Effects of farm management on ecology of virulent *Rhodococcus equi*

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Abstract

Environmental samples (air and soil) were collected from Thoroughbred breeding farms with different prevalences of *R. equi* pneumonia to increase our understanding of the ecology of virulent *R. equi* on horse farms. The airborne population of virulent *R. equi* was a major focus of this research, as inhalation of the pathogen from the environment is considered the primary route of pulmonary infection. Air sampling was performed using an air monitoring system with selective media to facilitate the recovery of *R. equi*, allowing quantitative measurement of airborne virulent *R. equi*. Polymerase chain reaction and DNA hybridisation techniques were used to evaluate environmental samples to identify and differentiate *R. equi*.

Associations were observed between the concentration of airborne virulent *R. equi*, the proportion of airborne *R. equi* that were virulent and the prevalence of disease due to *R. equi* on farms. No association was observed between the concentration of virulent *R. equi* in soil, the proportion of *R. equi* in soil that were virulent and the prevalence of disease due to *R. equi*. Environmental samples were taken at a variety of locations and at regular intervals to identify high risk areas. Elevated concentrations of virulent *R. equi* were detected in the middle and later periods of the breeding season, yet cases of *R. equi* pneumonia fell late in the season, illustrating the age specificity associated with the disease. The holding pens and lanes were associated with elevated airborne concentrations of virulent *R. equi* and these areas were typically dry and barren. Lower soil moisture and lower pasture heights were significantly associated with elevated airborne concentrations of virulent *R. equi*. Few environmental variables were associated with elevated concentrations of virulent *R. equi* in soil, although the data suggested that acidic soil conditions may lead to an elevated proportion of virulent *R. equi* in soil.

The management characteristics of farms were compared with disease prevalence and severity, using questionnaires completed by managers and veterinarians. An association between high disease prevalence and mortality was found, along with an association between low foal numbers and low disease prevalence. There was no

conclusive evidence that immunisation, parasite control or other health management practices in mares or foals reduced the prevalence or severity of *R. equi* pneumonia.

Isolation of virulent *R. equi* from the breath of clinically affected and healthy foals demonstrated for the first time the possibility for foal-to-foal aerosol transmission. The concentration of virulent *R. equi* in exhaled air was significantly greater than that seen in environmental air. These results implicate breath from infected foals as a source of spread of the pathogen within the herd.

The ecological links established between factors influencing air contamination and the epidemiology of the disease may be used to implement effective environmental and foal management strategies to reduce the level of virulent *R. equi* to which susceptible foals are exposed, thus reducing the prevalence and severity of *R. equi* pneumonia in the horse breeding industry.

Declaration

This is to certify that

I. this thesis comprises only my original work towards the PhD except where indicated in the preface,
II. due acknowledgement has been made in the text to all other material used,
III. this thesis is less than 100,000 words in length, exclusive of tables, maps, bibliographies and appendices.

Gary Muscatello

Preface

Serological analysis for exposure to virulent *R. equi* (ELISA) in 10 foals from which exhaled air samples were taken were carried out by Tongted Phumoonna at the the Institute of Medical and Veterinary Science Infectious Disease Laboratories under the supervision of Dr. Michael W. Heuzenroeder and Prof. Mary D. Barton of the Institute of Medical and Veterinary Science Infectious Disease Laboratories.

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Parts of this work were presented at the following conference presentations:

'Air sampling for rattles'

G. Muscatello, S. Church, K.G. Whithear and G.F. Browning, presented at the International Horse Industry Symposium. 4-6 July 2001, Sydney, Australia.

'Virulent *Rhodococcus equi* on horse farms: sources and spread of rattles'

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'Ecology of virulent *Rhodococcus equi* on Thoroughbred studs'

G. Muscatello, S. Church, K.G. Whithear, J.R. Gilkerson and G.F. Browning, presented at the 24th Bain-Fallon Memorial Lectures. 23-27 July 2002, Gold Coast, Qld, Australia.

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‘The ecology and epidemiology of virulent *Rhodococcus equi*’

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‘*Rhodococcus equi* pneumonia: environmental and management factors which influence disease prevalence’

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Abbreviations

The following abbreviations are used throughout this thesis:

AGID	agar gel immunodiffusion
°C	degrees Celsius
cfu	colony forming units
CR	count ratio
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphates
ELISA	enzyme-linked immunosorbent assay
EHV	equine herpesvirus
Ha	hectare
H_2O	water
IgG	immunoglobulin G
IV	intravenous
kb	kilobase pairs
kDa	kiloDalton
km/h	kilometres per hour
l	litre
mg	milligram
NSW	New South Wales

PBS	phosphate buffered saline
PCR	polymerase chain reaction
rpm	revolutions per minute
rrnA	16s rRNA gene
R_s	Spearman's correlation coefficient
SBA	sheep blood agar
SDS	sodium dodecyl sulphate
Vap	virulence associated protein
VFA	volatile fatty acid
Vic	Victoria

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Chapter 1

Review of literature

1.1. Introduction

Since its discovery by Magnusson (1923) *Rhodococcus equi* has been recognised as a significant pulmonary pathogen of foals worldwide. The primary disease manifestation of *R. equi* infection is severe pyogranulomatous pneumonia, but it is also a cause of lymphadenitis in pigs and ulcerative enteritis in foals (Takai *et al.* 1996a; Giguere and Prescott 1997). *R. equi* pneumonia has been referred to as 'rattles' in reference to the audible lung sounds in foals suffering from this disease. In humans, *R. equi* is an increasingly significant cause of disease in patients with acquired immune deficiency syndrome (AIDS) caused by infection with human immunodeficiency virus (HIV), with case fatality rates in these immunocompromised patients exceeding 50% (Kedlaya *et al.* 2001).

R. equi pneumonia is one of the most significant infectious diseases affecting the Australian Thoroughbred industry and is ranked among the four most important disease problems of the horse industry (Pilkington and Wilson 1993). However, the control of disease due to *R. equi* on Thoroughbred farms has to date been based largely on anecdotal evidence.

1.2. General characteristics

1.2.1. Genus *Rhodococcus*

The genus *Rhodococcus* belongs to the family *Actinomycetales*, which also includes the genera *Corynebacterium*, *Mycobacterium* and *Nocardia*. Rhodococci are widely distributed in the environment and have been isolated from soil, fresh water and

marine habitats, as well as from activated sludge foam, the gut contents of blood-sucking arthropods and herbivore dung (Goodfellow *et al.* 1998).

Rhodococci have diverse and unusual enzymatic properties, with considerable potential for biotechnological exploitation. The ability to degrade hydrocarbons is widespread in the genus *Rhodococcus* and strains of the species *R. erythropolis* and *R. globerulus* can degrade various chloroaromatic, nitroaromatic and polychlorobiphenyls (Warhurst and Fewson 1994). These enzymatic properties may be exploited to clean up organic pollutants and treat contaminated soil and effluent. Other rhodococci, including strains of *R. ruber*, *R. rhodochrous* and *R. opacus*, can utilize gaseous hydrocarbons as their sole carbon source. Petroleum companies may be able to exploit this capability to identify and tap subterranean petroleum deposits (Ivshina *et al.* 1982; Ashraf *et al.* 1994; Rosner *et al.* 1997).

1.2.2. Taxon *Rhodococcus equi*

Phylogenically, *R. equi* is located at the periphery of the *Rhodococcus* genus (Goodfellow *et al.* 1998). *R. equi* seems to be more closely related to some *Corynebacterium, Nocardia* and *Mycobacterium* species than to other members of its own genus and shares many phenotypic and genetic similarities with members of these genera. This phenotypic similarity has resulted in a number of nomenclature changes for this organism since its discovery. In 1977 Goodfellow and Alderson cast doubt on the correctness of the taxon *Corynebacterium equi* and suggested that the organism would be best classified in the genus *Rhodococcus,* where it has remained to the present day. Its relative isolation from the classical *Rhodococcus* species and its relationship with the genera *Nocardia* and *Mycobacterium* is recognised in its description as a nocardioform actinomycete (Barton *et al.* 1989). The potential of *R. equi* as a pathogen of animals and humans is also anomalous within the genus *Rhodococcus* and is more a trait of species within the genera *Nocardia* and *Mycobacterium* (Prescott 1987), although there are many saprophytes in these genera as well.

The similarity of *R. equi* to bacteria in the genera *Nocardia* and *Mycobacterium* was noted shortly after its discovery, when several researchers reported that the then

Corynebacterium equi was an acid-fast organism, which led to its reclassification as *Mycobacterium equi* during the 1930s and 1940s (Jensen 1934; Krasil'nikov 1938; Krasil'nikov 1941). Determination of the cell wall structure of *R. equi* confirmed the phenotypic link between *R. equi* and other intracellular bacteria within the *Mycobacterium-Nocardia* genera. These species are classified as the mycolata (Sutcliffe 1997), which characteristically have a cell wall architecture dominated by lipids, notably the high molecular weight, branched-chain mycolic acids.

Results from sequencing 30% of the chromosome of *R. equi* have reinforced its genetic links with pathogens in the genus *Mycobacterium*. There is extensive similarity between *R. equi* genes and those of *Mycobacterium tuberculosis*, and many of the homologous genes are putative or proven virulence genes in mycobacteria (Rahman *et al.* 2003).

The size of the *R. equi* genome has been estimated to be 4.4 Megabases (Mb) and it has a high guanosine and cytosine content, estimated to be in the order of 67-73% (Goodfellow *et al.* 1998; Rahman *et al.* 2003). Extensive strain typing of *R. equi* by pulsed field gel electrophoresis (PFGE) of restriction endonuclease digests of their genomes showed a high level of genomic heterogeneity within the taxon *R. equi* (Morton *et al.* 2001; Cohen *et al.* 2003). In a study of *R. equi* isolates from Australian Thoroughbred foals, 44 strains were identified amongst 209 clinical isolates (Morton *et al.* 2001). A second study from the USA examined 290 isolates of *R. equi* from 4 countries, including 218 virulent isolates from foals and 72 avirulent isolates from faeces, soil and respiratory tract samples (Cohen *et al.* 2003). There was significant diversity among isolates of *R. equi* and often less than 80% similarity in restriction endonuclease digestion patterns as determined by PFGE. *R. equi* isolates from the same farm were frequently shown to be different strains. Isolates from Ireland and the United States were significantly more similar to each other ($P<0.001$) than to those from Japan or Argentina. The genomic variation within the species was noted in earlier studies, which found that the restriction endonuclease digest patterns of some strains within the same capsular serotype showed only 40% similarity (Fuhrmann and Lammler 1997). Despite the extensive evidence of chromosomal variation within the taxon, none of these variations have been linked directly

with virulence (Cohen *et al.* 2003). Such diversity in the chromosome of the species may lead taxonomists to re-examine the classification of *R. equi* in the future. The determination of the chromosomal sequence of *R. equi* may resolve some of these issues and reveal its true taxonomic position, as well as identifying the chromosomal genes that play a role in virulence.

1.2.3. Identifying *R. equi*

R. equi are Gram-positive coccobacilli and have a rod-coccus life cycle. In the early stages of growth in culture the bacteria are predominantly rods and as the culture ages cocci become the more dominant form (Fuhrmann *et al.* 1997). *R. equi* typically grow aerobically and will grow well on or in a variety of standard laboratory media, including sheep blood agar (SBA), Luria-Bertani (LB) broth and agar, tryptic soy broth and agar, and brain-heart infusion broth (BHIB).

On SBA *R. equi* colonies incubated at 37°C for greater than 48 hours will produce mucoid, irregularly shaped colonies between 3 and 5 mm in diameter and with age these colonies typically turn salmon-pink in colour. *R. equi* are typically catalase and urease positive and are unable to ferment lactose or glucose. *R. equi* are considered acid-fast bacteria and can be identified within alveolar macrophages in tracheal lavages of foals using an acid-fast stain.

R. equi produces a substance known as ‘equi’ factor, which interacts with phospholipase D of *Corynebacterium pseudotuberculosis* and the β-toxin of *Staphylococcus aureus* to completely haemolyse erythrocytes, a CAMP reaction (Bernheimer *et al.* 1980; Prescott *et al.* 1982). ‘Equi’ factor is highly antigenic, and consists of a cholesterol oxidase and a phospholipase C exoenzyme (Machang’u and Prescott 1991). CAMP reactions have also been noted between *R. equi* and *Listeria monocytogenes*, but appear to be dependent on the strain of *L. monocytogenes* used (Skalka *et al.* 1982; McKellar 1994).

Agar media selective for *R. equi* have been extensively used in ecological studies. The first *R. equi* selective agar (NANAT) was developed in 1979, and contained nalidixic

acid, novobiocin, actidione (cycloheximide) and potassium tellurite in a trypticase soy agar base (Woolcock *et al.* 1979). This medium inhibited the growth of a variety of Gram-positive and Gram-negative bacteria, but not the growth of pseudomonads and some coryneforms. NANAT medium allowed selective growth and identification of individual *R. equi* colonies, enabling isolation from the mixed bacterial population in the environment or the faeces of horses. In 1995 a more selective medium was developed (von Graevenitz and Punter-Streit 1995). This medium contained ceftazidime and novobiocin in a Mueller-Hinton agar base (CAZ-NB), and inhibited more nocardioforms than NANAT agar.

1.3. Virulent *R. equi*

1.3.1. Search for virulence factors

The cell wall proteins of many Gram-positive pathogens are important determinants of pathogenicity and virulence (Navarre and Schneewind 1999). Initial research into the virulence of *R. equi* focused on the cell wall after it was observed that capsular antigens prevented a phagosome-lysosome fusion in foal macrophages (Yager *et al.* 1987). Unfortunately, neither the definition of 7 capsular serotypes by Prescott (1981), nor 27 capsular serotypes by Nakazawa *et al.* (1983), revealed any association between specific serotypes and virulence.

