow-through was discarded and this wash step was repeated.
7. 10 µl of Milli-Q $H_2O$ was added directly to the column matrix

8. Columns were placed into 1.5 mL tubes and centrifuged again at 10,000 rpm for 45 sec to elute DNA.

### 5.1.9 Plasmid Isolations/Purifications

Plasmid Isolation Method A

1. A few µL of broth stored at -80ºC was streaked onto plates and colonies grew overnight.
2. Loops full of bacteria were transferred to 10 mL of BHI broth which were grown overnight at 37ºC in a shaking incubator.
3. Bacteria were transferred to 1.7 mL Eppendorf tubes, spun down in a centrifuge at 16k for 30 sec and then the pellets were resuspended in 50 µl of TE.
4. 300 µl of TENS Buffer (0.1M NaOH, 0.2% SDS, 1mM EDTA, 10mM Tris pH 7.5) was added to the tubes and were put in 80ºC for 5 min. 150 µl of 3M NaOAc was added to the tubes; they were vortexed and spun for 2 min at 16k.
5. Using a flat ended toothpick the pellets were removed and discarded. 300 µl of Phenol: Chloroform was added to each tube, after vortexing the tubes they were centrifuged at 16k for 5 min.
6. The supernatant was removed and transferred to separate fresh tubes. Approximately equal amounts of 100% isopropanol was put into each tube and left on the bench for 5 min.

7. After spinning them down in the centrifuge for 10 min, pellets were washed with 1 mL of 70% Ethanol.
8. Once all the ethanol was discarded and pellets were air-dried for about 10 min depending on the size of the pellets TE was added and the concentration of DNA was measured using a NanoDrop.

Plasmid Isolation Method B

This variation involved using colonies grown at 37ºC on plates rather than broth and the use of CTAB.

Plasmid Isolation Method C (Ocampo-Sosa *et al*. 2007)

1. Bacteria were grown at 30ºC overnight using Brain Heart Infusion (BHI) broth.
2. They were harvested by centrifugation at 16k for 10 min at 4ºC followed by washing with 1 X TBE and then their transfer to 1.7 mL Eppendorf tubes.
3. After transfer they were centrifuged again under the same conditions.
4. The pellets were resuspended in 200 µl of SETL solution (15% Sucrose, 0.01M EDTA, 25mmol/L Tris-HCl (pH 8.0), 5mg/ml Lysozyme) and incubated for about 2.5 h at 37ºC.
5. 400 µl of Lysis Solution was added then tubes were inverted 250 times until a homogenous viscous suspension was obtained.

6. 300 µl of 5 M prechilled potassium acetate (60ml 5M KOAc, 11.5ml Glacial Acetic Acid, 28.5ml dd $H_2O$) was added after which tubes were inverted again about a 100 times and then left on ice for 30 min.
7. The tubes were centrifuged for 10 min at 4ºC, the supernatants were transferred to fresh 1.7 mL tubes and 10 µl of a 10mg/mL solution of RNase {896.7µl dd $H_2O$, 3.3µl 3M NaOAc (pH 5.2), 100µl 1M Tris-Cl (pH 7.4)} was added.
8. 500 µl of phenol: chloroform: isoamyl alcohol was added after incubation at 37ºC for 30 min.
9. The tubes were gently inverted about a 100 times and then centrifuged under the same conditions.
10. The supernatant was carefully discarded and the plasmid DNA was washed twice with 1 mL of 70% Ethanol.
11. The pellet was air-dried for 15 min and resuspended in appropriate amounts of TE.

Plasmid Isolation Method D (Birnboim & Doly 1988)

1. 5 mL of bacterial colonies were grown in BHI broth for 2 days.
2. 1 mL of cells were centrifuged at 16k for 10 min and then resuspended in 200µl of Solution I (0.05M Tris-HCl pH 8.0, 0.01M EDTA pH 8.0, 0.5M NaCl, 20%Sucrose, 5mg/ml Lysozyme).

3. These cells were incubated at 37ºC for 2.5 h after which 400 µl of Solution II (2% SDS, 0.3M NaOH) was added to the tubes.
4. The tubes were put on ice for 60 min followed by centrifugation for 5 min.
5. The supernatant was transferred to fresh tubes. 2 µl of RNase was added to each tube and the incubated at 37ºC for 30 min.
6. 500 µl of phenol: chloroform: isoamyl alcohol was added after which the tubes were inverted a few times then centrifuged at 4ºC for 10 min.
7. The supernatant was added to fresh tubes and roughly equal amounts of Isopropanol were added to them.
8. After centrifugation at 4ºC for 10 min the supernatant was discarded, the DNA was washed with 1 mL of 70% Ethanol and once all the ethanol was discarded and air-dried for 15 min, the pellets were resuspended in TE.

Tracey's plasmid purification method

1. A 1.2% agarose gel in a 0.7 mL Eppendorf tube containing a hole was placed over a 0.7 mL tube to collect all the purified plasmid.
2. 10 µl of the isolated plasmid solution was put in the tube just above the agarose and kept overnight to allow plasmid to seep through slowly followed by 10 µl of TE.

### 5.1.10 Restriction Digestion of Plasmid

NcoI

1. 5 µl of DNA (0.2-1 µg/µl), 2 µl of NcoI (2-10U), 2 µl of Restriction enzyme 10X buffer 3 and 11 µl of Milli-Q $H_20$ was added to a 1.7 mL tube.
2. The contents were mixed gently by pipetting and then centrifuged briefly at 12,000 x g in a micro centrifuge.
3. The tube was incubated at 37ºC in a thermomixer for approximately 4 h, a restriction digestion with overnight incubation was also carried out.

EcoRI

The same protocol as above was followed except for the replacement of the restriction enzyme and the buffer.

### 5.1.11 Multiple primer Analysis

1. Primer DR REP IR was used in combination with other primers in an extended PCR to amplify regions of VP positive plasmid isolate 2.3.
2. PCR conditions were 94ºC for 2 min, one cycle of 53.5 ºC for 20 s, 45 ºC for 30 s, 68 ºC for 2 min, 39 cycles of 94 ºC for 20 s, 45 ºC for 30 s, 68 ºC for 2 min and a final step at 68 ºC for 5 min.
3. A gradient PCR was performed after which 2 annealing temperatures were selected.

## 5.2 Appendix: Additional Results and Discussions

### 5.2.1 Colony Hybridisation

In order to find an easier way of deciphering the incidence of virulent colonies amongst a large number of single colonies grown on plates, radioactive labelling of genomic DNA with a VapA probe was attempted. The results showed that the VapA probe had bound to all the VP (virulence plasmid) positive colonies that were swabbed on an agar plate by a single VP positive colony whereas VP negative isolates did not bind to the VapA probe. Thus the VapA test for VP positivity was an efficient one.

### 5.2.2 Staining with DAPI and Gelstar to identify VP positive isolates

DAPI (4', 6-diamidino-2-phenylindole) is known to form fluorescent complexes with double stranded DNA. It was tested to see if it would bind to circular double stranded plasmids from VP positive isolates differently and be able to show a visual difference between VP positive and VP negative *R. equi* samples based on fluorescence. A number of dilutions of *R. equi* positive and *R. equi* negative bacteria were stained with DAPI but no significant differences were found in the staining of either of the bacteria on observation under a microscope. All the bacteria were stained blue under UV light.

### 5.2.3 PCR Optimization

In order to increase specificity of PCR a temperature gradient was trialled to determine the optimal PCR temperature as well as the adjustment of template DNA

concentrations. Negative and positive controls were used for all trials. The negative control consisted of Milli-Q $H_2O$ in the place of DNA where as the positive control used sample DNA known to amplify well.

### 5.2.4 Temperature Optimization

The choice of annealing temperatures in PCR is related to the concentration of the DNA template. If the concentration of the template DNA is low then the annealing temperature should be low. If the temperature is increased there will be a lot of unspecific binding. When primer length is increased so should the annealing temperature to get higher specificity for the PCR reaction. Thus the annealing temperature was increased by 5 ºC from 55 ºC to 60 ºC when the TraA primer was used to amplify a product size of 959-bp in comparison to when the VapA primer was used at 55 ºC to amplify a product size of 286-bp. Performing a temperature gradient, as has been done for the multiple primer analysis, assisted in choosing the right annealing temperature for amplifying the specific sequences.

### 5.2.5 Post-PCR DNA Processing

Some samples contained multiple PCR products of different sizes due to non-specific binding, which prevented direct sequencing. When this occurred, the PCR products of desired sizes had to be excised from the electrophoresis gel. The annealing temperature could be adjusted to obtain single products but even when single desired products were obtained in an electrophoresis gel and the remaining PCR products were purified the sequencing data showed the presence of a smaller product. This suggested that since there was a small quantity of the undesired product it was not

visible in the electrophoresis gel. It was better to band stab the desired products for all samples from an electrophoresis gel whether there was non-specific binding or not in order to prevent any contamination with undesired products. Gel band extractions performed by the Zymoclean™ Gel DNA recovery Kit seemed to present better quality sequences in comparison to using the freeze and squeeze method and the Illustra™ Extraction Kit.

### 5.2.6 Plasmid Isolations

Before carrying out any plasmid isolation method it was ensured that the bacteria were tested for the presence of plasmid by the VapA or TraA tests due to its ability to be lost during growth in media (von Bargen & Haas 2009). A number of different methods were used in an attempt to isolate plasmids from bacterial samples and a number of difficulties were faced while doing so. Isolations with Method A showed very less or no plasmid DNA and a smear of chromosomal DNA was generated at 50,000-bp as seen in figure. It was alleged that method A needed to be modified in order to yield better quality and more quantity of plasmid. Since *R. equi* bacteria have a thick polysaccharide capsule which is known to be a hindrance when isolating plasmids (Prescott 1991) it was thought that perhaps in Method A the cells were not being lysed therefore, in one of the later steps, CTAB was added to the bacteria as it would dissolve any polysaccharide that may not have dissolved in earlier steps and allow cell lysis. Bacteria used for isolations in Method A were taken from broth thus in order to see if growth media might have been involved in making cells more susceptible to plasmid isolations, plasmids were isolated from bacteria grown on both

broth and agar plates. The modification made to Method A was Method B, which used bacteria grown on agar plates instead of broth and involved the addition of CTAB. Neither the CTAB nor the growth media seemed to improve the plasmid isolations even when repeated a number of times however more chromosomal DNA was generated as seen in figure. The next method tested was Method C, which has been quite frequently used since the 90s, and was followed exactly from a published paper (Ocampo-Sosa *et al*. 2007) but repeated attempts did not yield any positive results. Another method, Method D obtained from a paper by Birnboim and Dolly (Birnboim & Doly 1988) was tested and just as before repeated attempts failed to show plasmid DNA. The SDS and NaOH used in Method C and D were known to break the membrane's phospholipid bilayer and denature proteins involved in maintaining the structure of the cell membrane. It seemed that although the cells were being lysed and DNA was being released, at some point the DNA was getting lost. The last method tested and also the most successful was Method E which was long drawn out which involved using TENs Buffer and the addition of an extra precipitation step earlier on in the isolation procedure where 20% PEG or 100% Isopropanol was used. Comparisons of the two proved 20% PEG to be a better candidate for the extra precipitation step as it successfully showed the possible plasmid DNA band as shown in figure. Repeated attempts of Plasmid isolations with Method E yielded far too much chromosomal DNA and none if little plasmid DNA as shown in figure. It was possible that the chromosomal DNA band was large enough to be able to obscure the presence of any plasmid DNA. λDNA, which is 48,512-bp

in size, was used as a control for indication of chromosomal DNA in the electrophoresis gel.

### 5.2.7 Plasmid Visualisation in electrophoresis gels

After plasmids were isolated, success of the isolation was confirmed by PCR with the VapA or TraA primers to ensure the presence of plasmids. Having had success in VapA/TraA amplification in isolated plasmid samples, it was necessary to visualise plasmids on an electrophoresis gel so that bands could be excised and sequenced for characterisation. Due to the lack of visibility of plasmid bands in electrophoresis gels; buffers, agarose concentrations, voltages and running times were investigated. As the size of the plasmid increases its resolution in an agarose gel electrophoresis decreases (Schleef 2001). Thus to improve the resolution of the plasmid, electrophoresis had to be carried out at a low voltage, for a long time period (approximately 10 h) and a low concentration of agarose (0.7%). UV irradiation can create single strand breaks in supercoiled DNA molecules which results in monomers and dimers (Schleef 2001). The increasing size of plasmid makes resolution between monomers and dimers insufficient thus exposure to UV was minimized. The supercoiled DNA migrates the fastest in the gel due to its tight conformation followed by nicked DNA and then its linear form (Schleef 2001). In Figure 5.1 electrophoresis has been carried out for 6h but for 10 h in Figure 5.2, where it was observed that the supercoiled plasmid is further than the linear or nicked DNA. At higher DNA concentrations the fluorescence intensity can reach a saturation level whereas at lower DNA concentrations no band is detected (Schleef 2001) thus monitoring DNA

concentration is also an important factor in resolution. In conclusion, gel staining of large plasmids generally requires a lot of experience and perseverance (Schleef 2001).

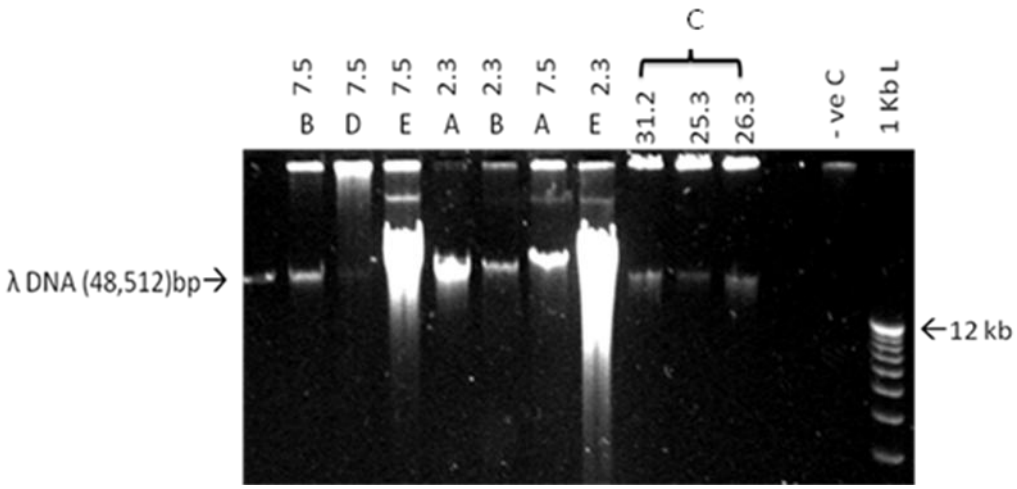



Figure 5.1: Electrophoresis gel at 6 h of running at 40V.

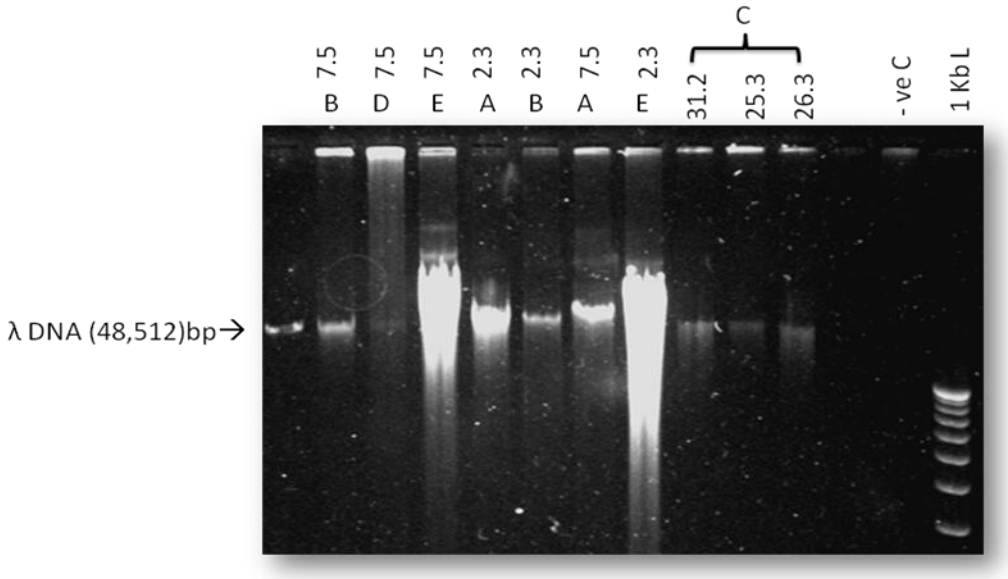



Figure 5.2: Electrophoresis gel at 10 h of running at 40V.

Electrophoresis gel figures at 6 h and 10 h using Methods A, B, C, D and E. The numbers are the isolates used for plasmid isolation.

The successful Method E was repeated again for five other plasmid positive strains but failed to reveal the expected results that were obtained earlier. Since the gels were being electrophoresed for long periods of time, the possibility that EtBr used for staining the DNA was obscuring the results by affecting the visibility of plasmids and changing its topology was considered and the plasmid isolation with method E was repeated but instead of adding EtBr to the gel before electrophoresis it was added to the buffer the gel was soaked in for 20 min after electrophoresis. As observed in the figure below the chromosomal DNA band was less bright which was achieved by reducing the time of EtBr exposure. However, plasmid visibility was not significantly improved and out of the 5 Virulence Positive (VP) isolates tested, three failed to show even a faint presence of the plasmid. SYBR green was used to stain plasmid DNA to see if improved plasmid visualisation since is known to have a sensitivity of higher than 25 times that of EtBr and does not intercalate between DNA bases. SYBR green did not prove to be any more useful than EtBr in allowing better visualisation of plasmid DNA as seen in Figure 5.3.

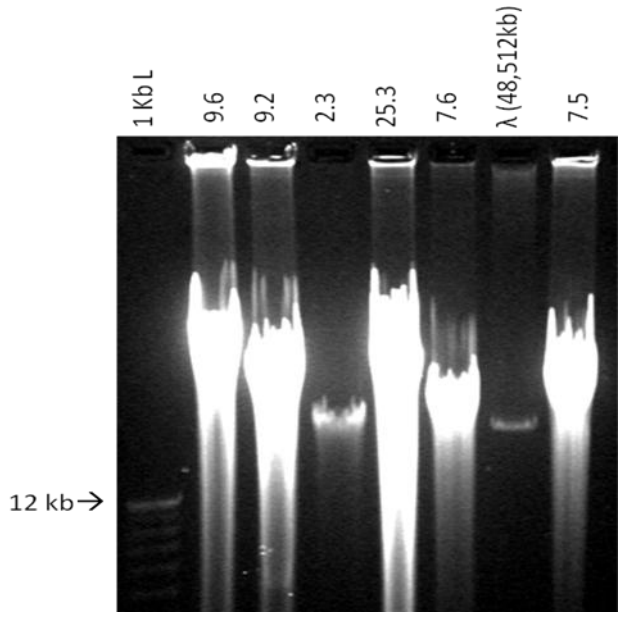



Figure 5.3: Using SYBR green to stain plasmid DNA from 6 isolates, isolated by Method E.