The lipid-rich cell wall of *R. equi* contains long chain mycolic acids. In *M. tuberculosis* it is the mycolic acid glycolipids that are thought to be responsible for the formation of granulomatous lesions in the lung of the host (Johnston *et al.* 1983; Ishino *et al.* 1992). The mycolic acids in the cell wall of *R. equi* are important factors in the induction of a granulomatous response in the host (Gotoh *et al.* 1991). *R. equi* recovered from an active lymph node abscess induced a granulomatous response more rapidly when inoculated into the livers of mice than those recovered from a tonsil of a healthy pig. The chain lengths of the mycolic acids in the *R. equi* from the abscess were longer than those of the *R. equi* from the healthy tonsil. Furthermore, intravenous inoculation of purified glycolipids yielded a similar granulomatous response in the liver (Gotoh *et al.* 1991). This led to a suggestion that pathogenic strains of *R. equi* (virulent *R. equi*) have longer mycolic acid chains than avirulent strains. However, as there have not been definitive studies to

establish that virulence is related directly to longer mycolic acid chain length, these findings can only be interpreted as being suggestive that the mycolic acid content and type may be important in the intracellular survival of the organism and in the formation of granulomatous lesions.

Other *R. equi* cell wall proteins that have homologues in the mycobacterial cell wall have also been suggested to be important virulence factors. Notable amongst these is the recent discovery that the cell wall lipoglycan of *R. equi*, lipoarabinomannan (ReqLAM), binds and interacts with macrophage receptors, suggesting that it may affect bacterial uptake into macrophages (Garton *et al.* 2002). ReqLAM was shown to induce early cytokine responses to *R. equi* infection by macrophages (Garton *et al.* 2002). The lipoarabinomannan with mannosyl extensions (ManLAM) found in the cell wall of *M. tuberculosis* has been shown to have many properties that may influence the pathogenicity of *M. tuberculosis*, including mediation of internalisation by adherence to mannose receptors and evasion of bactericidal mechanisms of macrophages (Strohmeier and Fenton 1999; Astarie-Dequeker *et al.* 1999). The interactions between ReqLAM and macrophage cell wall receptors, including mannose receptors and other collectins, may play important roles in the pathogenesis of disease caused by *R. equi* (Garton *et al.* 2002).

Despite the evidence that a variety of cell wall proteins may have some role in pathogenicity, most recent research has focused on the role of a 15-17 kiloDalton (kDa) lipoprotein that has been shown to be a major virulence factor of *R. equi* (Chirino-Trejo and Prescott 1987).

1.3.2. 15-17 kDa lipoprotein and virulence

Isolates of *R. equi* from foals with clinical disease were shown to express a 15-17 kDa lipoprotein. This protein was absent in the apathogenic type stain (ATCC 6939), which suggested an association between the expression of the 15-17 kDa lipoprotein and virulence (Chirino-Trejo and Prescott 1987). The pathogenicities of these two types of *R. equi* were compared in a mouse model (Takai *et al*. 1991a). Doses of 10^6 of all isolates that expressed the 15-17 kDa protein killed mice, while doses of greater than 10^8 of isolates not expressing the 15-17 kDa protein were unable to kill mice (Takai *et al.* 1991a).

The same study demonstrated that sera obtained from foals that were naturally infected with *R. equi* contained antibodies that reacted strongly with the 15-17 kDa protein, whilst sera from healthy foals did not. *R. equi* isolates that express this 15-17 kDa antigen also possess a plasmid of approximately 80-85 kb (Takai *et al.* 1991b; Tkachuk-Saad and Prescott 1991).

1.3.3. Virulence associated proteins

a. Virulence associated protein A (VapA)

The region encoding the 15-17 kDa virulence associated protein was mapped to the E3 fragment on an 85 kb plasmid (pREAT701) from a virulent *R. equi* strain (ATCC33701) (Kanno *et al.* 1993). A single gene predicted to encode a 19 kDa precursor of 189 amino acids, with at least five signal peptidase cleavage sites, was identified on the E3 fragment (Sekizaki *et al.* 1995). Analysis of the amino acid sequence showed that, if the signal peptidase cleaved the precursor at the predicted positions, the resultant peptides would range from 15 to 17 kDa, in agreement with the sizes of proteins detected by immunoblotting (Takai *et al*. 1991a,b). The protein encoded by the gene was named virulence associated protein A (VapA), and the gene *vapA.*

Studies using immunoblotting to detect the presence of the VapA and polymerase chain reaction (PCR) assays to detect *vapA* in *R. equi* recovered from tracheal aspirates from foals with pneumonia have demonstrated that the majority of these isolates contain the *vapA* gene or express the VapA protein (Haites *et al.* 1997; Hashikura *et al.* 2000). In one study 151/154 (98%) *R. equi* isolates from infected foals contained the *vapA* gene (Haites *et al.* 1997), while in the other study 649/655 (99.1%) isolates recovered from tracheal aspirates from clinical cases of pneumonia expressed VapA (Hashikura *et al.* 2000).

The expression of VapA has been shown to be temperature and pH dependent, with high expression of VapA when cells were grown at temperatures between 34°C and 41°C. Optimal expression of VapA occurred at 38°C and at a pH of 6.5, similar to conditions experienced within the host (Takai *et al.* 1992, 1996b). This protein has been shown to be

susceptible to trypsin digestion and accessible to biotin labelling, suggesting that it is located on the cell surface (Takai *et al.* 1992). Phase partitioning of whole cell proteins of a virulent strain of *R. equi* (103) using Triton X-114 and radiolabelling with ^{3}H palmitic acid demonstrated that VapA was a lipoprotein (Tan *et al.* 1995).

b. The Vap family

The *vapA* gene is part of a family, members of which share extensive DNA sequence similarity (Takai *et al.* 2000). The Vap proteins have extensive amino acid sequence identity at the C-terminus but not the N-terminus. VapC, D and E share approximately 40% overall amino acid sequence identity with VapA, but have approximately 80% amino acid sequence similarity at the C-terminus (Byrne *et al.* 2001). The N-terminus of VapA is the outermost part of the protein on the cell surface, so if the other Vaps are cell-surface expressed, the diversity at the N-terminus may generate antigenic variation among virulent *R. equi* (Takai *et al.* 2000). Attempts to identify VapC, D and E by immunoblotting analysis of *R. equi* cellular antigen preparations from cultures in brain heart infusion broth (BHIB) containing 0.1% yeast extract were unsuccessful. However, immunoblotting analysis of culture supernatants was able to identify all three of these Vap proteins. This indicates that VapC, D and E are secreted proteins, unlike VapA (Byrne *et al.* 2001). Even though these proteins may have a different biological function to VapA, VapA, C, D and E appear to be coordinately regulated. The effect of temperature on expression of VapC, D and E is similar to its effect on expression of VapA, with all of these Vap proteins expressed well at 37°C, but minimally at 30°C (Byrne *et al.* 2001).

A study using microarrays to investigate the expression of virulence plasmid genes in equine macrophages found that expression of all *vap* genes was significantly induced when *R. equi* were grown in macrophages, compared to growth *in vitro* at 30°C and pH 7 (Ren and Prescott 2003). This study also investigated the influence of environmental factors on virulence plasmid gene expression. All *vap* genes were shown to be induced at 37°C, under conditions of restricted iron or calcium, or at pH 5, whilst they were down-regulated under conditions of restricted magnesium (Ren and Prescott 2003). Two studies have shown expression of *vap* genes to be up-regulated by acidic conditions and the

presence of H_2O_2 (Benoit *et al.* 2001, 2002). These results demonstrate that expression of virulence genes is up-regulated by conditions that resemble those found within the phagosome of macrophages. They suggest that the Vap family of proteins are maximally expressed within the intracellular environment of the host and are regulated by a common pathway. Thus, the virulence plasmid and its virulence genes may be a genetic adaptation of the bacteria to the intracellular environment within the host. However, there may be environmental niches outside the host with similar conditions (low pH, oxidative stress, poor nutrient availability, high temperatures) that also facilitate the survival and replication of virulent strains.

Studies on an *R. equi* deletion mutant, from which an 8 kb DNA region of the virulence plasmid spanning 5 *vap* genes (*vapA, vapC, vapD*, *vapE* and *vapF*) had been removed, found that the mutant was unable to multiply in mice and was rapidly cleared (Jain *et al.* 2003). The mutant strain was then complemented with each of the missing *vap* genes and the complemented strains tested in the mouse model for restoration of virulence. The mutant strain complemented with *vapA* was restored to full virulence, whereas the other four *vap* genes were unable to restore virulence. Prior to this study virulence had been attributed to the entire plasmid or genes within the pathogenicity island (Giguere *et al.* 1999a; Takai *et al.* 2000). These findings suggest that *vapA* is an essential virulence gene and that the protein encoded by this gene is the primary virulence determinant within the Vap family of proteins.

1.3.4. Virulence plasmids

a. Geographic diversity

A variety of virulence plasmids have been identified (Table 1.1) on the basis of size and distinct restriction endonuclease digestion patterns.

Table 1.1: Restriction endonuclease digestion patterns of plasmids of *R. equi*

Name	Size (kb)	Strain	Country	Type of pattern when digested with:				Reference
				*Eco*RI	*Eco*T22I	*Hind*III	BamHI	
85 kb type I	85	ATCC33701	Canada	1	1	1	1	Takai *et al.* (1993a, b)
85 kb type II	85	96E35	France	1	1	2	1	Rahal *et al.* (1999)
85 kb type III	85	T47-2	USA	2	1	1	1	Takai *et al.* (2001a)
85 kb type IV	85	T43	USA	3	1	1	1	Takai *et al.* (2001a)
87 kb type I	87	222	Canada	4	2	1	1	Nicholson and Prescott (1997)
87 kb type II	87	96B6	Japan	5	2	3	2	Takai *et al.* (1999)
87 kb type III	87	Brazil	Brazil	11	6	5	1	Ribeiro *et al.* (2005)
90 kb type I	90	90	Japan	6	1	4	3	Takai *et al.* (1993a, b)
90 kb type II	90	S11-8 (Kiso)	Japan	7	1	1	1	Takai *et al.* (2001b)
90 kb type III	90	Kuma 83-3	Japan	8	3	4	3	Takai *et al.* (2001c)
90 kb type IV	90	Kuma 83-10	Japan	9	4	4	3	Takai *et al.* (2001c)
90 kb type V	90	J21-2	Korea	10	5	5	4	Takai *et al.* (2003a)

The variation in plasmid type seems to be related to geographic origin of the strain. Some plasmid types (the 90 kb types I–V, 85 kb type II and 87 kb type III) have only been found in restricted geographic areas, while others, such as the 85 kb type I and 87 kb type I plasmids, have been found in many countries from different continents (Takai *et al.* 1999, 2001c, 2003a). Despite the genotypic variation between plasmids, there is little evidence of a difference in virulence between the strains carrying them (Takai *et al.* 1999). The geographic specificity of some plasmids, however, allows plasmid typing to be used for investigating the molecular epidemiology of virulent strains of *R. equi* (Takai *et al.* 1999).

b. Sequence

The complete sequences of two 85 kb type I virulence plasmids have been determined (Takai *et al.* 2000). The plasmid is 80610 bp in length and is predicted to encode 64 open reading frames (ORFs), 22 of which are similar to genes of known function, and 7 to genes of unknown function, in other bacterial species. Thirty three ORFs were of unknown function and had no similarity to genes in other species, one had similarity to a gene in the transfer gene complex of plasmid RP4 and the final ORF was a putative replication protein found in the region devoted to replication and partition. The most significant feature of the sequenced virulence plasmid is the presence of a 27.5 kb pathogenicity island containing 7 *vap* genes. The *vap* genes are arranged as a cluster of 3 genes (*vapA, vapC, vapD*), a pair of genes (*vapE, vapF*) and two individual genes (*vapG, vapH*). Regions of extensive direct repeats of unknown, but possibly thermoregulative function, lie immediately upstream of the *vapA, vapC* and *vapD* cluster and the *vap E* and

vap F pair, but not the singular *vap* genes. The remainder of the plasmid consists of regions associated with conjugation, replication and partitioning (Takai *et al.* 2000).

1.4. Ecology and epidemiology

1.4.1. Life cycle of *R. equi*

Like many of the members of the genus *Rhodococcus, R. equi* is ubiquitous in soil and is commonly found in the faeces of grazing animals (Rowbotham and Cross 1977; Woolcock *et al.* 1980; Barton and Hughes 1982, 1984; Hughes and Sulaiman 1987). *R. equi* is routinely ingested by grazing animals, surviving and multiplying within the contents of the gastrointestinal tract and within the faecal material after the faeces is deposited (Barton and Hughes 1984; Hughes and Sulaiman 1987; Takai *et al.* 1994a). The soil concentration of *R. equi* is highest in areas where horses graze and increases with the length of time that the pasture has been grazed by horses (Woolcock *et al.* 1980; Prescott *et al.* 1984). This has led to the description of *R. equi* as a coprophilic soil-associated actinomycete (Barton 1991). Inhalation of organisms from a contaminated environment is considered to be the primary route of pneumonic infection in foals. Inhalation of aerosols generated from the respiratory tract of foals with pneumonia has not been considered previously as a possible route of transmission.

1.4.2. Habitat of *R. equi*

Examination of horse faeces has shown that the concentration of *R. equi* increases by up to 10^4 fold between one and two weeks after excretion from the horse (Barton and Hughes 1984). Growth was found to be substantially improved in soil enriched with horse faeces (Hughes and Sulaiman 1987). *R. equi* utilises a variety of volatile fatty acids (VFAs) (acetate, propionate and butyrate) found in significant quantities in the gut contents and faeces of herbivores (Phillipson 1977). Acetate and propionate are the two major VFA sources that *R. equi* use during proliferation in horse faeces and soil enriched with horse faeces (Hughes and Sulaiman 1987).

a. Growth and prevalence of virulent *R. equi* in equine faeces

R. equi is found in the faeces of adult horses and foals (Woolcock *et al.* 1980; Takai *et al.* 1986a). The average concentration of *R. equi* in the faeces of mares ranges from 10^2 to 10^3 colony forming units (cfu)/gram (Takai *et al.* 1987, 1994a). The concentration of *R. equi* in the faeces of healthy foals is greater than that seen in the mare, ranging from 10^4 to 10^5 cfu/gram. The concentration of *R. equi* in the faeces of foals is greatest between 4 and 10 weeks of age, after which the concentration gradually decreases to that seen in mares (Takai *et al.* 1986a, 1994a). Foals with *R. equi* pneumonia have an even higher faecal concentration of *R. equi* than healthy foals, with up to 10^8 cfu/gram detected in some foals (Takai *et al.* 1986a).

The prevalence of virulent *R. equi* in the faecal *R. equi* population in mares ranges from 3.6 to 14.6%, while in foal faeces the prevalence is slightly higher, ranging from 10.8 to 17.7% (Takai *et al.* 1994a). The prevalence of virulent *R. equi* in the faeces of foals also changes with age. The prevalence of virulent *R. equi* peaks at 25% of the faecal *R. equi* population in foals at 4 weeks of age and decreases gradually thereafter. By 14 weeks of age the proportion of virulent *R. equi* in the faecal *R. equi* population is similar to that in the adult horse (Takai *et al.* 1994a).

The intestinal contents of an infected foal were investigated to examine the prevalence of virulent *R. equi* in the intestinal *R. equi* population. Seventy *R. equi* isolates were recovered from various areas of the gastrointestinal tract, of which 35 (50%) were found to be virulent using an immunoblotting assay. This suggests that infected foals excrete a higher concentration of virulent *R. equi* in their faeces (Takai *et al.* 1994a).

b. Growth and prevalence of virulent *R. equi* in soil

R. equi is a soil saprophyte that is most commonly found in the superficial soil layer at concentrations of up to 10^4 cfu/gram, while very low concentrations of *R. equi* are detected in soil greater than 30 cm below the surface (Takai *et al.* 1986b). Early studies of *R. equi* ecology demonstrated that the isolation of *R. equi* from soil was not influenced by soil pH, but that there was a greater prevalence of *R. equi* in sand based soils than in clay

based soils (Barton and Hughes 1984). *R. equi* was infrequently isolated from wet soils, but when these areas were re-sampled under drier condition *R. equi* was frequently recovered (Barton and Hughes 1984).

The *in vitro* growth characteristics of *R. equi* have been investigated using Steven's minimal salt medium enriched with 0.1% sodium acetate (Hughes and Sulaiman 1987). *R. equi* was found to be an obligate aerobe with optimal growth at 30°C at pH 7.0-8.5. This study also compared the growth of one isolate in horse faeces alone, or in faeces mixed with various soil samples at different pH. Growth of *R. equi* was best in faeces alone and was reduced when soil was added to the faeces, but was better when the faeces was mixed with an alkaline soil than an acid soil. Growth in alkaline soil supplemented with 0.4% sodium acetate was as good as growth in faeces. This suggested that the multiplication of *R. equi* is favoured by a neutral or moderately alkaline soil (pH 6.0-8.5) and enhanced by the presence of faeces or supplementation with an appropriate VFA. As these studies were conducted prior to the discovery of virulent strains, it is unclear whether virulent *R. equi* in soil behave in this manner.

Studies in Japan have shown that the likelihood of clinical *R. equi* pneumonia on farms was not closely correlated with the concentration of total *R. equi* in the soil, but rather the prevalence of virulent *R. equi* in the soil (Takai *et al.* 1991c, 1994a; Takai 1997). These studies found a higher prevalence of virulent *R. equi* in soil from a farm on which 10-15% of foals had *R. equi* pneumonia than in soil from a farm with no history of *R. equi* pneumonia (Takai *et al.*1991c). The prevalence of virulent *R. equi* in the soil on farms with sporadic cases of *R. equi* pneumonia (cases every 2 or 3 years) lay between that seen on the farm with endemic disease and that without disease (Takai *et al.* 1994a).

1.4.3. Airborne *R. equi*

The risk of a foal contracting *R. equi* pneumonia is assumed to be related to the likelihood of the foal inhaling virulent *R. equi* from environment. The measurement of the airborne concentration of virulent *R. equi* may thus be a more reliable method of assessing the risk of *R. equi* pneumonia than measurements of soil concentrations. Studies in Japan have shown that the concentration of *R. equi* in the air in stables increases when weather

conditions are warm, dry and windy and that this coincides with increases in the incidence of disease on farms (Takai *et al.* 1987; Falcon *et al.* 1985). One Japanese study has looked at the prevalence of virulent *R. equi* amongst a small number of *R. equi* isolates from the air in stables and found that 4/51 isolates collected between May and June from a farm in Aomori with sporadic cases of *R. equi* pneumonia were virulent (Takai *et al.* 1994a).

As the primary route of infection is believed to be inhalation of virulent *R. equi*, management strategies to reduce the incidence of infection on farms have focused on reducing the airborne burden of the pathogen in the foals' environment (Prescott and Yager 1991; Giguere and Prescott 1997). Keeping large numbers of foals on bare, dusty paddocks that are heavily contaminated with manure has been suggested to increase the likelihood of foals encountering a heavy respiratory challenge with virulent *R. equi*. The excretion of high concentrations of virulent *R. equi* in the faeces of foals suffering from disease ensures that the soil is replenished with large numbers of virulent *R. equi* (Takai *et al.* 1994a). Strategies such as rotation of foal paddocks, collection of faeces, isolation of infected foals, irrigation of paddocks and avoidance of use of stables with dirt floors for foals may reduce the incidence and severity of *R. equi* pneumonia on farms (Clarke 1989; Chaffin *et al.* 2003a). Such strategies are likely to reduce the chance of dust formation and thus reduce the amount of virulent *R. equi* inhaled by foals. However, it is not known how such measures might affect the virulent *R. equi* population on a farm.

1.4.4. Disease ecology: prevalence, management and the environment

Farm management and environmental conditions have been hypothesised to play an important role in determining the prevalence and severity of disease due to *R. equi* (Prescott *et al.* 1984, Barton 1991, Giguere and Prescott 1997). *R. equi* pneumonia occurs endemically on some farms and sporadically, or not at all, on many farms (Prescott 1987). Prior to the discovery of virulent *R. equi*, farms with endemic disease were thought to contain a greater concentration of *R. equi* in soil than farms on which disease was not endemic. Early studies found a direct association between the prevalence of *R. equi* pneumonia and the concentration of *R. equi* in the soil (Prescott *et al.* 1984; Takai *et al.* 1986a). A Japanese study found that the concentration of *R. equi* in soil increased

dramatically in April and May (Spring) and remained high throughout the subsequent breeding season (Takai *et al.* 1986b). After the discovery of the virulence plasmid, these ecological studies were revisited, with attention focused on the concentration and proportion of virulent *R. equi* in the horses' environment. It was suggested that the concentration and prevalence of virulent *R. equi* (Table 1.2) are important risk factors for development of *R. equi* pneumonia on an individual farm (Takai 1997).

Table 1.2: Variation in incidence of *R. equi* pneumonia on farms associated with the prevalence of virulent *R. equi* in the environment (Takai 1997)

Disease	Soil contamination with *R. equi*	Proportion of *R. equi* that are virulent in soil and/or faeces
Endemic (cases annually)	Heavy	>20%
Sporadic (cases every 2-5 years)	Slight/moderate	5-10%
None	Low	<5%

A study performed in Texas found no significant relationship between detection of virulent *R. equi* in soil and the prevalence of *R. equi* pneumonia (defined as endemic or non-endemic) on individual farms (Martens *et al.* 2000). This was supported by studies in Japan that detected virulent *R. equi* in the soil of a farm on which disease was not endemic (Takai *et al* 1991c). Thus, the presence of virulent *R. equi* in the soil is seemingly not a significant determinant of the prevalence of *R. equi* pneumonia on the farm. Rather, it is the concentration of virulent *R. equi* or the proportion of *R. equi* in the soil that are virulent that may be important.

The potential for differences in the ecology of virulent strains was first noted when expression of virulence antigens was lost after repeated passage of a strain at 42°C (Chirino-Trejo and Prescott 1987). This was confirmed by further studies (Takai *et al.* 1991d, 1994b) that established that *R. equi* carrying the virulence plasmid grow slower at 38°C than their avirulent derivatives that have been cured of the plasmid, but that both virulent and avirulent strains grow at the same rate at 30°C. These studies did not examine differences at other temperatures or at different pH. Acid tolerance has recently been shown to be a trait of both avirulent and virulent strains of *R. equi* (Benoit *et al.* 2000).

No reports that examine the concentration of virulent *R. equi* in the soil, the geochemical characteristics of soil and possible correlations with the prevalence of *R. equi*

pneumonia on farms have been published. A recent study in Texas found no association between any surface soil geochemical characteristics and the prevalence of disease due to *R. equi* on farms (Martens *et al.* 2002a). However, this study did not attempt to isolate virulent *R. equi*, nor to quantify the virulent *R. equi* in soil samples, and hence these data do not show conclusively the effect of soil characteristics on the proliferation of virulent *R. equi* in soil, and on the subsequent risk of disease on a farm.

The management and characteristics of breeding farms where *R. equi* pneumonia is considered endemic and of those that do not have endemic disease have been evaluated in an attempt to identify risk factors associated with the development of *R. equi* pneumonia in foals (Chaffin *et al.* 2003a, b). High foal density, large acreage and a large population of transient mares were identified as significant risk factors associated for pneumonia caused by *R. equi* in foals (Chaffin *et al.* 2003b). Farms that generally failed to provide good preventative medicine (i.e. anthelmintic treatment, vaccination and administration of hyperimmune serum) did not have an increased risk of *R. equi* pneumonia in foals (Chaffin *et al.* 2003a). The observation that good health management does not influence the risk of *R. equi* pneumonia is in contrast to reports and studies in the past that have suggested that the implementation of parasite control and vaccination against respiratory viruses could reduce the incidence of *R. equi* pneumonia (Bain 1963; Derby and Bailie 1987; Clarke 1989).

There is little conclusive proof that any management strategy will reduce *R. equi* pneumonia by decreasing the likelihood of the foal being exposed to virulent *R. equi*. All observations relating to the effectiveness of changing management strategies have been based on anecdotal evidence. No study has shown conclusively that there is an association between the prevalence of *R. equi* pneumonia and the concentration and/or prevalence of virulent *R. equi* in the foals' environment.

1.5. Manifestations of disease due to *R. equi*

1.5.1. Disease in foals

a. *R. equi* pneumonia

R. equi is one of the most significant causes of pneumonia in foals throughout the world. *R. equi* pneumonia is seen in foals up to 6 months of age, with the majority of cases in foals between 1 and 3 months of age (Zink *et al.* 1986; Prescott 1991).

The prevalence of *R. equi* pneumonia appears to be increasing in all horse breeds (Martens *et al.* 1989a), with morbidity rates ranging from 5 to 17% worldwide and case fatality rates as high as 80% (Bain 1963; Hillidge 1986; Sweeney *et al.* 1987). In the United States approximately 3% of foal deaths are attributable to *R. equi* pneumonia (Madigan *et al.* 1991). Typically *R. equi* infection in the foal is manifested as a chronic suppurative bronchopneumonia with extensive abscessation and associated suppurative lymphadenitis (Zink *et al.* 1986). The infected foal may display some, but not necessarily all, of the following clinical signs: pyrexia, illthrift, tachycardia, tachypnoea and coughing (Giguere and Prescott 1997). Thoracic auscultation typically reveals areas of lung consolidation, crackling, wheezing and rattling sounds, with pleural effusion in some cases. On radiographic examination a prominent alveolar pattern, characterised by ill-defined regional consolidation, has been reported as the most common finding (Falcon *et al.* 1985). The insidious onset of the disease, coupled with the ability of foals to compensate for the progressive loss of lung function, means that significant lung pathology exists before clinical signs are apparent. These factors make the early clinical diagnosis of *R. equi* pneumonia difficult. Thoracic ultrasonographic examination has been used with increasing frequency as a diagnostic tool to detect lung abscesses in foals with no obvious clinical signs of *R. equi* pneumonia. Lung abscesses appear as variably sized, well defined, hypo-echoic nodules with no small airways or vessels within the centre. “Gas shadowing” may be seen at the interface between the aerated lung and the far wall of the abscess (Ramirez *et al.* 2004). Detection of abscesses by ultrasonographic examination has allowed clinicians to make early presumptive diagnoses and commence treatment before clinical signs are obvious, thus minimising the development of significant lung pathology.

The most common haematological change reported in foals infected with *R. equi* is a neutrophilic leucocytosis with hyperfibrinogenaemia (Hillidge 1986). While hyperfibrinogenaemia is the most common laboratory finding in foals with *R. equi* pneumonia, leucocytosis has been shown to be of a greater diagnostic value and was also of greater diagnostic value than an agar gel immunodiffusion (AGID) serological test (Giguere *et al.* 2003a).

Experimental infection studies have shown that the respiratory route is most likely to result in the progressive pulmonary lesions associated with clinical disease (Barton and Embury 1987; Martens *et al.* 1989b; Hooper-McGrevy *et al.* 2001). The pathogenesis of *R. equi* pneumonia in the foal involves the inhalation of virulent *R. equi* into the alveoli, where it is ingested by macrophages. It survives phagocytosis by preventing the fusion of the phagosome with the lysosome. *R. equi* replicates efficiently within the phagosome of the macrophage (Hietala and Ardens 1987; Zink *et al.* 1987). Ultimately, replication in the macrophage leads to cell death, resulting in necrotic damage and severe lung pathology (Luhrmann *et al.* 2004).

A purulent bronchopneumonia with noticeable abscessation is typically seen at *post mortem*. The lung abscesses are usually discrete, encapsulated, variable in size and scattered throughout the lung (Knottenbelt and Pascoe 1994). Abscesses may also be seen in the pulmonary lymph nodes. Typical abscesses contain a central necrotic zone surrounded by a zone infiltrated with activated macrophages, neutrophils and multinucleated giant cells. The macrophages contain numerous intracellular bacteria. The interalveolar and interlobular septa of the affected lung tissue are intact but oedematous. The alveolar septa are congested and hypercellular, while the intralobular septa contain a few macrophages, lymphocytes and plasma cells. The bronchioles are filled with exudate containing macrophages, neutrophils, necrotic debris and mucus. Most bacteria are contained within macrophages, surrounded by material that stains positive using the PAS (periodic acid-Schiff) technique. The lymph nodes draining the affected lung tissue are often swollen and may contain abscesses. Oedema and hypoplastic follicles often surround abscesses within these lymph nodes (Ishino *et al.* 1992).