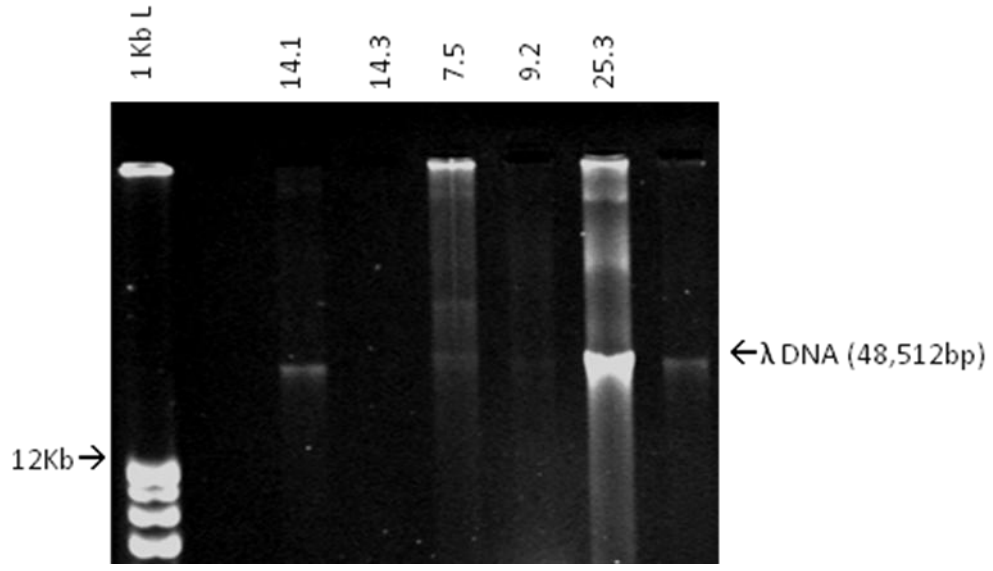



Figure 5.4: Staining five isolated plasmid samples with EtBr after electrophoresis is complete.

### 5.2.8 Restriction Digestion

When the plasmid was cut with restriction enzyme EcoR1 the banding pattern was very faint and the lane in the gel appeared to be smeared as in Figure 5.5, this suggested that the plasmid product was not clean. Thus it would be pointless to use restriction enzyme digests of purified plasmid samples that were of poor quality and yield since all of them would have different levels of purity and that could obscure banding patterns of digests, thereby showing RFLPs in plasmids without there being a true difference between them.

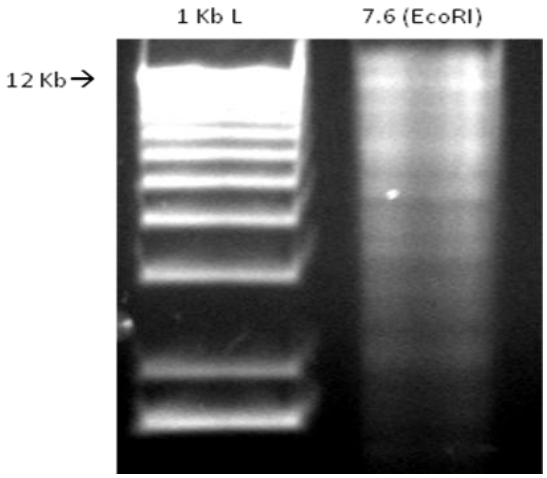



Figure 5.5: Restriction digestion *R. equi* isolate 7.6 with EcoRI after being electrophoresed for 5 h at 50V.

### 5.2.9 Multiple primer Analysis

When using a combination of primers in an extended PCR it was decided to use primers DR REP IR and Tra F since the reaction produced on prominent band

compared to the others. DR REP IR and Tra F together were used to amplify those regions in VP positive isolates to see if any polymorphisms occurred. To find the optimal PCR annealing temperature for using the 2 primers PCR reactions were carried out across temperature gradients. Temperature 53.5 and 43 ºC were chosen based on the multiple banding pattern that appears in Figure 5.6 as it would help recognise polymorphisms in isolates. When the two annealing temperatures were put to the test a vast number of banding patterns were observed as seen in Figure 5.7 with different band intensities as seen in figure suggesting non-specific binding. A better method was required for plasmid characterisation; hence variable regions were selected for RFLP analysis as discussed in the results and discussion of the Research method section.

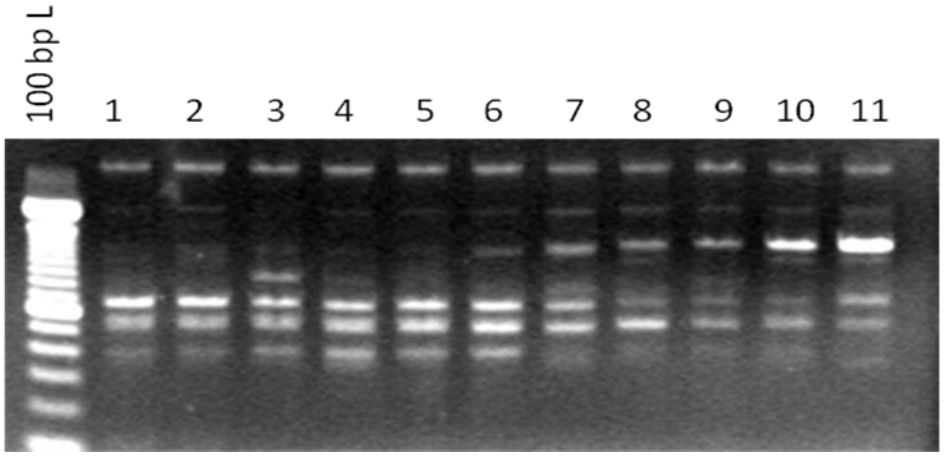



Figure 5.6: Temperature Gradient PCR.

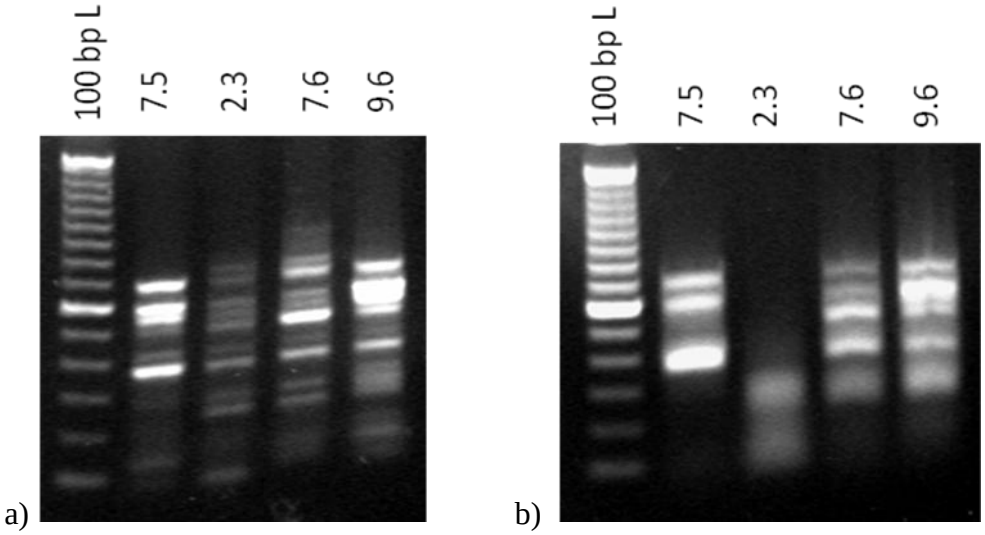



Figure 5.7 PCR amplifications of plasmid isolations from four isolates with primers DR REP IR and TraF. Annealing temperature in a) is 53.5 ºC and annealing temperature in b) is 43 ºC.