Some foals present with an acute form of the disease. These foals may die without any obvious clinical signs of respiratory disease or within a day or two of detection of clinical signs. On post-mortem examination these acutely affected foals are found to have an interstitial pneumonia with diffuse miliary pyogranulomatous lung lesions (Martens *et al.* 1982).

Attempts have been made to examine the long-term effect of *R. equi* pneumonia. Radiographic and haematological examinations, bronchoalveolar lavage (BAL) and pulmonary function tests were performed on five horses with prior history of *R. equi* pneumonia and the results compared to age-matched controls with no history of significant respiratory disease. No significant difference in pulmonary function was found between the two groups (Ainsworth *et al.* 1993). This supported earlier observations on a group of six foals experimentally infected with *R. equi*, all of which developed clinical signs of pneumonia and pulmonary lesions and then recovered with the aid of hyperimmune plasma (Martens *et al.* 1989b). Lung lesions (area of lung consolidation) reduced appreciably over time or had disappeared in all six surviving foals by three months after infection. These two studies suggest that the lung tissue is repaired once a foal has recovered from *R. equi* bronchopneumonia and that the foal can recover fully, with no obvious long-term effects. Interestingly, one foal, which had been infected with *R. equi* (Martens *et al.* 1989b), had developed pneumonia and had apparently recovered, was found to have a sterile lung abscess at necropsy 12 months after infection. The lesion, although sterile, was of sufficient size to have the potential to impair pulmonary function.

Despite the evidence that lung function returns to normal after *R. equi* infection, there is conflicting evidence on the effect of the disease on the foal's future as a racehorse. Results from one study suggest that racing performance of adult horses is unaffected by a history of *R. equi* pneumonia as a foal (Bernard *et al.* 1991). However, the likelihood of racing as a two-year old and the earning potential of a racehorse is deleteriously affected by a history of *R. equi* pneumonia as a foal (Ainsworth *et al.* 1998).

b. *R. equi* enteritis

Foals suffering from *R. equi* enteritis show signs of weight loss, pyrexia and diarrhoea. The majority of foals with *R. equi* enteritis also have *R. equi* pneumonia. Studies have reported that 40-60% of foals with disease due to *R. equi* have both pneumonic and enteric forms, but that only a small proportion (4-20%) of foals with disease due to *R. equi* exhibit only the enteric form (Hutchins *et al.* 1980; Zink *et al.* 1986). It is thought that the majority of cases of *R. equi* enteritis occur as a sequela of *R. equi* pneumonia, due to the ingestion of sputum containing bacteria from the respiratory tract (Johnson *et al.* 1983).

R. equi enteritis is characterised by a multifocal ulcerative enterocolitis and typhlitis over the areas of the Peyer's patches, with granulomatous or suppurative inflammation of the mesenteric and/or colonic lymph nodes (Zink *et al.* 1986).

c. Less common forms of disease due to *R. equi*

R. equi have occasionally been reported as the cause of disease outside the respiratory and gastrointestinal tracts. A recent retrospective study of cases of *R. equi* infection in 109 horses revealed that 82 (75.2%) involved the lower respiratory tract, with the remainder involving mesenteric lymph nodes, joints, uterus, abdominal viscera, vertebrae or skin, or abscesses from unspecified locations. The majority of the lower respiratory tract infections in this study were seen in Thoroughbred foals (65.9%), with remainder of cases spread over a variety of other breeds including Saddlebreds, Quarterhorses, Arabians, Tennessee Walking Horses, Rocky Mountain Horses and Standardbreds (Poonacha *et al.* 1998). Non-septic, possibly immune-mediated, polysynovitis has been reported in approximately one third of cases of *R. equi* pneumonia (Sweeney *et al.* 1987). Septic arthritis and osteomyelitis, presumably as a consequence of haematogenous spread of *R. equi*, has been reported with and without concurrent respiratory or enteric disease due to *R. equi* (Firth *et al.* 1993; Chaffin *et al.* 1995; Paradis 1997). *R. equi* has been reported to cause subcutaneous and cutaneous abscesses, ulcerative lymphangitis and cellulitis (Dewes 1972; Smith and Jang 1980; Zink *et al.* 1986; Pedrizet and Scott 1987; Paradis 1997). Other diseases associated with *R. equi* infection in foals

include hepatic and renal abscessation, uveitis, panophthalmitis and nephritis (Blogg *et al.* 1983; Ellenberg and Genetzki 1986; Beech and Sweeney 1991).

1.5.2. Disease due to *R. equi* in the adult horse

Disease due to *R. equi* is rare in the adult horse, although cases of pneumonic and enteric disease have been reported (Roberts *et al.* 1980; Zink *et al.* 1986). *R. equi* pneumonia has been documented in immunocompromised adult horses (Freestone *et al.* 1987). *R. equi* is a recognised cause of lymphangitis, primarily of the lower limbs, in adult horses. Percutaneous invasion by the larvae of *Strongyloides westeri* has been suggested to facilitate entry of *R. equi*, explaining the classical distribution of lymphangitis on the lower limbs of foals and adult horses (Dewes 1989). *R. equi* has also been implicated as an occasional cause of equine abortion, endometritis and placentitis (Zink *et al.* 1986; Fitzgerald and Yamini 1995; Poonacha *et al.* 1998; Patterson-Kane *et al.* 2002).

1.5.3. Disease due to *R. equi* in other species

R. equi has also been reported to cause disease in pigs and immunocompromised humans. In pigs infection is associated with a granulomatous lymphadenitis of cervical lymphatic tissue (Karlson *et al.* 1940; Gotoh *et al.* 1991). However, as *R. equi* can be isolated from healthy porcine lymphatic tissue, the details of the pathogenesis of this disease in pigs remain unclear (Karlson *et al.* 1940; Gotoh *et al.* 1991; Takai *et al.* 1996a; Lammler *et al.* 1997). In humans *R. equi* causes cavitary pneumonia, in which gray-white cavities filled with necrotic material are found in the lungs (Islam *et al.* 2003). The detection of *R. equi* pneumonia in the human population has increased as a consequence of the HIV-AIDS pandemic. Common clinical signs of *R. equi* pneumonia in humans include pyrexia, coughing and expectoration (Torres-Tortosa *et al.* 2003). The prognosis for humans with *R. equi* pneumonia depends, in part, on the immune status of the patient. In patients with AIDS, the case fatality rate ranges from 50% to 55%, compared with 20% to 25% in immunocompromised patients without AIDS, and 10% in immunocompetent patients (Kedlaya *et al.* 2001).

Three types of *R. equi* (virulent, intermediately virulent and avirulent) have been isolated from pigs and humans. The majority of isolates from pigs and humans are intermediately virulent (Takai *et al.* 1995a, 1996a; Makrai *et al.* 2002). Intermediately virulent isolates express a 20 kDa antigen named VapB. Isolates expressing VapB were shown to kill mice at a dose of 10^7 cfu, tenfold higher than the fatal dose of those expressing VapA (Takai *et al.* 1995a, 1996a). Like VapA, VapB is located on the cell surface and is susceptible to proteolysis by trypsin (Takai *et al.* 1996b). VapB expression is regulated by pH and temperature in the same way as VapA. Expression of VapB is maximal at 38°C and pH 6.5 (Takai *et al.* 1996b). Comparisons of the *vapA* and *vapB* genes found 83.6% nucleic acid sequence identity and 76% amino acid sequence identity, with the greatest similarity (94%) in the carboxyl terminal region (Byrne *et al.* 2001). Antigenic cross-reactivity between VapA and VapB has been demonstrated using immunoblots and serum from infected foals (Takai *et al.* 1995a). Isolates expressing VapB contain a virulence plasmid that confers an intermediate level of virulence. Up to seven different plasmid types, ranging in size from 79 kb to 100 kb, have been found in isolates expressing VapB (Takai *et al.* 1995a, 1996a). Isolates expressing VapB have never been recovered from foals with pneumonia or from horse breeding farms.

R. equi infections have been described in other species, including cattle, sheep, goats, llamas, cats and dogs, but disease in these species is extremely rare, with lesions confined to lymph node abscessation or wound infection (Addo and Dennis 1977; Hong and Donahue 1995; Elliott *et al* 1986, Poonacha *et al.* 1998; Flynn *et al.* 2001). While pneumonia is rare in these species, one case report described two litters of kittens that were housed separately in the same cattery and that both had *R. equi* pneumonia (Gunew 2002).

Not a great deal is known of the types of *R. equi* isolated from species other than horses, pigs and humans. Neither virulent nor intermediately virulent *R. equi* have been detected among *R. equi* isolates obtained from bovine lymph node lesions (Flynn *et al.* 2001). An examination of *R. equi* isolates from dogs and cats found that five of nine cat isolates and one of nine dog isolates were VapA positive and contained an 85 kb type I or an 87 kb type I virulence plasmid (Takai *et al.* 2003b)

1.6. Humoral immunity

Foals acquire antibody to *R. equi* passively, either from their dam via ingestion of colostrum in the first 24 hours post partum, or by intravenous administration of hyperimmune serum or plasma. Hyperimmune serum appears to promote phagocytosis and killing of *R. equi* by equine macrophages and neutrophils *in vitro* (Hietala and Ardans 1987; Martens *et al.* 1987). The protective effects of hyperimmune and normal serum have been demonstrated in both experimental and naturally occurring *R. equi* infections, and are manifested as prevention of disease or a significant reduction in the severity of pneumonia (Martens *et al.* 1989b; Madigan *et al.* 1991; Higuchi *et al.* 1999; Perkins *et al.* 2002). The protective component of hyperimmune serum is currently unknown. Antibody is considered to be a key factor, but other non-specific factors in plasma (cytokines, complement factors, fibronectin) are likely to contribute (Giguere and Prescott 1998). This may explain the failure to protect the foal by hyperimmunising mares (Martens *et al.* 1991) and the failure to protect against naturally occurring *R. equi* pneumonia by administration of hyperimmune serum to foals in some studies (Hurley and Begg 1995; Giguere *et al.* 2002). In Argentina, control of *R. equi* pneumonia using a combination of mare vaccination and administration of hyperimmune serum to foals has been reported. Mares were vaccinated subcutaneously with a vaccine containing solubilised *R. equi* antigens, including VapA, during the last 2 months of pregnancy. The concentration of anti-*R. equi* antibody in foals at 2 days of age was monitored by AGID. Foals with low anti-*R. equi* antibody concentrations were given an intravenous (IV) dose of hyperimmune serum from donors vaccinated with *R. equi*. All foals were administered hyperimmune serum at 25 days of age, irrespective of previous antibody concentrations (Becu *et al.* 1997).

Antibody may play a critical role in limiting the earliest stages of infection, before intracellular infection is established. Once *R. equi* enters the alveolar macrophage it is no longer freely accessible to antibody, and experimental challenge studies have shown that administration of hyperimmune serum to foals once infection has been established does not appear to change the course of disease (Chaffin *et al.* 1991).

1.7. Cell mediated immunity

Due to the intracellular nature of *R. equi*, cell mediated immune mechanisms are thought to be of major importance in resistance to, and clearance of, *R. equi* infections. Much of what is known of cell mediated immunity against *R. equi* has come from studies in immunocompromised strains of mice.

1.7.1. Murine models, immunodeficiency and susceptibility

Immunocompetent strains of mice challenged via the respiratory route with virulent *R. equi* develop a transient inflammatory response that results in clearance of the bacteria from the lungs and resolution of lesions within 21 days of infection (Yager *et al.* 1991; Kanaly *et al.* 1993), presumably due to induction of a protective primary immune response (Bowles *et al.* 1989; Yager *et al.* 1991). Intravenous inoculation of immunocompetent mice with *R. equi* can result in significant mortalities, with the mortality rate dependent on the dose and virulence of the strain (Takai *et al.* 1991a, 1995a, 1996a). The relevance of the difference in the response to intravenous inoculation compared to that to the respiratory route of inoculation is unclear.

Mice with severe combined immunodeficiency, lacking both T and B lymphocytes (*scid/scid*), and athymic mice lacking T lymphocytes (*nu/nu*), are unable to eliminate *R. equi* from the lung following respiratory challenge and develop significant pulmonary lesions (Bowles *et al.* 1987; Yager *et al.* 1991). Complement (C5) deficient A/J mice and natural killer cell deficient C57 B1/6J.bg/bg (beige) mice are capable of resolving a pulmonary infection with *R. equi*. This suggests that deficiencies in complement components, phagocyte function and NK (natural killer) cell function do not impair pulmonary clearance of *R. equi* (Yager *et al.* 1991). In adoptive transfer experiments, splenocytes from immunocompetent mice previously infected with *R. equi* were transferred to SCID/beige mice, which were then inoculated intranasally with *R. equi*. The transfused mice showed significant protection (Balson *et al.* 1991). These findings suggest that immunodeficiency, principally affecting T lymphocytes, can be a critical factor that predisposes the host to *R. equi* pneumonia.