## 5.3 Appendix: Gel Photos

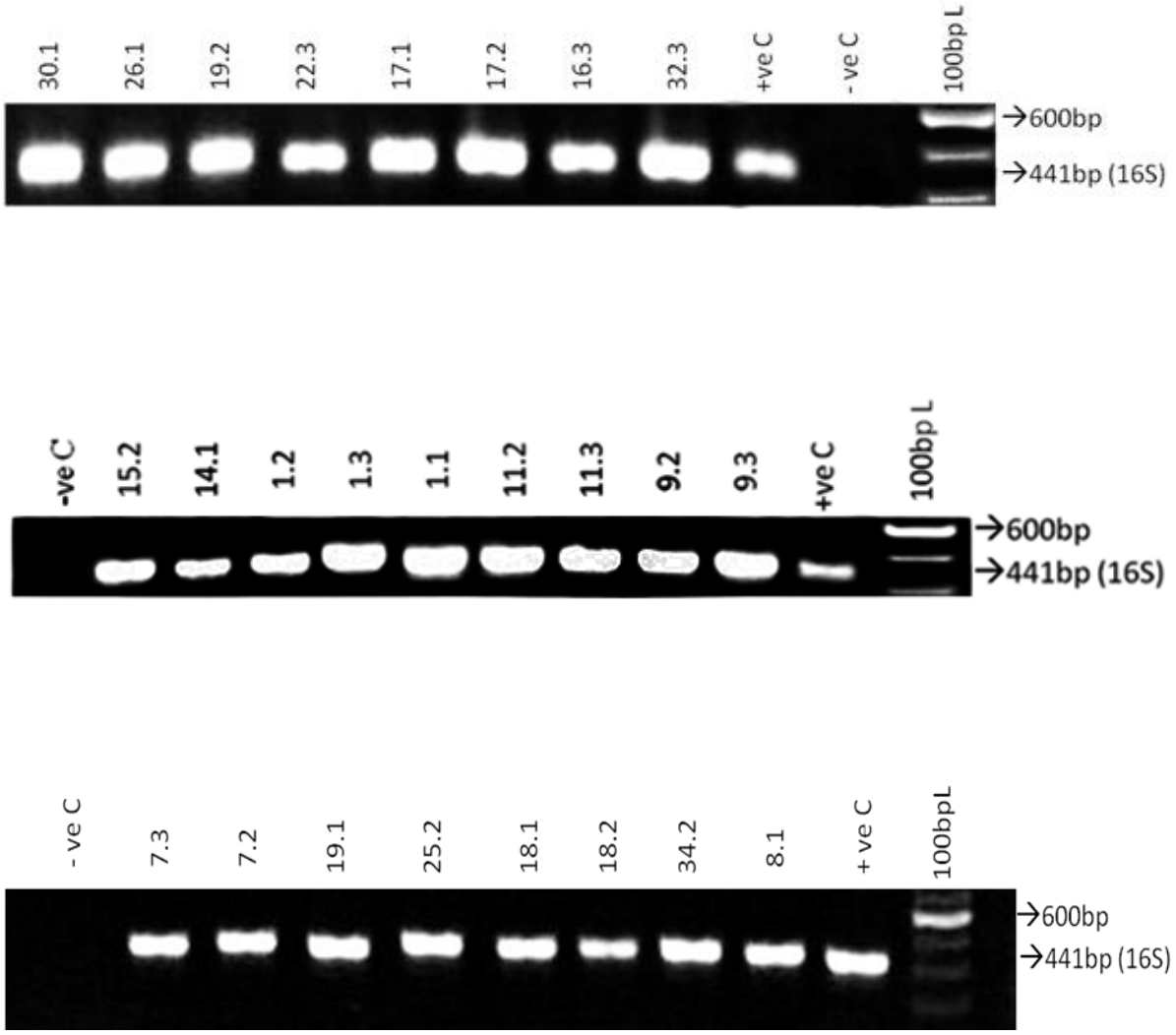
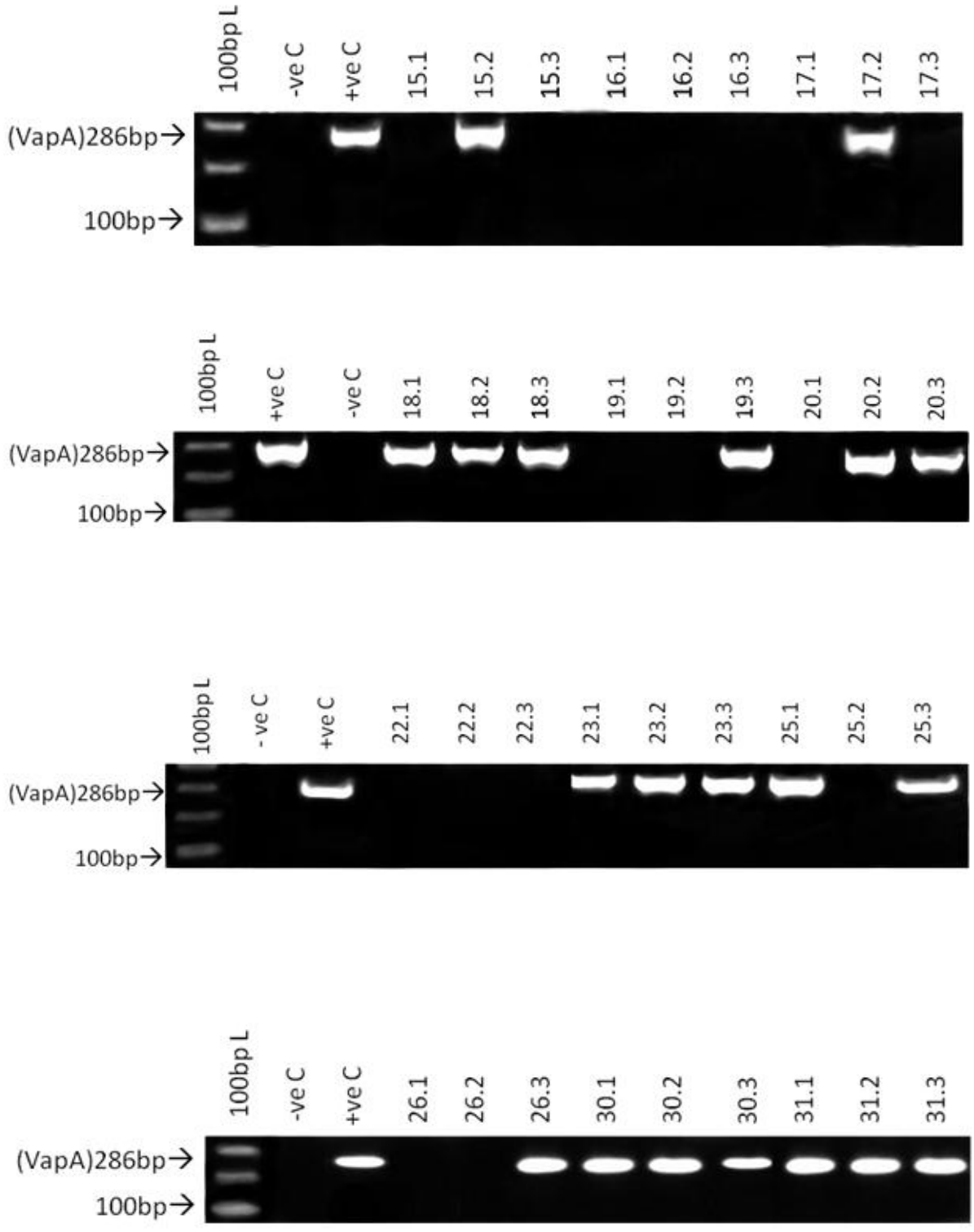



Figure 5.8: 16S confirmation of *R. equi* identification


100bp L
-ve C
+ve C
15.1
15.2
15.3
16.1
16.2
16.3
17.1
17.2
17.3
(VapA)286bp→
100bp→
100bp L
+ve C
-ve C
18.1
18.2
18.3
19.1
19.2
19.3
20.1
20.2
20.3
(VapA)286bp→
100bp→
100bp L
- ve C
+ve C
22.1
22.2
22.3
23.1
23.2
23.3
25.1
25.2
25.3
(VapA)286bp→
100bp→
100bp L
-ve C
+ve C
26.1
26.2
26.3
30.1
30.2
30.3
31.1
31.2
31.3
(VapA)286bp→
100bp→