1.7.2. T lymphocytes, cytokines and clearance of *R. equi*

To further dissect the cell mediated immune response to *R. equi* infection, BALB/c mice depleted of $CD4^+$ and/or $CD8^+$ T lymphocytes were inoculated intravenously with *R. equi* (Nordmann *et al.* 1992). The spleens and livers were harvested from these mice 11 days after infection and the number of *R. equi* in these organs determined. Depletion of either subset resulted in a significant increase in the number of bacteria in the spleen and liver, indicating that both $CD4^+$ and $CD8^+$ T cells participated in the clearance of *R. equi*. Another study used transgenic 'knockout' mice to examine the role of $CD4^+$ and $CD8^+$ T lymphocytes in clearance of *R. equi* after pulmonary infection (Kanaly *et al.* 1993). The results of this study conclusively demonstrated the critical role of $CD4^+$ T lymphocytes, as transgenic mice deficient in $CD8^+$ T lymphocytes cleared virulent *R. equi* from lungs, while infection persisted in transgenic mice deficient in $CD4^+$ T lymphocytes and led to the formation of granulomas. In humans, *R. equi* pneumonia is predominantly seen in patients with HIV-AIDS. These people have very low numbers of $CD4^+$ T lymphocytes. Thus $CD4^+$ T lymphocytes appear to play a critical role in clearing *R. equi* from the lungs in mice and man, with $CD8^+$ cells having a less significant role.

In mice, $CD4^+$ lymphocytes can be divided into T helper 1 (Th1) and T helper 2 (Th2) subsets based on distinct, non-overlapping patterns of cytokine production (Mosmann and Coffman 1989; Mosmann and Sad 1996). Th1 cells produce interferon gamma (IFN-γ) and interleukin-2 (IL-2), whilst Th2 cells produce IL-4, IL-5 and IL-10. In mice and humans many infectious agents preferentially induce Th1 (type 1) or Th2 (type 2) responses (Bretscher *et al.* 1992). The balance between type 1 and type 2 responses in the host can determine the outcome of infection, including the ability of the infected host to control an intracellular pathogen (Gajewski and Fitch 1988; Heinzel *et al.* 1989; Scott 1991, Yamamura *et al.* 1991). This effect is mediated by the cytokines produced by the specific T helper cells.

Pathogens that survive and replicate within the macrophage are able to alter the capacity of the macrophage to destroy microbes. This effect is mediated by cytokines (Trinchieri *et al.* 1993). The cytokine IFN-γ is a major macrophage activation factor,

capable of upregulating a number of pathways for microbial killing (Mosmann and Coffman 1989). In addition IFN-γ has been demonstrated to stimulate phagosome-lysosome fusion and enhances expression of Fc receptors (Nathan *et al.* 1983). These mechanisms can be enhanced by other cytokines, such as IL-2 and TNF-α (tumour necrosis factor alpha), whilst others, such as IL-10, can down-regulate macrophage function (Trinchieri *et al.* 1993). In the murine model, *R. equi* clearance appears to be mediated by a Th1-like response, with IFN-γ a primary mediator presumably leading to activation of pulmonary macrophages (Nordmann *et al.* 1993; Kanaly *et al.* 1995). Adoptive transfer of $CD4^+$ T cells from mice that had developed Th1 responses after infection with *R. equi* into nude mice can promote clearance of *R. equi* from the lung (Kanaly *et al.* 1996). The expression of IFN-γ mRNA in cells in the bronchial lymph nodes was noted in the mice transfused with $CD4^+$ Th1 cell. Nude mice transfused with $CD4^+$ T cells from mice that developed Th2 responses after infection with *R. equi* failed to clear *R. equi* from the lung and subsequently developed large lung abscesses.

In mice the shift to either a Th1 or Th2 response to infection appears to be regulated by the response of the innate immune system at the time of initial exposure (Sharton and Scott 1993; Ladel *et al.* 1996). IL-12 and IL-4 appear to be early inducers of Th1 and Th2 responses, respectively (Kaufmann, 1995). If Th1 responses are protective in foals, IL-12 is likely to be an important mediator.

1.7.3. Pulmonary clearance of *R. equi* in the adult horse

Studies on adult horses experimentally infected with virulent *R. equi* have demonstrated that elevated concentrations of $CD4^+$ and $CD8^+$ cells, and IFN-γ expressing cells, in bronchoalveolar fluid samples are associated with pulmonary clearance of virulent *R. equi* (Hines *et al.* 2001; Lopez *et al.* 2002; Hines *et al.* 2003). In one study, twelve adult horses were challenged with virulent *R. equi* by intrabronchial inoculation of the right lung. The clearance of *R. equi* from the lung was associated with an influx of leucocytes, primarily lymphocytes, at 7 and 14 days after inoculation. The number of lymphocytes recovered in bronchoalveolar lavage fluid (BALF) from the contralateral lung was significantly less than in that recovered from the inoculated lung. Significant elevations

were seen in $CD4^+$ and $CD8^+$ lymphocytes in BALF from the right lung 7 days after infection. The left and right lungs were then inoculated with soluble *R. equi* antigen or recombinant VapA. The right lung response was more marked than the left, and the right lung was more sensitive to the antigens than the left (Hines *et al.* 2001). This study suggests that the cellular response to virulent *R. equi* challenge may be compartmentalised.

1.7.4. T lymphocyte populations and cytokine expression in the lung and peripheral blood of foals

Foals up to 10 weeks of age have been shown to have a significantly lower proportion of T lymphocytes and higher proportion of macrophages in BALF than their dams (Balson *et al.* 1997). Lymphocyte subset analysis of these samples showed that the difference can be attributed to lower numbers of $CD8^+$ lymphocytes in foals. The proportion of $CD4^+$ cells was also lower in foals, but this difference was only significant in the samples from foals less than one week of age. Studies examining circulating T lymphocytes in the foal have noted that the number of circulating $CD4^+$ and $CD8^+$ T cells in peripheral blood increase linearly up to 3 months of age (Flaminio *et al.* 1999). Studies of the $CD4^+$:$CD8^+$ ratio in the blood in affected and unaffected foals have illustrated that foals with *R. equi* pneumonia have a significantly lower $CD4^+$:$CD8^+$ ratio (Chaffin *et al.* 2004). Examination of cytokine expression in peripheral blood mononuclear cells (PBMC) of foals during the first four weeks of life has shown that they have significantly increased expression of IFN-γ, IL-1α and transforming growth factor beta 1 (TGF-β1) with age (Boyd *et al.* 2003). The relative deficiency of T lymphocytes in the lungs, the increasing numbers of circulating T lymphocytes by 3 months of age and the evidence of enhanced cytokine expression are all suggestive of a naïve immune system developing in response to environmental antigenic stimuli. These observations may also, in part, explain the age-related susceptibility of foals to *R. equi* pneumonia. The association between a low $CD4^+$:$CD8^+$ ratio and *R. equi* pneumonia implies a central role for $CD4^+$ T cells in immunity to the disease and that a $CD4^+$ Th1 cell response is critical in the foal's ability to clear the infection. Studies comparing the cytokine profiles in the lungs of foals experimentally infected with virulent *R. equi* with those of foals infected with an avirulent strain showed that there was downregulation of IFN-γ in $CD4^+$ lymphocytes from the

bronchial lymph nodes and upregulation of IL-10 in lung tissue, suggesting that there was an ineffective Th2 response in foals infected with the virulent strain (Giguere *et al.* 1999b) and that virulent *R. equi* may have an immunomodulating effect on cell-mediated imunity, promoting the development of lung pathology in the foal.

1.8. Immunology of *R. equi* infections

1.8.1. Immunogenic virulence associated proteins

Most horses have antibodies to *R. equi* (Hietala *et al.* 1985; Takai *et al.* 1985). As virulent *R. equi* occurs in most horse breeding farm environments, regardless of the prevalence of disease (Takai *et al.* 1991c; Martens *et al.* 2000), most adult horses develop some degree of an immune response against it. Most foals acquire antibodies against VapA from their dam via colostrum and also seroconvert after vaccination or natural exposure to virulent *R. equi* (Takai *et al.* 1996c; Fontanals *et al.* 1997; Prescott *et al.* 1997). Foals that recover from *R. equi* pneumonia develop a strong antibody response to VapA. Foals that do not experience clinical signs also produce antibody to VapA, presumably due to exposure through the gastrointestinal tract and/or subclinical respiratory tract infection. Serum samples from foals that have died of *R. equi* pneumonia fail to react with VapA in immunoblots, implying that the ability of the foal to mount a VapA antibody response may determine the outcome of a respiratory infection with *R. equi* (Fontanals *et al.*1997).

An avirulent *R. equi* containing a shuttle vector (pMH1), into which the *vapA* gene had been inserted, was used to investigate the role of the virulence plasmid and the VapA proteins in intracellular survival and virulence of *R. equi* (Giguere *et al.* 1999a). This recombinant *R. equi* did not induce lung pathology and was effectively cleared from the lungs of experimentally infected mice or foals. No expression of VapA was detected by immunostaining lung tissue of the animals infected with the recombinant strain. The results of this study show that the *vapA* gene alone is not sufficient to confer virulence for horses on *R. equi*. No serological testing was performed on the animals infected with the recombinant and the animals were not challenged with virulent *R. equi.*

Infected foals also produce antibody against other Vaps. Immunoblotting assays have demonstrated that VapC, VapD and VapE can be recognised by infected foals (Byrne *et al.* 2001; Hooper-McGrevy *et al.* 2001). Foals that received purified anti-VapA and anti-VapC immunoglobulins intravenously had less severe clinical signs and a later onset of *R. equi* pneumonia after experimental infection than untreated foals. However Vap specific immunoglobulins are not totally protective against disease (Hooper-McGrevy *et al.* 2001).

Adult horses challenged with virulent *R. equi* have an anamnestic increase in IgG of all isotypes against *R. equi* cells and VapA (Lopez *et al.* 2002). The titres of the IgG isotypes that preferentially opsonise bacteria and fix complement (IgGa and IgGb) were shown to be dramatically enhanced after challenge. Thus the adult horse appears to generate a protective immune response, suggesting the potential for immunisation of the foal through the preferential stimulation of specific IgG isotypes against VapA. The antibody response to VapA and other Vap proteins may mediate an effector mechanism that prevents the progression of *R. equi* pneumonia in the foal (Hooper-McGrevy *et al.* 2001)

1.8.2. Antibody and the age related incidence of *R. equi* pneumonia

Foals acquire maternally derived antibody against *R. equi* from colostrum in the first hours of life. The age related susceptibility of foals to *R. equi* pneumonia is thought to be associated with waning of this maternally derived antibody (Hines and Hietala 1996; Ainsworth 1999). The concentration of maternal antibody in the foal declines from a maximum at 24 hours post-suckling until 8-12 weeks of age, when antibody production by the foal causes immunoglobulin concentrations to rise until the foal reaches 4-6 months of age (Hietala *et al.* 1985). The period of lowest circulating antibody concentration in the foal corresponds to the age at which the prevalence of *R. equi* pneumonia is highest (Zink *et al.* 1986; Prescott 1991).

An alternative hypothesis to explain the age-related incidence of *R. equi* pneumonia has been proposed (Horowitz *et al.* 2001). These investigators observed that the age of onset of clinical signs of *R. equi* pneumonia and the age at death due to *R. equi* pneumonia in foals followed a log-normal distribution and therefore fitted Sartwell's model. Sartwell

observed that the incubation period of an infectious disease originating from a point-source exposure follows a log-normal distribution (Sartwell 1950). Horowitz *et al.* hypothesised that, as the only common event for all foals in their study was birth, the period immediately after birth was the time at which the point-source exposure to *R. equi* occurred. An incubation period of 49 days was estimated in this study. This estimate is much greater than that observed in experimental infections of foals, where dose dependent incubation periods of between 6 and 13 days have been observed following a single intratracheal inoculation (Wada *et al.* 1997) and an incubation period of approximately 18 days has been seen in foals repetitively inoculated intranasally with *R. equi* (Barton and Embury 1987). Given that experimental models suggest a shorter incubation period, an alternative hypothesis might be that the log-normal distribution of the age of onset of clinical signs and the age at death reflects not a point-source exposure to *R. equi*, but rather the point-source exposure to the protective effects of colostrum. Thus the analysis of Horowitz *et al.* may rather reinforce the very significant role that transfer of colostral antibody plays in the time of onset and severity of *R. equi* pneumonia.

1.9. Immunisation of foals against *R. equi* pneumonia

Oral and parenteral administration of killed *R. equi,* live virulent *R. equi* and VapA enriched protein preparations have been trialled and shown to be either ineffective or impractical for immunoprophylaxis (Prescott *et al.* 1979, Chirino-Trejo *et al.* 1987, Prescott *et al.* 1997). The oral immunisation of foals with live virulent *R. equi* provided very effective protection against experimental infection with an aerosol of virulent *R. equi* (Chirino-Trejo *et al.* 1987). The use of such an oral vaccine would result in widespread dissemination of virulent *R. equi* in the environment and thus is impractical and potentially dangerous. However, the capacity to induce protective immunity by administering the live pathogen strongly suggests the importance of cell mediated immunity in protection against *R. equi*. The incidental ingestion of virulent *R. equi* from the environment by foals may provide some degree of protection against *R. equi* pneumonia.

European researchers have suggested that infection with equine herpesvirus type 2 (EHV 2) is a predisposing factor for *R. equi* pneumonia in foals (Nordengrahn *et al.* 1996). Foals on farms with endemic *R. equi* pneumonia were frequently infected with EHV 2

(Palfi *et al.* 1978; Belak *et al.* 1980) and it has been suggested that this viral infection may temporarily suppress cell mediated immunity, thus increasing susceptibility to *R. equi* (Nordengrahn *et al.* 1996; Jensen-Waern *et al.* 1998). Immunisation of foals and mares with killed *R. equi* and a combined *R. equi*/EHV 2 vaccine was found to protect foals from *R. equi* pneumonia on farms with endemic disease (Varga *et al.* 1997). Vaccination against EHV 2 only was found to reduce the prevalence and severity of *R. equi* pneumonia (Nordengrahn *et al.* 1996). EHV 2 vaccination may reduce EHV 2 mediated suppression of the cell mediated immune response and hence decrease the foal's susceptibility to *R. equi* pneumonia.

Both cellular and humoral immunity seem to influence the outcome of infection and protection from disease. Effective protection of foals through immunisation is likely to require induction of an appropriate immune response involving both arms of the immune system and, importantly, will need to be established within the first month of the foal's life. Exploring methods to enhance the immune response of the foal to the pathogen, possibly using immunisation strategies that are not only antigen-specific but that also drive specific cytokine responses (i.e. IFN-γ and IL-2) so as to produce an antigen-specific Th1 cell response, may be the key to effective immunoprophylaxis against disease caused by *R. equi*.

1.10. Diagnosis

There is currently no single diagnostic tool available that is highly specific or sensitive for the diagnosis of *R. equi* infection. Diagnosis of *R. equi* pneumonia is based on clinical signs, haematological abnormalities, radiographic and/or ultrasonographic evidence of lung pathology, and recovery of *R. equi* from tracheal aspirates.

Early recognition of foals with *R. equi* pneumonia, and their subsequent isolation and treatment, will reduce losses, minimise the spread of virulent *R. equi* and limit the cost of therapy. On a Canadian farm with endemic disease, twice weekly auscultation of the lungs allowed early diagnosis of *R. equi* pneumonia and successful prevention of mortality (Prescott *et al.* 1989). However, this may not be practical in an extensive farming situation in Australia.

1.10.1. Ultrasonographic examination of lungs

Ultrasonography is widely available on many large horse farms. Its use as a diagnostic aid for detecting cases of *R. equi* pneumonia is gaining popularity, especially on farms with a high prevalence of disease.

Ultrasonographic examination allows visualisation of the lateral pleural surface of the lung and presumptive diagnosis. Early diagnosis is likely to promote early treatment, ultimately reducing mortality rates and the duration of treatment (J.F. Freestone, personal communication). Despite its increased usage, ultrasonography has limitations. The lesions induced by *R. equi* are relatively specific, but not pathognomonic, and other bacterial causes of pneumonia in foals may cause similar lesions. Only lesions on the periphery of the lung are detectable, although most abscessation due to *R. equi* infection involves the periphery of the lung (Reef 1991). However, cases may be missed if abscessation only occurs in the deep lung parenchyma or on the mediastinal surface (Reef 1998; Ramirez *et al.* 2004).

1.10.2. Serological assays

A variety of serological tests have been investigated as diagnostic tools for detection of *R. equi* pneumonia in foals. These tests include agar gel immunodiffusion (AGID), synergistic haemolysis inhibition (SHI) and enzyme-linked immunosorbent assays (ELISA).

The serological diagnosis of *R. equi* infection is problematic due to the ubiquitous distribution of both virulent and avirulent *R. equi* in the environment and, thus, the widespread exposure of foals, with subsequent antibody production, in the absence of clinical disease. The inability of these tests to differentiate between maternally derived antibodies and those produced by the foal after exposure further confound interpretation. Studies conducted to evaluate the clinical usefulness of several serological tests have found them to be an unreliable means of diagnosis (Martens *et al.* 2002b; Giguere *et al.* 2003b). Recently an ELISA using an eleven amino acid peptide epitope of VapA has shown promise as an effective serological assay for detection of active *R. equi* infection in foals.

Antibody in serum samples from 51 diseased foals all recognised this linear B cell epitope of VapA, whilst antibody in the sera from 3 horses that had recovered from *R. equi* infection 10 months previously and 16 foals with no history of disease due to *R. equi* did not (Vanniasinkam *et al.* 2001).

1.10.3. Culture of *R. equi* from the respiratory tract

Treatment of *R. equi* pneumonia is often expensive and antibiotics used to treat *R. equi* may not be effective against other causes of lung abscessation. Therefore, culture of tracheal lavage fluid is still recommended for confirmation of the aetiology, even when clinical, haematological, radiographic and ultrasonographic signs suggest *R. equi* pneumonia (Lavoie *et al.* 1994). Culture from tracheal aspirates is considered the 'gold standard' for definitive diagnosis of *R. equi* pneumonia.

The preferred method for obtaining a tracheal aspirate is an invasive transtracheal approach. This approach bypasses the upper respiratory tract and is commonly used for the evaluation of pulmonary disease in the horse (Mansmann and Knight 1972; Beech 1981). A nasotracheal technique for obtaining tracheal aspirates for bacteriological culture has been evaluated and yielded rates of recovery of *R. equi* from foals with clinical signs of *R. equi* pneumonia similar to those obtained using the transtracheal method. Both of these techniques have been demonstrated to achieve sub-optimal recovery of *R. equi* from clinically affected foals, with only 64% of foals with radiographic evidence of lung abscessation and 62% of foals positive for culture of *R. equi* at necropsy yielding positive cultures from tracheal lavage fluid in an early study (Hillidge 1987). In a more recent survey a similar proportion of foals with clinical signs of *R. equi* pneumonia yielded positive cultures for *R. equi* from tracheal lavage fluid obtained by either the transtracheal or the nasotracheal method (66.7% and 61.4%, respectively) (Hashikura *et al.* 2000). There is always a likelihood that pneumonia is caused by other organisms. *Streptococcus* spp., *Staphylococcus* spp., *Escherichia coli, Bacillus* spp., *Mucor* spp. and *Aspergillus* spp. are all potential respiratory pathogens that have been isolated from respiratory tract samples from foals with pneumonia (Hashikura *et al.* 2000). The majority of these organisms have been cultured from foals older than 2 months of age (Lavoie *et al.* 1994).

R. equi may also be recovered from the respiratory tract of asymptomatic foals. In one study 77/216 (35.6%) foals without signs of respiratory disease yielded tracheal lavages that were positive on culture for *R. equi* (Ardens *et al.* 1986). As this study was performed prior to the recognition of virulent and avirulent *R. equi*, these results need to be interpreted carefully. The recovery of *R. equi* from the respiratory tract of a foal, especially when virulent and avirulent isolates are not distinguished, needs to be interpreted in the context of clinical signs, haemotological, radiographic and ultrasonographic findings. However, the failure to recover the *R. equi* from tracheal aspirates does not eliminate a diagnosis of *R. equi* pneumonia (Hillidge, 1987).

1.10.4. Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) assays amplify small quantities of DNA recovered from a sample. The *vapA* gene of virulent *R. equi* has been detected in tracheal lavage fluid (Sellon *et al.* 1997). The *vapA* PCR has been compared to serology (AGID) and microbiological culture, with clinical diagnosis by the attending clinician on the basis of all available information about each case used as the standard for the calculation of sensitivity and specificity (Sellon *et al.* 2001). The study was performed on tracheal lavage fluid from 46 foals. The *vapA* PCR had a diagnostic sensitivity of 100% and a specificity of 90.6%, whilst the sensitivity and specificity for standard microbiological culture of tracheal lavage fluid were 57.1% and 93.8%, respectively. AGID had a sensitivity of 62.5% and a specificity of 75.9%. Thus, PCR on tracheal aspirates is more sensitive than the ‘gold standard’ of microbiological culture for diagnosing *R. equi* pneumonia. The added advantage of PCR is that results are available more rapidly (within 24-48 hours) than microbiological culture, which can take up to 7 days for definitive identification.

1.11. Treatment

R. equi are sensitive to a wide range of antimicrobial agents *in vitro,* but their intracellular habitat prevents many antimicrobials reaching the site of infection. All 17 foals with *R. equi* pneumonia in one study were treated with a combination of penicillin and gentamicin and all of the foals died, even though all isolates were sensitive to gentamicin *in vitro* (Sweeney *et al.* 1987).

1.11.1. Erythromycin and rifampicin

The combination of erythromycin and rifampicin is the antimicrobial treatment of choice for *R. equi* infections in foals. Its use has led to a dramatic reduction in foal mortality (Hillidge 1987; Sweeney *et al.* 1987). Although these two agents are bacteriostatic against *R. equi in vitro*, they are highly effective *in vivo* (Nordmann and Ronco 1992). They have synergistic effects both *in vitro* and *in vivo* (Prescott and Nicholson 1984; Nordmann *et al.* 1992). When administered in combination, the synergistic action of the two agents also reduces the likelihood of *R. equi* developing resistance to either antimicrobial (Nordmann and Ronco 1992), and in particular rifampicin (Takai *et al.* 1997). The recommended dosage regimen for the combination antimicrobial therapy is 25 mg erythromycin/kg orally every 8 or 12 hours and 5 mg rifampicin/kg orally every 12 hours or 10 mg/kg every 24 hours. Treatment duration may range from 4 to 9 weeks (Hillidge 1987). Veterinarians have observed a reduction in treatment duration when early diagnoses have been made, for example by using routine ultrasonographic examination (J.F. Freestone, personal communication).

The administration of erythromycin alone has been reported to result in successful treatment of foals with *R. equi* pneumonia (Wilson 1992), but is not recommended, in part due to the likelihood of development of resistance, but also because of the poor efficacy of erythromycin in the intracellular environment, even though high concentrations accumulate within granulocytes and alveolar macrophages (Prescott and Sweeney 1985; Maurin and Raoult 1993). In order to reduce the likelihood of antimicrobial resistance, use of rifampicin alone is also discouraged (Nordmann and Ronco 1992; Takai *et al.* 1997). Rifampicin resistance was seen in *R. equi* isolated from a foal after unsuccessful therapy for a month with rifampicin alone (Takai *et al.* 1997). Even though the vast majority of *R. equi* isolates from infected foals are sensitive to erythromycin and rifampicin, strains resistant to either drug alone have been encountered (Giguere and Prescott 1997; Takai *et al.*1997). Resistance to both rifampicin and erythromycin has also been observed during the treatment of *R. equi* pneumonia in a 10-month-old foal with reactive arthritis (Kenney *et al.* 1994).

A variety of adverse drug reactions have been associated with the administration of erythromycin and rifampicin to foals. Most of these adverse reactions are minor, but some are life threatening. Faecal softening is commonly seen in foals given erythromycin and rifampicin, but this effect is usually self-limiting and does not necessitate cessation of therapy (Giguere and Prescott 1997). The initiation of treatment with erythromycin and rifampicin may lead to partial anorexia, mild colic and bruxism, signs associated with gastric ulceration. These signs usually resolve after temporary cessation of treatment (Prescott and Hoffman 1993). During very hot weather an idiosyncratic reaction, characterised by severe hyperthermia, tachypnoea and increased circulating concentrations of liver enzymes, has been reported in foals treated with erythromycin (Wilson 1992; Prescott and Hoffman 1993). Death often occurs as a consequence of this hyperthermic reaction. This adverse reaction to erythromycin is a concern to veterinarians treating foals for *R. equi* pneumonia in warm climates. Mild to severe cases of colitis have been reported in mares nursing foals that are being treated with erythromycin, presumably due to coprophagic behaviour and thus ingestion of sufficient active erythromycin to alter the intestinal flora of the mare (Wilson 1992, Gustafsson *et al.* 1997). *Clostridium difficile* has been suggested as the aetiological agent in these cases of erythromycin-induced colitis in Sweden (Gustafsson *et al.* 1997).

1.11.2. Azithromycin and other new antimicrobial alternatives

An azalide antimicrobial commonly used in human medicine (Whitman and Tunkel 1992), azithromycin, is currently being advocated as a useful alternative to erythromycin in combination erythromycin-rifampicin therapy for foals diagnosed with *R. equi* pneumonia (Jacks *et al.* 2001). Azithromycin has higher oral bioavailability, a larger apparent volume of distribution, and better tissue and phagocytic cell uptake than erythromycin (Girard *et al.* 1987; Baldwin *et al.* 1990; Foulds *et al.* 1990; Shepard and Falkner 1990). It has a longer elimination half-life, allowing once daily dosage and a shortened duration of treatment (Girard *et al.* 1987; Foulds *et al.* 1990; Shepard and Falkner 1990). In humans, adverse drug reactions seem to be less frequent with azithromycin than erythromycin. In the foal intravenous administration of azithromycin has induced a range of transient adverse effects immediately after injection, including yawning, trembling, ataxia and

weakness (Jacks *et al.* 2001). Cases of azithromycin-induced hyperthermia in foals treated for *R. equi* pneumonia have been reported anecdotally in Australia (J.F. Freestone, personal communication). Cross-resistance occurs between erythromycin and azithromycin, so azithromycin is unlikely to be effective against an erythromycin resistant strain.

The high and sustained intracellular concentration achieved by azithromycin is credited with its high *in vivo* efficacy against several intracellular pathogens, including *Legionella* spp., *Salmonella* spp., *E. coli* Shigella spp. and *Mycobacterium avium* (Butler and Girard 1993; Rakita *et al.* 1994; Koletar *et al.* 1999; Frenck *et al.* 2000; Jonas *et al.* 2000). The suggested dosage of azithromycin for the treatment of *R. equi* pneumonia in foals is 10 mg/kg orally once a day for 5 days then every second day (Jacks *et al.* 2001). Its use in combination with rifampicin is encouraged as this increases the efficacy of treatment and discourages the development of drug resistance.

Clarithromycin, an antimicrobial related to azithromycin, has also been suggested as an alternative to erythromycin. Clarithromycin was shown to be more active than azithromycin against *R. equi*, with a minimal inhibitory concentration (MIC) for 90% of isolates of 0.12 μg/ml, compared to 1 μg/ml for azithromycin (Jacks *et al.* 2003). It has a shorter half-life (4.81 hours) than azithromycin, but no adverse reactions have been observed in foals administered this drug. Pharmacokinetic studies suggest a dosage regimen of 7.5 mg/kg every 12 hours would be sufficient for treatment of a foal with *R. equi* pneumonia (S. Jacks, personal communications). As for other macrolides, its use of clarithromycin in combination with rifampicin would be expected to be beneficial.

R. equi are highly susceptible *in vitro* to the imidazoles bifonazole, clotrimazole, econazole and miconazole, with MICs of under 1 μg/ml (Dabbs *et al.* 2003). Thus, the imidazoles may be useful for treatment of erythromycin and rifampicin resistant strains.

1.12. Aims of work reported in this thesis

The work reported in this thesis aimed to investigate the effects of the horse breeding farm environment on the ecology of virulent *R. equi* and the epidemiology of *R. equi* pneumonia. The development of a technique to measure environmental concentrations of virulent *R. equi* on farms was central to this investigation. The traditional methods of soil sampling and a novel air sampling method were used to assess the environmental virulent *R. equi* population on farms. These measurements formed the basis of an investigation of the relationship between the concentration of virulent *R. equi* and the proportion of *R. equi* that were virulent on the farm and disease prevalence and severity. Environmental and management variables were compared to the environmental burden of virulent *R. equi* and the disease prevalence and severity on farms to identify factors of importance in the ecology of the organism and the prevalence and severity of disease on farms.