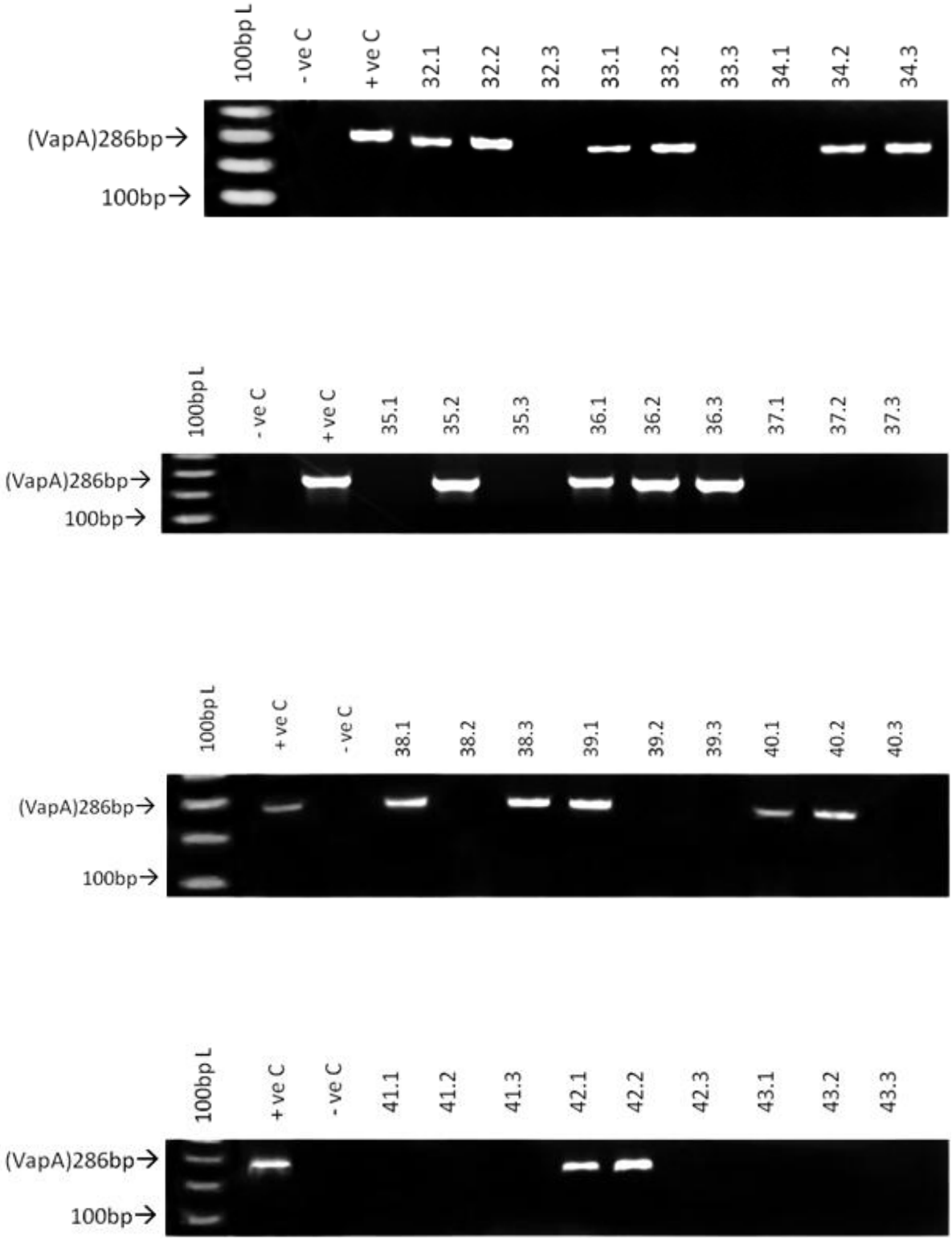
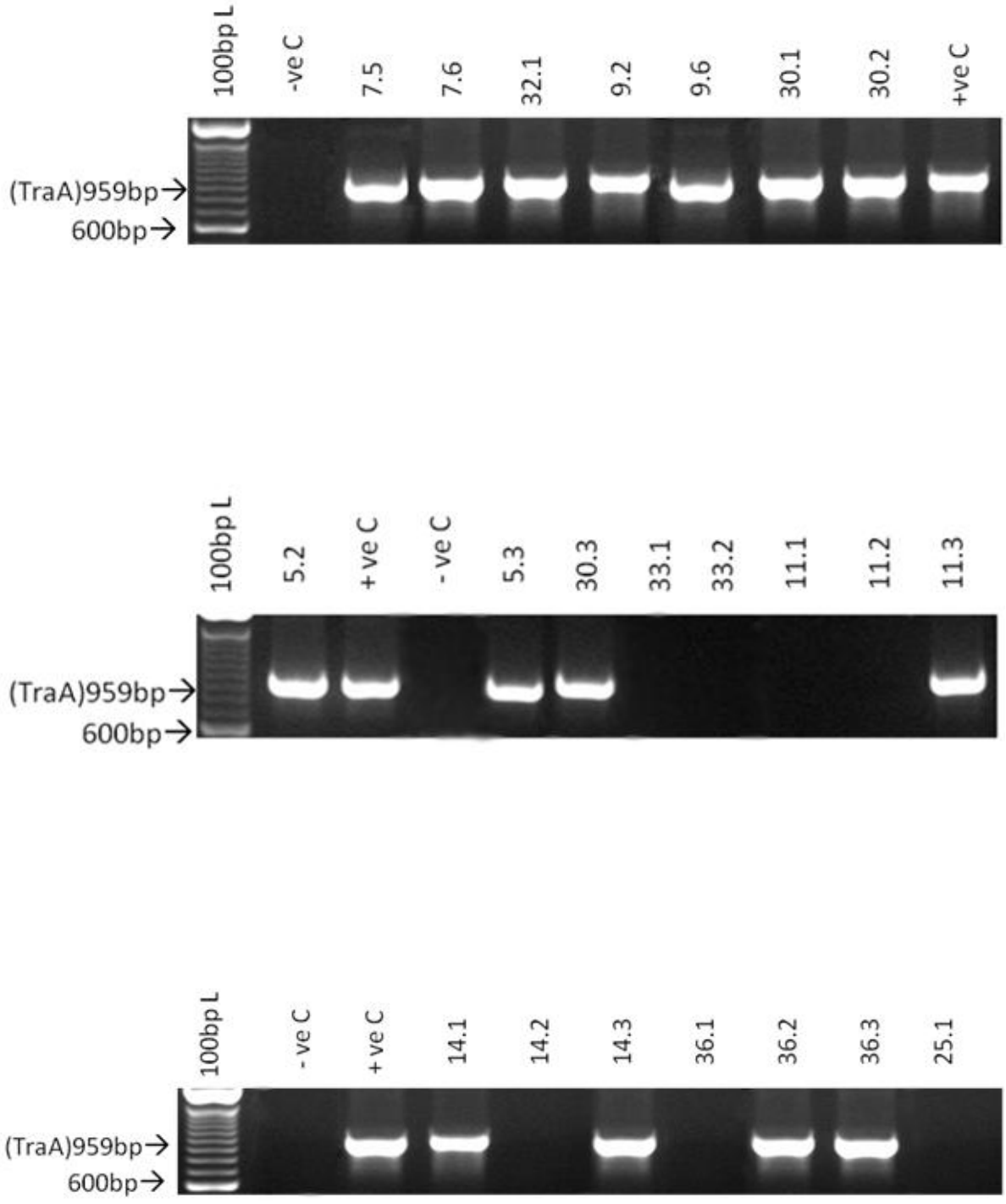



Figure 5.9: VapA analysis of virulence plasmid identification in *R. equi* isolates.


100bp L
-ve C
7.5
7.6
32.1
9.2
9.6
30.1
30.2
+ve C
(TraA)959bp
600bp
100bp L
5.2
+ ve C
- ve C
5.3
30.3
33.1
33.2
11.1
11.2
11.3
(TraA)959bp
600bp
100bp L
- ve C
+ ve C
14.1
14.2
14.3
36.1
36.2
36.3
25.1
(TraA)959bp
600bp

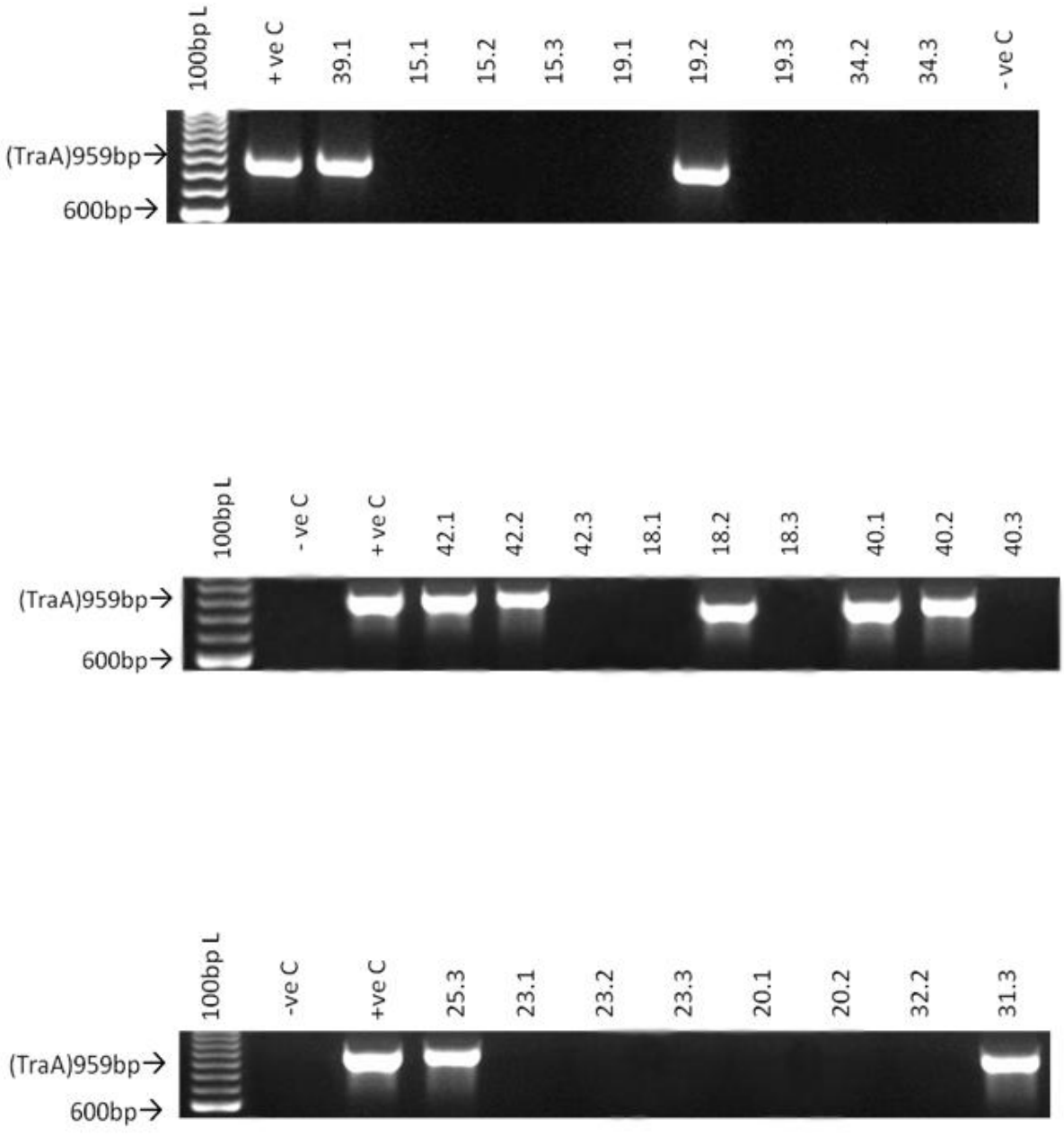



Figure 5.10: TraA confirmation for presence of large plasmids in isolates.

## 5.4 Appendix: BLAST Analysis of PI and PII

PI- 7.5

| Accession | Description | Max score | Total score | Query coverage | E value | Max ident |
|---|---|---|---|---|---|---|
| AM947677.1 | *Rhodococcus* vapA-type virulence plasmid (pVAPA1037), strain 103S | 1620 | 1620 | 100% | 0 | 100% |
| AP001204.1 | *Rhodococcus* plasmid pREAT701 DNA, complete sequence | 1620 | 1620 | 100% | 0 | 100% |
| AF116907.2 | *Rhodococcus* virulence plasmid, complete sequence | 1620 | 1620 | 100% | 0 | 100% |
| AM947676.1 | *Rhodococcus* vapB-type virulence plasmid (pVAPB1593), strain PAM1593 | 252 | 252 | 24% | 5.00E-68 | 88% |

PI-9.6

| Accession | Description | Max score | Total score | Query coverage | E value | Max ident |
|---|---|---|---|---|---|---|
| AM947677.1 | *Rhodococcus* vapA-type virulence plasmid (pVAPA1037), strain 103S | 1602 | 1602 | 100% | 0 | 100% |
| AP001204.1 | *Rhodococcus* plasmid pREAT701 DNA, complete sequence | 1602 | 1602 | 100% | 0 | 100% |
| AF116907.2 | *Rhodococcus* virulence plasmid, complete sequence | 1602 | 1602 | 100% | 0 | 100% |
| AM947676.1 | *Rhodococcus* vapB-type virulence plasmid (pVAPB1593), strain PAM1593 | 252 | 252 | 24% | 4.00E-68 | 88% |

PI-14.3

| Accession | Description | Max score | Total score | Query coverage | E value | Max ident |
|---|---|---|---|---|---|---|
| AM947677.1 | *Rhodococcus* vapA-type virulence plasmid (pVAPA1037), strain 103S | 1430 | 1430 | 100% | 0 | 99% |
| AP001204.1 | *Rhodococcus* plasmid pREAT701 DNA, complete sequence | 1430 | 1430 | 100% | 0 | 99% |
| AF116907.2 | *Rhodococcus* virulence plasmid, complete sequence | 1430 | 1430 | 100% | 0 | 99% |
| AM947676.1 | *Rhodococcus* vapB-type virulence plasmid (pVAPB1593), strain PAM1593 | 252 | 252 | 26% | 4.00E-68 | 88% |

PI-32.1

| Accession | Description | Max score | Total score | Query coverage | E value | Max ident |
|---|---|---|---|---|---|---|
| AM947677.1 | *Rhodococcus* vapA-type virulence plasmid (pVAPA1037), strain 103S | 1391 | 1391 | 100% | 0 | 99% |
| AP001204.1 | *Rhodococcus* plasmid pREAT701 DNA, complete sequence | 1391 | 1391 | 100% | 0 | 99% |
| AF116907.2 | *Rhodococcus* virulence plasmid, complete sequence | 1391 | 1391 | 100% | 0 | 99% |
| AM947676.1 | *Rhodococcus* vapB-type virulence plasmid (pVAPB1593), strain PAM1593 | 252 | 252 | 27% | 4.00E-68 | 88% |

PI-30.1

| **Accession** | **Description** | Max score | Total score | Query coverage | E value | Max ident |
|---|---|---|---|---|---|---|
| AM947677.1 | *Rhodococcus* vapA-type virulence plasmid (pVAPA1037), strain 103S | 1526 | 1526 | 100% | 0 | 100% |
| AP001204.1 | *Rhodococcus* plasmid pREAT701 DNA, complete sequence | 1526 | 1526 | 100% | 0 | 100% |
| AF116907.2 | *Rhodococcus* virulence plasmid, complete sequence | 1526 | 1526 | 100% | 0 | 100% |
| AM947676.1 | *Rhodococcus* vapB-type virulence plasmid (pVAPB1593), strain PAM1593 | 252 | 252 | 25% | 4.00E-68 | 88% |

PI-7.6

| **Accession** | **Description** | Max score | Total score | Query coverage | E value | Max ident |
|---|---|---|---|---|---|---|
| AM947677.1 | *Rhodococcus* vapA-type virulence plasmid (pVAPA1037), strain 103S | 1441 | 1441 | 100% | 0 | 99% |
| AP001204.1 | *Rhodococcus* plasmid pREAT701 DNA, complete sequence | 1441 | 1441 | 100% | 0 | 99% |
| AF116907.2 | *Rhodococcus* virulence plasmid, complete sequence | 1441 | 1441 | 100% | 0 | 99% |
| AM947676.1 | *Rhodococcus* vapB-type virulence plasmid (pVAPB1593), strain PAM1593 | 252 | 252 | 26% | 4.00E-68 | 88% |

PI-9.2

| **Accession** | **Description** | Max score | Total score | Query coverage | E value | Max ident |
|---|---|---|---|---|---|---|
| AM947677.1 | *Rhodococcus* vapA-type virulence plasmid (pVAPA1037), strain 103S | 1437 | 1437 | 100% | 0 | 99% |
| AP001204.1 | *Rhodococcus* plasmid pREAT701 DNA, complete sequence | 1437 | 1437 | 100% | 0 | 99% |
| AF116907.2 | *Rhodococcus* virulence plasmid, complete sequence | 1437 | 1437 | 100% | 0 | 99% |
| AM947676.1 | *Rhodococcus* vapB-type virulence plasmid (pVAPB1593), strain PAM1593 | 252 | 252 | 26% | 4.00E-68 | 88% |

PI-30.3

| **Accession** | **Description** | Max score | Total score | Query coverage | E value | Max ident |
|---|---|---|---|---|---|---|
| AM947677.1 | *Rhodococcus* vapA-type virulence plasmid (pVAPA1037), strain 103S | 1358 | 1358 | 100% | 0 | 99% |
| AP001204.1 | *Rhodococcus* plasmid pREAT701 DNA, complete sequence | 1358 | 1358 | 100% | 0 | 99% |
| AF116907.2 | *Rhodococcus* virulence plasmid, complete sequence | 1358 | 1358 | 100% | 0 | 99% |
| AM947676.1 | *Rhodococcus* vapB-type virulence plasmid (pVAPB1593), strain PAM1593 | 252 | 252 | 28% | 4.00E-68 | 88% |

PI-30.2

| **Accession** | **Description** | Max score | Total score | Query coverage | E value | Max ident |
|---|---|---|---|---|---|---|
| AM947677.1 | *Rhodococcus* vapA-type virulence plasmid (pVAPA1037), strain 103S | 1325 | 1325 | 100% | 0 | 98% |
| AP001204.1 | *Rhodococcus* plasmid pREAT701 DNA, complete sequence | 1325 | 1325 | 100% | 0 | 98% |
| AF116907.2 | *Rhodococcus* virulence plasmid, complete sequence | 1325 | 1325 | 100% | 0 | 98% |
| AM947676.1 | *Rhodococcus* vapB-type virulence plasmid (pVAPB1593), strain PAM1593 | 252 | 252 | 28% | 4.00E-68 | 88% |

PI-18.2

| **Accession** | **Description** | Max score | Total score | Query coverage | E value | Max ident |
|---|---|---|---|---|---|---|
| AM947677.1 | *Rhodococcus* vapA-type virulence plasmid (pVAPA1037), strain 103S | 1312 | 1312 | 100% | 0 | 99% |
| AP001204.1 | *Rhodococcus* plasmid pREAT701 DNA, complete sequence | 1312 | 1312 | 100% | 0 | 99% |
| AF116907.2 | *Rhodococcus* virulence plasmid, complete sequence | 1312 | 1312 | 100% | 0 | 99% |
| AM947676.1 | *Rhodococcus* vapB-type virulence plasmid (pVAPB1593), strain PAM1593 | 252 | 252 | 29% | 4.00E-68 | 88% |

PI-25.3

| **Accession** | **Description** | Max score | Total score | Query coverage | E value | Max ident |
|---|---|---|---|---|---|---|
| AM947677.1 | *Rhodococcus* vapA-type virulence plasmid (pVAPA1037), strain 103S | 1336 | 1336 | 100% | 0 | 98% |
| AP001204.1 | *Rhodococcus* plasmid pREAT701 DNA, complete sequence | 1336 | 1336 | 100% | 0 | 98% |
| AF116907.2 | *Rhodococcus* virulence plasmid, complete sequence | 1336 | 1336 | 100% | 0 | 98% |
| AM947676.1 | *Rhodococcus* vapB-type virulence plasmid (pVAPB1593), strain PAM1593 | 252 | 252 | 27% | 4.00E-68 | 88% |

PI-39.1

| **Accession** | **Description** | Max score | Total score | Query coverage | E value | Max ident |
|---|---|---|---|---|---|---|
| AM947677.1 | *Rhodococcus* vapA-type virulence plasmid (pVAPA1037), strain 103S | 1526 | 1526 | 100% | 0 | 100% |
| AP001204.1 | *Rhodococcus* plasmid pREAT701 DNA, complete sequence | 1526 | 1526 | 100% | 0 | 100% |
| AF116907.2 | *Rhodococcus* virulence plasmid, complete sequence | 1526 | 1526 | 100% | 0 | 100% |
| AM947676.1 | *Rhodococcus* vapB-type virulence plasmid (pVAPB1593), strain PAM1593 | 252 | 252 | 25% | 4.00E-68 | 88% |

PI-14.1

| Accession | Description | Max score | Total score | Query coverage | E value | Max ident |
|---|---|---|---|---|---|---|
| AM947677.1 | *Rhodococcus* vapA-type virulence plasmid (pVAPA1037), strain 103S | 1430 | 1430 | 100% | 0 | 99% |
| AP001204.1 | *Rhodococcus* plasmid pREAT701 DNA, complete sequence | 1430 | 1430 | 100% | 0 | 99% |
| AF116907.2 | *Rhodococcus* virulence plasmid, complete sequence | 1430 | 1430 | 100% | 0 | 99% |
| AM947676.1 | *Rhodococcus* vapB-type virulence plasmid (pVAPB1593), strain PAM1593 | 252 | 252 | 26% | 4.00E-68 | 88% |

PII-36.3

| **Accession** | **Description** | Max score | Total score | Query coverage | E value | Max ident |
|---|---|---|---|---|---|---|
| AM947676.1 | *Rhodococcus* vapB-type virulence plasmid (pVAPB1593), strain PAM1593 | 394 | 394 | 30% | 8.00E-111 | 88% |
| AM947677.1 | *Rhodococcus* vapA-type virulence plasmid (pVAPA1037), strain 103S | 224 | 503 | 31% | 1.00E-59 | 98% |
| AP001204.1 | *Rhodococcus* plasmid pREAT701 DNA, complete sequence | 224 | 503 | 31% | 1.00E-59 | 98% |
| AF116907.2 | *Rhodococcus* virulence plasmid, complete sequence | 224 | 503 | 31% | 1.00E-59 | 98% |