The scope of the project allowed a comparison of *R. equi* selective medium to be performed. The development of an air sampling method also allowed an investigation of an alternative route of transmission of virulent *R. equi* in a herd.

Chapter 2

Identification and differentiation of avirulent and virulent *R. equi* using selective media and colony blotting DNA hybridisation

2.1. Introduction

The use of selective agar media has facilitated the isolation of *R. equi* from environmental samples (Woolcock *et al.* 1979; von Graevenitz and Punter-Streit 1995) but does not allow differentiation of virulent and avirulent isolates. Determination of the virulence of an *R. equi* isolate has required the use of PCR to detect the presence of the virulence associated gene *vapA* or an immunoblotting assay to detect the 15-17 kDa VapA protein (Takai *et al.* 1993b; Haites *et al.* 1997).

The use of PCR and immunoblotting techniques to investigate the concentration and proportion of virulent *R. equi* in environmental samples is labour intensive and thus limits the number of bacterial isolates able to be assessed in any environmental or ecological study of *R. equi* on horse farms.

The aim of the work described in this chapter was to develop a technique enabling individual colonies to be identified as *R. equi* and then to characterise these colonies as virulent or avirulent using simple colony blotting and DNA hybridisation techniques.

2.2. Materials and methods

2.2.1. Development of method using defined cultures

a. Bacteria and media

Two *R. equi* isolates obtained from tracheal aspirates from foals (Haites *et al.* 1997) were used in this study. Isolate 7 was a *R. equi* isolate that contained the virulence plasmid, as confirmed by PCR for the *vapA* gene. Isolate 128 was an *R. equi* isolate that did not contain the *vapA* gene. Both isolates were PCR positive for the *rrnA* gene, using primers that amplified either the whole gene or the conserved region of the gene (Sellon *et al.* 1997; Morton *et al.* 2001). Isolates were identified as *R. equi* on the basis of formation of typical mucoid salmon-pink colonies on SBA after growth at 37°C for 48 hours, production of catalase and urease, and an inability to ferment lactose.

Laboratory strains of *Corynebacterium renale* (isolate number CC144), *Corynebacterium pseudotuberculosis* (isolate number CC68) and *Nocardia asteroides* (isolate number CC23) were used as negative controls in this experiment to assess the specificity of the *rrnA* gene probe.

All bacteria were grown in 10 ml LB broth for 24 to 48 hours in a shaking incubator at 37°C. The resulting cultures were diluted 10^5 fold and 50 µl was spread onto separate sheep blood agar (SBA) plates. *R. equi* isolates were spread on one half of each plate and one of the other strains was plated on the other half, with the exception of *C. renale*. Each half plate contained between 10 and 200 colonies.

The isolates were also plated onto two different *R. equi* selective agar media. These were the NANAT medium (Woolcock *et al.* 1979) and the CAZ-NB medium (von Graevenitz and Punter-Streit 1995).

b. Colony blotting

Plates were incubated at 37°C for 48 hours and colonies were then blotted onto circular nylon membranes (Hybond-N+, Amersham Pharmacia Biotech, Buckinghamshire, UK) by applying the membranes to the surface of the agar for at least 1 minute. The membranes were than transferred into a 500 ml glass beaker containing 5 ml of 10 mM tris (hydroxymethyl) aminomethane hydrogen chloride (Tris-HCl), 1 mM ethylenediaminetetraacetic acid (EDTA) (pH 8.0) and lysozyme at 80 mg/ml (lysis buffer). The membranes were incubated in lysis buffer at 37°C for 10 minutes in a shaking incubator at 200 rpm to facilitate lysis of the cell walls. Membranes were transferred, colony side up, onto Whatman 3MM paper soaked with denaturing solution (0.5 M NaOH, 1.5 M NaCl) for 5 minutes, then transferred onto Whatman 3MM paper soaked with neutralising solution (1.5 M NaCl, 0.5 M Tris-HCl (pH 7.4)) for 5 minutes before transfer onto Whatman 3MM paper soaked with 2 x SSC (20 x SSC is 3 M NaCl and 0.3 M sodium citrate) solution. Membranes were left to air dry for at least 30 minutes, wrapped in Cling Wrap and then placed onto an ultraviolet transilluminator, colony side down, for 5 minutes to fix the DNA to the membrane.

c. Production of radiolabelled PCR products

PCR products from the *vapA* and *rrnA* genes were generated from individual colonies of virulent and avirulent *R. equi* strains as described previously (Haites *et al.* 1997; Morton *et al.* 2001). The template for PCR was prepared by emulsifying bacterial colonies that had been grown at 37°C for at least 48 hours on SBA in the PCR reaction mixture. The PCR reaction contained 0.4 U *Taq* polymerase (Roche Diagnostic GmBH, Mannheim, Germany) in 25 μl of buffer provided by the manufacturer, with the addition of $MgCl_2$ to 3 mM. Each dNTP was included at a concentration of 0.4 mM, and forward and reverse primers (Table 2.1) at 2.5 μM each.

Table 2.1: Oligonucleotide primers used to differentiate virulent and avirulent *R. equi*

Primer name	Gene	Sequence (5' to 3')	Location
VapA Forward	*vapA*	GACTCTTCACAAGACGGT	6
VapA Reverse	*vapA*	TAGGCGTTGTTGTGCCAGCTA	569
rrnA Forward	*R. equi rrnA*	GCTTAACACATGCAAGTCGAAC	29
rrnA Reverse	*R. equi rrnA*	CCGGTACGGCTACCTTGTTA	1479

Genbank accession numbers for *vapA* and *R. equi rrnA* sequences are D21236 and X80614 respectively

All PCRs were performed in a Hybaid thermocycler (Hybaid, Ashford, UK). To produce the *vapA* product the cycler was programmed for 40 cycles of 94°C for 1.5 minutes, 57°C for 1 minute and 72°C for 2 minutes. For the *rrnA* product the cycler was programmed for 40 cycles of 94°C for 1 minute, 52°C for 1 minute and 72°C for 1.5 minutes. PCR products were purified using a QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). The PCR products were ligated to the pGEM-T vector (Promega, Madison, WI, USA) and the ligated products were used to transform *E. coli* DH5α cells by electroporation. Transformants were selected by inoculation onto LB plates containing 100 μg ampicillin/ml and 32 μg 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal)/ml and incubation overnight at 37°C. Clones of *E. coli* carrying plasmids containing each PCR product were stored in 50% glycerol at -70°C. Plasmid DNA was purified from these clones using the method of Del Sal *et al.* (1989). PCRs using the purified plasmid DNA as template were performed as described above.

The PCR products were purified using a QIAquick PCR Purification Kit, electrophoresed in a 1% agarose gel and compared to known markers in order to determine the concentration of DNA in the purified product.

The two PCR products were then used to construct ^{32}P labelled probes using a Random Primed DNA Labelling kit (Roche Diagnostic GmBH, Mannheim, Germany) according to the manufacturer's recommendations.

d. DNA hybridisation

Colony blots were incubated for 2 hours at 68°C in Church buffer (0.5 M Na_2HPO_4 (pH 7.2), 1% bovine serum albumin (BSA), 1 mM EDTA, 7% sodium dodecylsulphate (SDS)). The denatured probe (either *vapA* or *rrnA*) was added and allowed to hybridise to membranes overnight at 68°C. Membranes were then washed three times for 10 minutes each at 68°C in 2 x SSC, 0.1% SDS, then a further three times for 10 minutes each at 68°C in 1 x SSC, 0.1% SDS, and finally for 10 minutes at 68°C in 0.1 x SSC, 0.1% SDS, then autoradiographed. Probes were stripped from membranes by boiling in 0.1% SDS to enable hybridisation with the alternate probe.

2.2.2. Development of method using environmental samples

a. Air sampling

A portable air sampling system (M Air T, Millipore, Saint-Quentin-Yveline, France) loaded with a cassette containing selective agar (NANAT) was placed 5 cm above the soil surface. Samples were taken in the holding pens (small yards used to hold horses temporarily while awaiting veterinary or farriery treatment) and lanes (paths along which horses were moved to and from pens and paddocks) on two Thoroughbred horse farms in northeastern Victoria early in the 2000 foaling season (farm A and B). A 500 litre (l) air sample was collected onto the selective media and the cassettes then incubated at 37°C for 48 hours.

b. Colony blotting of environmental samples

The cassettes used for air sampling were filled to the rim to form a meniscus. Blotting of colonies could thus be performed by placing plates colony side down on a nylon membrane sheet. Four sample cassettes and two control plates containing avirulent and virulent *R. equi* were blotted onto a single 16 x 21 cm nylon membrane sheet. This sheet was treated as described above (Section 2.2.1) with the following modifications. The sheet was incubated in 20 ml of lysis buffer in a plastic container of the same dimensions as the sheet. To prevent excess smearing of colonies during shaking a second nylon

membrane was placed over the blotted membrane. This membrane was discarded once lysis was complete. The membrane was then treated as described previously with denaturing, neutralising and SSC solutions. The cassettes were retained and stored at 4°C for further analysis of colonies.

2.3. Results

2.3.1. Development of blotting and hybridisation method on defined cultures

Colony blotting and DNA hybridisation using the *vapA* PCR product as a radiolabelled probe differentiated the virulent *R. equi* strain from the avirulent strain. Both strains were detected by the *rrnA* PCR product probe (Figure 2.1).

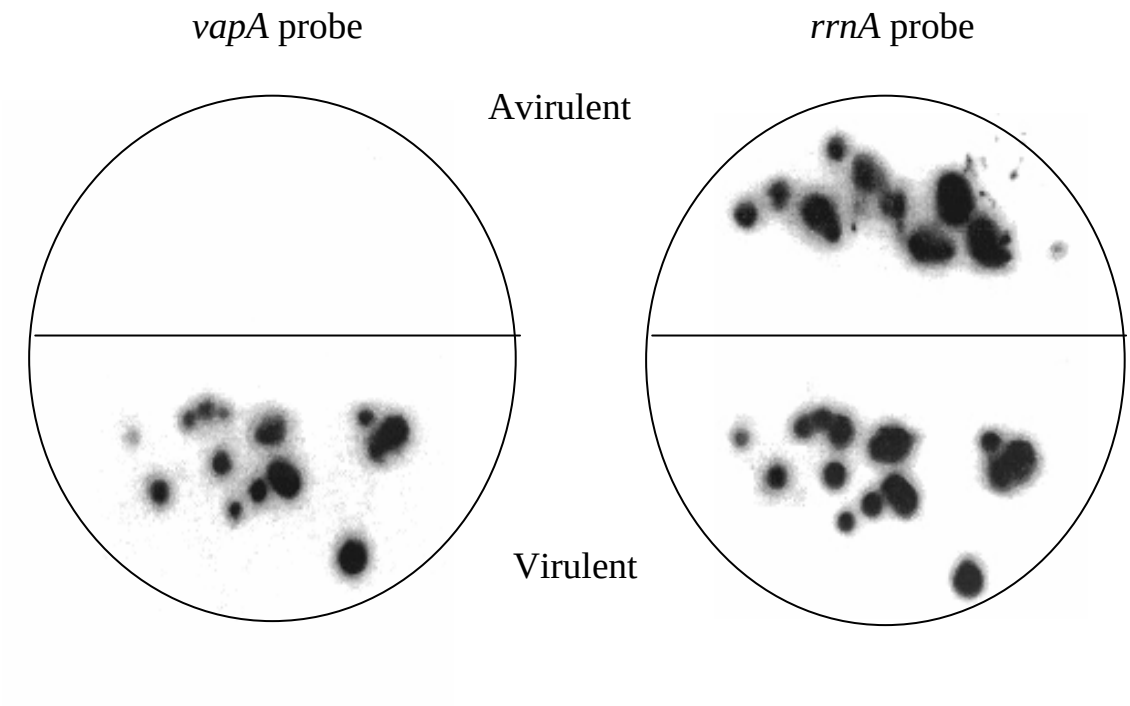

Figure 2.1: Hybridisation of *rrnA* and *vapA* probes to colony blots of virulent and avirulent *R. equi*.

The *vapA* probe did not hybridise to any of the 3 other actinomycetes tested. Similarly the *rrnA* probe did not recognise the three other actinomycetes (Figure 2.2). Of the 3 actinomycetes, only *N. asteroides* were able to grow on the selective media.

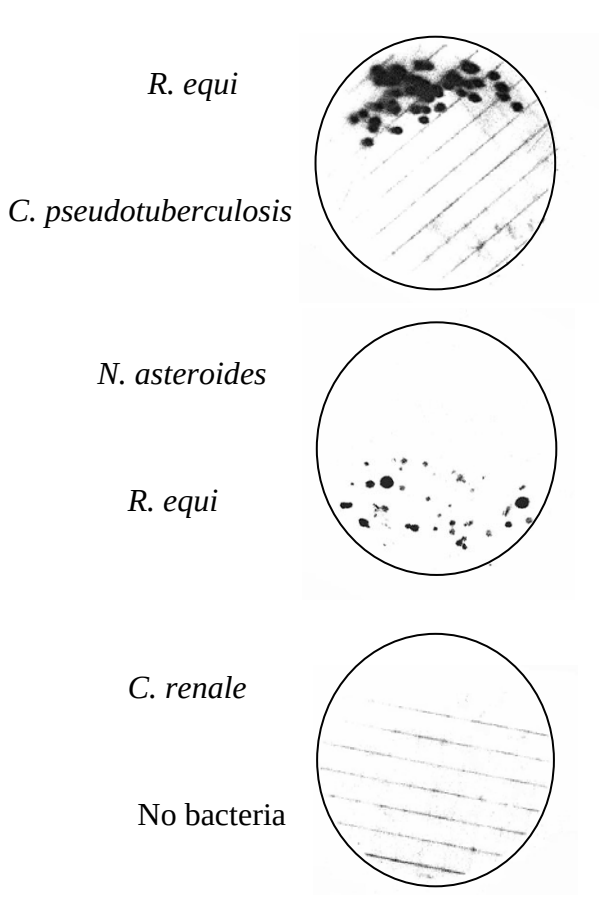

Figure 2.2: Hybridisation of *R. equi rrnA* probe to *R. equi, C. pseudotuberculosis, C. renale* and *N. asteroides*.