PII-31.3

| **Accession** | **Description** | Max score | Total score | Query coverage | E value | Max ident |
|---|---|---|---|---|---|---|
| AM947676.1 | *Rhodococcus* vapB-type virulence plasmid (pVAPB1593), strain PAM1593 | 394 | 394 | 32% | 8.00E-111 | 88% |
| AM947677.1 | *Rhodococcus* vapA-type virulence plasmid (pVAPA1037), strain 103S | 224 | 470 | 31% | 1.00E-59 | 98% |
| AP001204.1 | *Rhodococcus* plasmid pREAT701 DNA, complete sequence | 224 | 470 | 31% | 1.00E-59 | 98% |
| AF116907.2 | *Rhodococcus* virulence plasmid, complete sequence | 224 | 470 | 31% | 1.00E-59 | 98% |

PII-19.2

| **Accession** | **Description** | Max score | Total score | Query coverage | E value | Max ident |
|---|---|---|---|---|---|---|
| AM947676.1 | *Rhodococcus* vapB-type virulence plasmid (pVAPB1593), strain PAM1593 | 394 | 394 | 34% | 7.00E-111 | 88% |
| AM947677.1 | *Rhodococcus* vapA-type virulence plasmid (pVAPA1037), strain 103S | 224 | 400 | 29% | 1.00E-59 | 98% |
| AP001204.1 | *Rhodococcus* plasmid pREAT701 DNA, complete sequence | 224 | 400 | 29% | 1.00E-59 | 98% |
| AF116907.2 | *Rhodococcus* virulence plasmid, complete sequence | 224 | 400 | 29% | 1.00E-59 | 98% |

PII-36.2

| **Accession** | **Description** | Max score | Total score | Query coverage | E value | Max ident |
|---|---|---|---|---|---|---|
| AM947676.1 | *Rhodococcus* vapB-type virulence plasmid (pVAPB1593), strain PAM1593 | 394 | 394 | 32% | 8.00E-111 | 88% |
| AM947677.1 | *Rhodococcus* vapA-type virulence plasmid (pVAPA1037), strain 103S | 224 | 442 | 30% | 1.00E-59 | 98% |
| AP001204.1 | *Rhodococcus* plasmid pREAT701 DNA, complete sequence | 224 | 442 | 30% | 1.00E-59 | 98% |
| AF116907.2 | *Rhodococcus* virulence plasmid, complete sequence | 224 | 442 | 30% | 1.00E-59 | 98% |

PII-40.1

| **Accession** | **Description** | Max score | Total score | Query coverage | E value | Max ident |
|---|---|---|---|---|---|---|
| AM947676.1 | *Rhodococcus* vapB-type virulence plasmid (pVAPB1593), strain PAM1593 | 394 | 394 | 31% | 8.00E-111 | 88% |
| AM947677.1 | *Rhodococcus* vapA-type virulence plasmid (pVAPA1037), strain 103S | 224 | 466 | 31% | 1.00E-59 | 98% |
| AP001204.1 | *Rhodococcus* plasmid pREAT701 DNA, complete sequence | 224 | 466 | 31% | 1.00E-59 | 98% |
| AF116907.2 | *Rhodococcus* virulence plasmid, complete sequence | 224 | 466 | 31% | 1.00E-59 | 98% |

PII-42.1

| **Accession** | **Description** | Max score | Total score | Query coverage | E value | Max ident |
|---|---|---|---|---|---|---|
| AM947676.1 | *Rhodococcus* vapB-type virulence plasmid (pVAPB1593), strain PAM1593 | 390 | 390 | 35% | 9.00E-110 | 90% |
| AM947677.1 | *Rhodococcus* vapA-type virulence plasmid (pVAPA1037), strain 103S | 207 | 465 | 37% | 1.00E-54 | 100% |
| AP001204.1 | *Rhodococcus* plasmid pREAT701 DNA, complete sequence | 207 | 465 | 37% | 1.00E-54 | 100% |
| AF116907.2 | *Rhodococcus* virulence plasmid, complete sequence | 207 | 465 | 37% | 1.00E-54 | 100% |

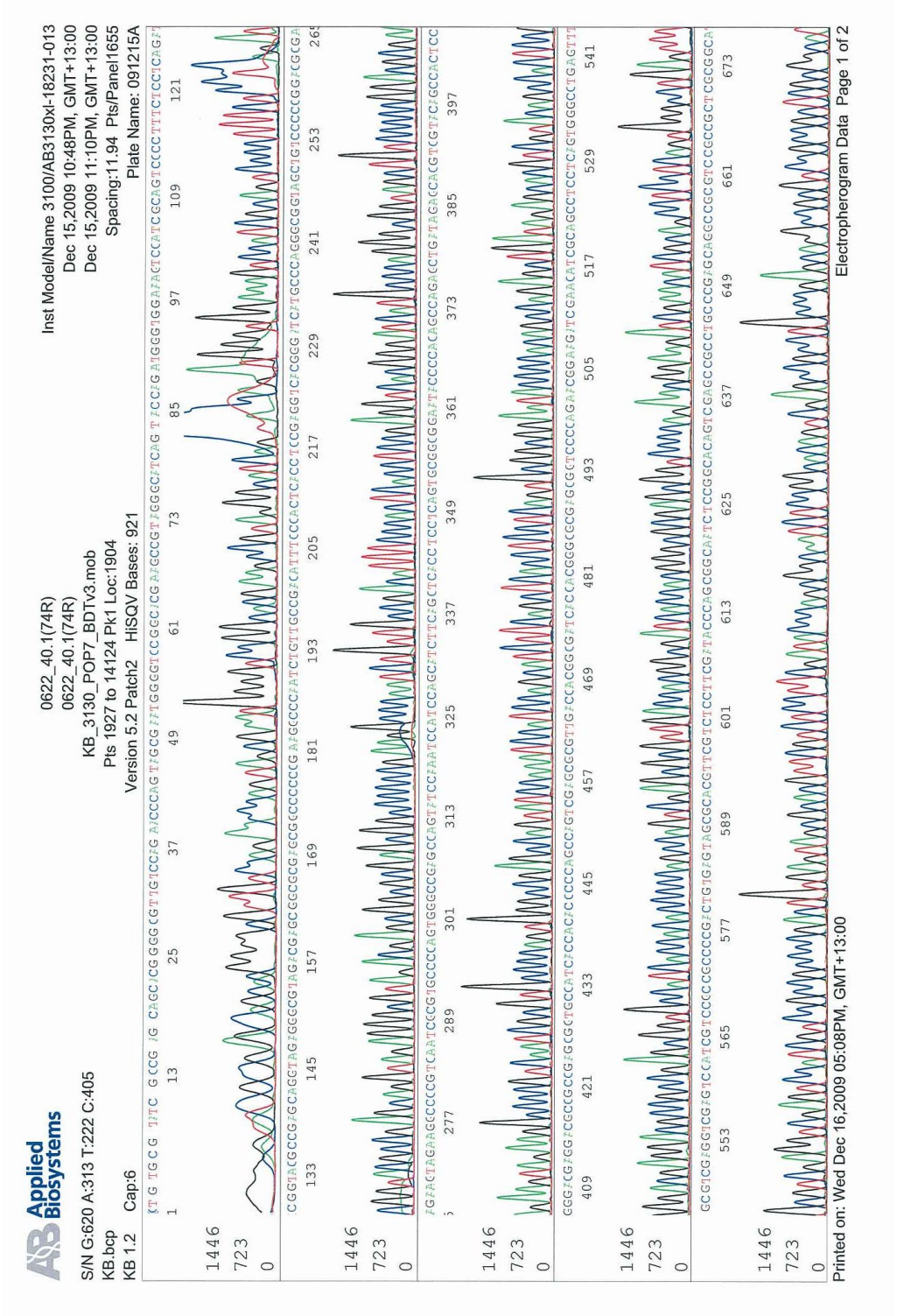
PII-40.2

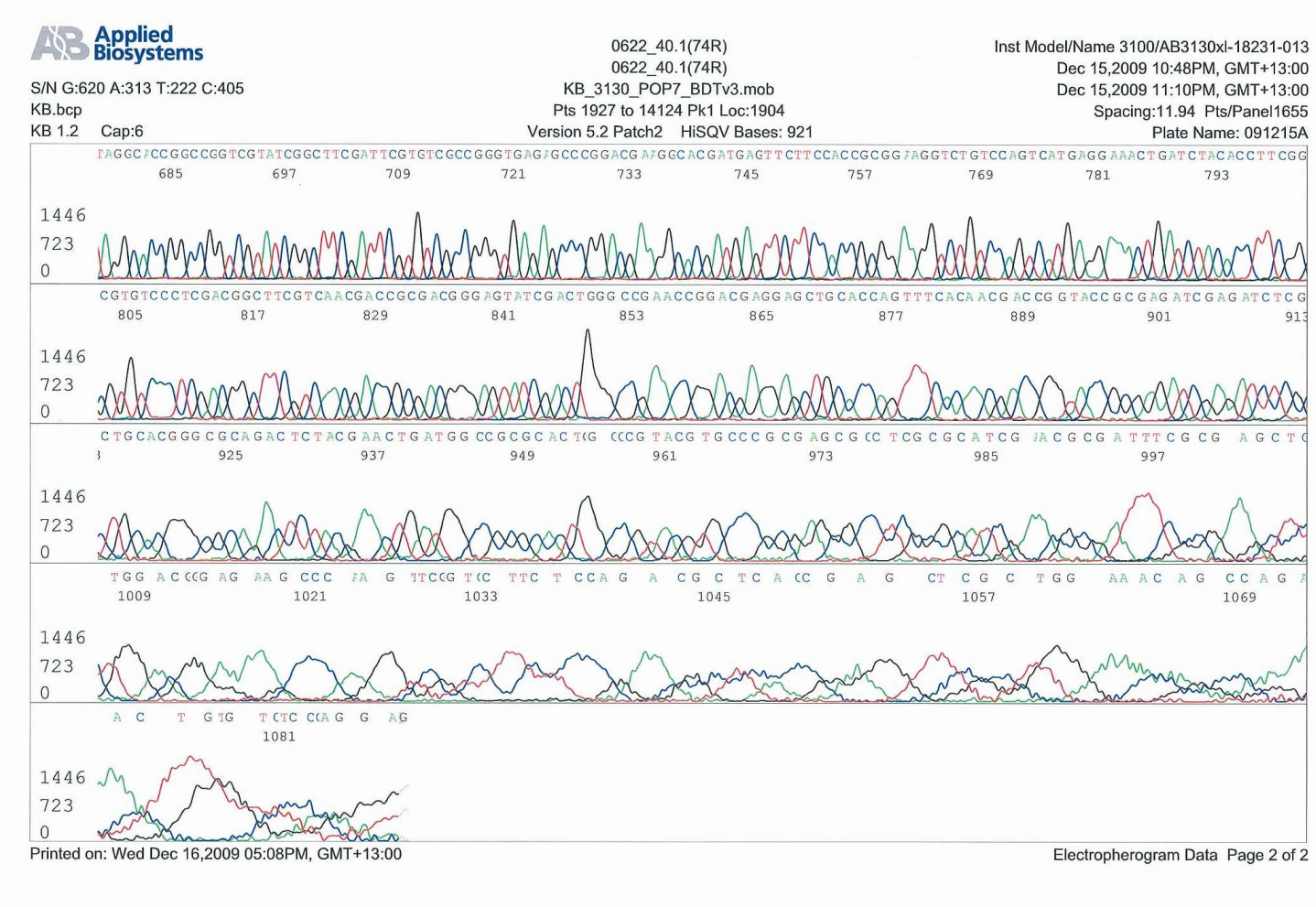
| **Accession** | **Description** | Max score | Total score | Query coverage | E value | Max ident |
|---|---|---|---|---|---|---|
| AM947676.1 | *Rhodococcus* vapB-type virulence plasmid (pVAPB1593), strain PAM1593 | 394 | 394 | 37% | 7.00E-111 | 88% |
| AM947677.1 | *Rhodococcus* vapA-type virulence plasmid (pVAPA1037), strain 103S | 224 | 398 | 31% | 9.00E-60 | 98% |
| AP001204.1 | *Rhodococcus* plasmid pREAT701 DNA, complete sequence | 224 | 398 | 31% | 9.00E-60 | 98% |
| AF116907.2 | *Rhodococcus* virulence plasmid, complete sequence | 224 | 398 | 31% | 9.00E-60 | 98% |

PII-42.2

| **Accession** | **Description** | Max score | Total score | Query coverage | E value | Max ident |
|---|---|---|---|---|---|---|
| AM947676.1 | *Rhodococcus* vapB-type virulence plasmid (pVAPB1593), strain PAM1593 | 394 | 394 | 36% | 7.00E-111 | 88% |
| AM947677.1 | *Rhodococcus* vapA-type virulence plasmid (pVAPA1037), strain 103S | 224 | 494 | 37% | 1.00E-59 | 98% |
| AP001204.1 | *Rhodococcus* plasmid pREAT701 DNA, complete sequence | 224 | 494 | 37% | 1.00E-59 | 98% |
| AF116907.2 | *Rhodococcus* virulence plasmid, complete sequence | 224 | 494 | 37% | 1.00E-59 | 98% |

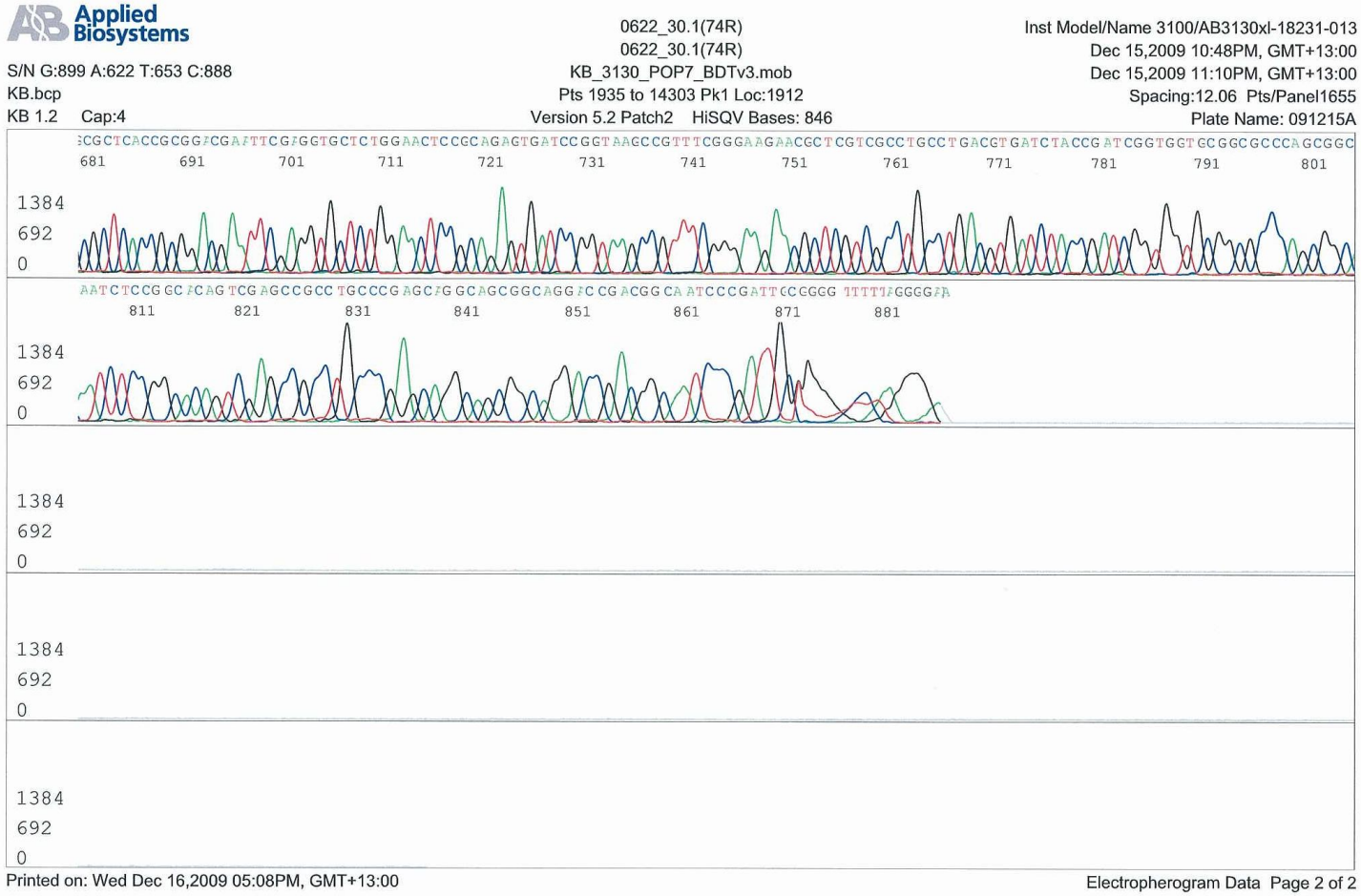
## 5.5 Appendix: Electropherograms: PII-40.1




Applied Biosystems
S/N G:620 A:313 T:222 C:405
KB.bcp
KB 1.2 Cap:6
0622_40.1(74R)
0622_40.1(74R)
KB_3130_POP7_BDTv3.mob
Pts 1927 to 14124 Pk1 Loc:1904
Version 5.2 Patch2 HiSQV Bases: 921
Inst Model/Name 3100/AB3130xl-18231-013
Dec 15,2009 10:48PM, GMT+13:00
Dec 15,2009 11:10PM, GMT+13:00
Spacing:11.94 Pts/Panel1655
Plate Name: 091215A
Printed on: Wed Dec 16,2009 05:08PM, GMT+13:00
Electropherogram Data Page 2 of 2

PI-30.1


Applied Biosystems
0622_30.1(74R)
0622_30.1(74R)
KB_3130_POP7_BDTv3.mob
Pts 1935 to 14303 Pk1 Loc:1912
Version 5.2 Patch2 HiSQV Bases: 846
Inst Model/Name 3100/AB3130xl-18231-013
Dec 15,2009 10:48PM, GMT+13:00
Dec 15,2009 11:10PM, GMT+13:00
Spacing:12.06 Pts/Panel1655
Plate Name: 091215A
S/N G:899 A:622 T:653 C:888
KB.bcp
KB 1.2 Cap:4
1384
692
0
Printed on: Wed Dec 16,2009 05:08PM, GMT+13:00
Electropherogram Data Page 1 of 2


Applied Biosystems
S/N G:899 A:622 T:653 C:888
KB.bcp
KB 1.2 Cap:4
0622_30.1(74R)
0622_30.1(74R)
KB_3130_POP7_BDTv3.mob
Pts 1935 to 14303 Pk1 Loc:1912
Version 5.2 Patch2 HiSQV Bases: 846
Inst Model/Name 3100/AB3130xl-18231-013
Dec 15,2009 10:48PM, GMT+13:00
Dec 15,2009 11:10PM, GMT+13:00
Spacing:12.06 Pts/Panel1655
Plate Name: 091215A
681 691 701 711 721 731 741 751 761 771 781 791 801
811 821 831 841 851 861 871 881
1384
692
0
Printed on: Wed Dec 16,2009 05:08PM, GMT+13:00
Electropherogram Data Page 2 of 2