2.3.2. Development of hybridisation technique using environmental samples

The capacity of this technique to identify and differentiate *R. equi* was further assessed on environmental samples from horse farms. Environmental air samples from the holding pen area of farm A yielded 15 bacterial colonies and those from the lane yielded 23 colonies. Environmental air samples from the holding pen area of farm B yielded 106 bacterial colonies and those from the lane yielded 5 colonies. The *rrnA* probe detected 4 colonies in samples from farm A (3 from the holding pen and 1 from the lane) and 10 colonies in samples from farm B (9 from the holding pen and 1 from the lane) (Figure 2.3).

Of the 14 colonies that hybridised to the *rrnA* probe, six did not have typical *R. equi* colonial morphology.

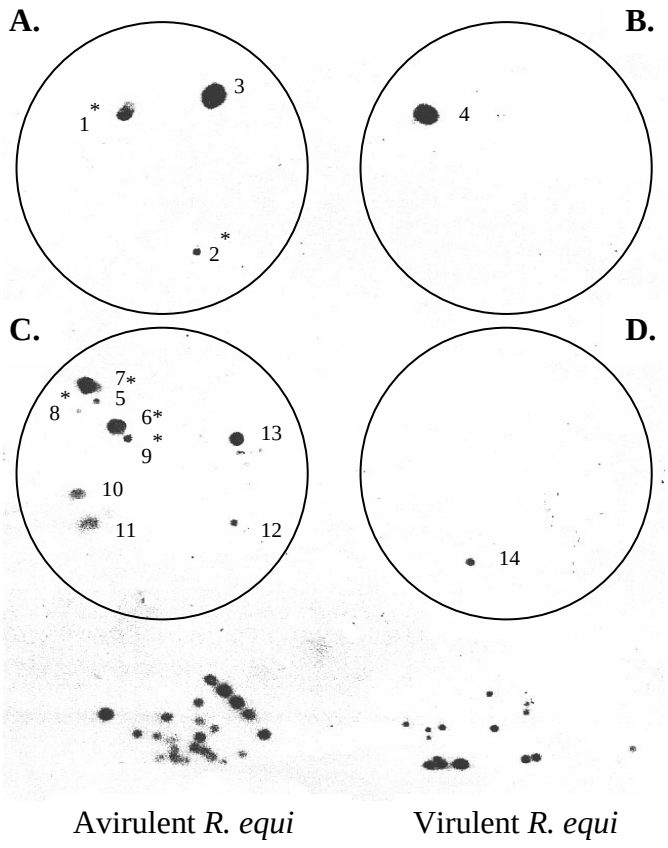

Figure 2.3: Hybridisation of *R. equi rrnA* probe to blots of bacteria cultured from environmental air samples.
Hybridisation was conducted at 68°C, and the final wash was in 0.1 x SSC, 0.1% SDS. Environments assessed were a pen (A) and lane (B) on farm A and a pen (C) and lane (D) on farm B. Half plates of virulent and avirulent *R. equi* were also blotted. Those colonies with an asterisk had a colonial morphology that differed from *R. equi*. Colonies 1, 2, 5 and 6 were used to modify the hybridisation technique. Colonies 8 and 9 had the same morphology as colony 5.

R. equi colonies are typically mucoid and grey on NANAT media. The atypical colonies detected by the *rrnA* probe were black, discrete and circular, with one type having a defined halo in its centre (isolates 1 and 2). Reprobing with the *vapA* probe showed that none of these atypical isolates contained the virulence plasmid, while one typical *R. equi* colony on the blot from the holding pen on farm B bound the *vapA* probe (Figure 2.4), a result confirmed by performing PCR on an emulsified colony of the isolate after subculture on SBA.

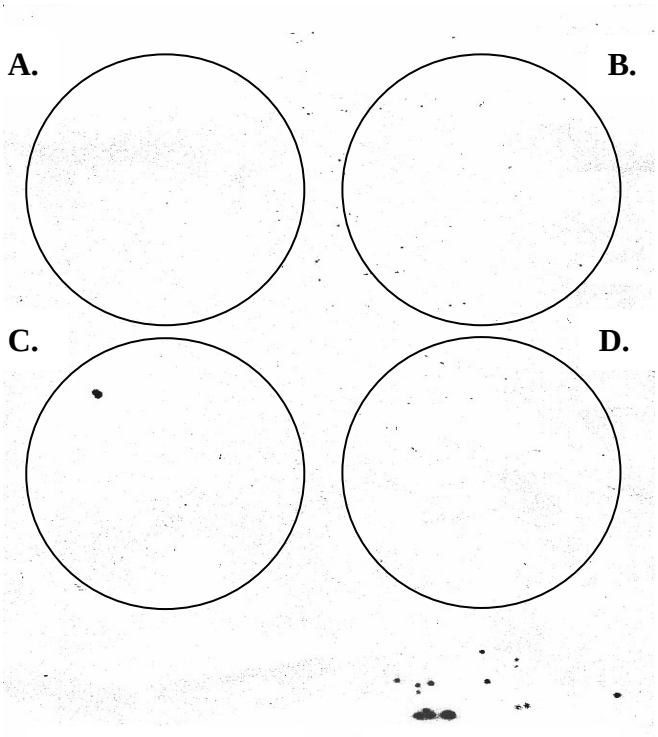

Figure 2.4: Hybridisation of *vapA* probe to blots of colonies from environmental air sample cultures. Hybridisation was conducted at 68°C, and the final wash was in 0.1 x SSC, 0.1% SDS. Environments assessed were a pen (A) and lane (B) on farm A and a pen (C) and lane (D) on farm B. Half plates of virulent and avirulent *R. equi* were also blotted. Only a single positive colony was detected.

2.3.3. Identification of cross-reactive environmental bacteria

Two isolates from each farm, representing two atypical colony types found to be cross-reactive with the *rrnA* probe, were recovered from the blotted agar cassettes, inoculated into sterile LB broth and incubated for 24 to 48 hours at 37°C in a shaking incubator at 200 rpm.

The broth cultures of each of the atypical isolates were diluted 10^5 fold and 50 µl of each was spread on cassettes containing NANAT media. Virulent and avirulent *R. equi* were also spread on cassettes containing NANAT media and the cassettes incubated at 37°C for 48 hours. Each cassette yielded between 1 and 200 colonies. These six cassettes were blotted onto a single membrane and probed with the *rrnA* probe at varying hybridisation conditions in order to optimise the differentiation of *R. equi*.

Gram smears, subculture onto SBA and selected biochemical assays were performed to aid identification of these isolates. In addition, two different PCR assays for the *rrnA* gene were performed on emulsified colonies of the isolates, as described by Sellon *et al.* (1997) and Morton *et al.* (2001). The primers used in the PCR described by Sellon *et al.* (1997) for the amplification of the conserved region of the *R. equi rrnA* gene were (forward) 5' TCGTCCGTGAAAACTTGGG 3' and (reverse) 5' CGACCACAAGG GGGCCGT 3', corresponding to bases 556 to 574 and 997 to 979, respectively, of the *rrnA* sequence. The PCR reaction was constituted as described previously.

PCR using universal *rrnA* primers was also performed on broth cultures of the unknown bacteria to facilitate identification by DNA sequencing. Bacteria were inoculated into 10 ml of tryptic soy broth and incubated for 24 hours in a shaking incubator at 37°C. One millilitre of the culture was centrifuged at 13,000 g for 1 minute, the supernatant removed and pellets resuspended in 1 ml of distilled H_2O. Two microlitres of this solution was used as PCR template in a 25 µl PCR reaction constituted as described previously. The universal *rrnA* primers used were those described at http://lyco.lycoming.edu/~newman/courses/bio32199/unknownlab.pdf for amplification of all major prokaryotic groups. The primers were 5' CGGCCCAGACTCCTACGGGAGGCAGCAG 3' and 5' GCGTGGACT

ACCAGGGTATCTAATCC 3', which amplify approximately 460 bp of the *rrnA* gene. The PCRs for both the conserved region of the *R. equi rrnA* gene and the region of the prokaryote *rrnA* gene were performed in a Hybaid thermocycler. For both reactions the cycler was programmed for 40 cycles of 94°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes.

Agarose gel electrophoresis with ethidium bromide staining was used to examine the PCR products, which were then extracted and purified using a QIAEX II Gel Extraction Kit. Nucleotide sequencing of the PCR products was performed using a Big Dye terminator cycle sequencing version 3 kit (Applied Biochemistry, Foster City, CA, USA), with the sequencing products run and analysed by the Australian Genomic Research Facility (AGRF) DNA sequencing laboratory. Sequences were then compared to those in the GenBank database using BLASTN to find species with similar *rrnA* sequences.

Both of the selective agar media used have been shown previously to allow growth of organisms other than *R. equi*. NANAT medium has been reported to allow growth of *Candida* species, coryneforms and some pseudomonads, whilst CAZ-NB medium allows growth of some *Corynebacterium* species, *Nocardia* species, *Mycobacterium* species and other *Rhodococcus* species (Woolcock *et al.* 1979; von Graevenitz and Punter-Streit 1995).

All four atypical isolates identified by the *rrnA* probe were Gram-positive bacilli. Two were small and uniform (isolates 1 and 2), whilst the other two were pleomorphic and often branching (isolates 5 and 6). Three of the four isolates (isolates 1, 2 and 6) produced pink, circular, non-mucoid colonies on SBA, whilst colonies of the other isolate (isolate 5) lacked colour on SBA. Isolate 5 was the only isolate capable of growing on CAZ-NB medium. All four isolates were catalase positive, but they did not ferment glucose and were non-haemolytic on SBA. Isolates 1 and 2 were urease positive and yielded PCR products using the *R. equi rrnA* PCR assay of Morton *et al.* (2001). None of the isolates yielded PCR products using the *R. equi rrnA* PCR assay of Sellon *et al.* (1997). None of the four isolates produced a CAMP-like reaction when inoculated onto SBA adjacent to *Listeria monocytogenes*, while all colonies that had typical *R. equi* colonial morphology produced a CAMP-like reaction when inoculated onto SBA adjacent to *L. monocytogenes*.

All four cross-reactive isolates yielded PCR products using the universal *rrnA* primers. DNA sequencing of the PCR products revealed that the sequences of isolates 1 and 2 were identical and were very similar (99%) to the corresponding region of the *rrnA* gene of *Corynebacterium ammoniagenes* (GenBank accession number X82056) (Figure 2.5a), with 88.7% sequence identity with the *rrnA* gene of *R. equi* (Figure 2.6). The *rrnA* gene sequence of isolate 5 was very similar (97%) to the corresponding region of the *rrnA* gene of *Bacillus fumarioli* (GenBank accession number AJ250057) (Figure 2.5b), with 79.1% sequence identity to the *rrnA* gene of *R. equi* (Figure 2.6). The sequence of isolate 6 was very similar (99%) to the *rrnA* gene of *Rhodococcus rhodochrous* (GenBank accession number X79288) (Figure 2.5c), with 94.6% sequence identity to the *rrnA* gene of *R. equi* (Figure 2.6). All of these species have been documented to be either soil or faecal inhabitants (Jones and Collins 1986; Lechavailer 1986; Logan *et al.* 2000).

```
C. ammoniagenes          AGGCGGCGACGGGTATCCGGCCTGAGAGGGTGTACGGACACATTGGGACTGAGATACGGC
                    241  ---------+---------+---------+---------+---------+---------+
Isolate 1 & 2            ------------------------------------------------------------

C. ammoniagenes          CCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCA
                    301  ---------+---------+---------+---------+---------+---------+
Isolate 1 & 2            ----------------------------------------------C·············

C. ammoniagenes          GCGACGCCGCGTGGGGGATGACGGCCTTCGGGTTGTAAACTCCTTTCGCTANNNNNNNNN
                    361  ---------+---------+---------+---------+---------+---------+
Isolate 1 & 2            ····················A···························TCGACGAAG

C. ammoniagenes          NNN-NNGGTGGTGACGGTAGGTAGATAAGAAGCACCGGCTAACTACGTGCCAGCAGCCGC
                    421  ---------+---------+---------+---------+---------+---------+
Isolate 1 & 2            CCACTTT···N·····N···A·A···N··N·····T·····················

C. ammoniagenes          GGTAATACGTAGGGTGCAAGCGTTGTCCGGAATTACTGGGCGTAAAGAGCTCGTAGGTGG
                    481  ---------+---------+---------+---------+---------+---------+
Isolate 1 & 2            ··N·····················································

C. ammoniagenes          TTTGTCGCGTCGTCTGTGAAATCCCGGGGCTTAACTTCGGGCGTGCAGGCGATACGGGCA
                    541  ---------+---------+---------+---------+---------+---------+
Isolate 1 & 2            ····················································

C. ammoniagenes          TAACTTGAGTGCTGTAGGGGAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATAT
                    601  ---------+---------+---------+---------+---------+---------+
Isolate 1 & 2            ····················································

C. ammoniagenes          CAGGAGGAACACCGATGGCGAAGGCAGGTCTCTGGGCAGTAACTGACGCTGAGGAGCGAA
                    661  ---------+---------+---------+---------+---------+---------+
Isolate 1 & 2            ·····································T··················

C. ammoniagenes          AGCATGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGGTGGGCGCT
                    721  ---------+---------+---------+---------+---------+---------+
Isolate 1 & 2            ------------------------------------------------------------
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Figure 2.5a: Sequence alignment and comparison of the partial sequence of the *rrnA* gene of environmental isolates 1 and 2 with that of *C. ammoniagenes*.
Sequences underlined indicate universal primers.

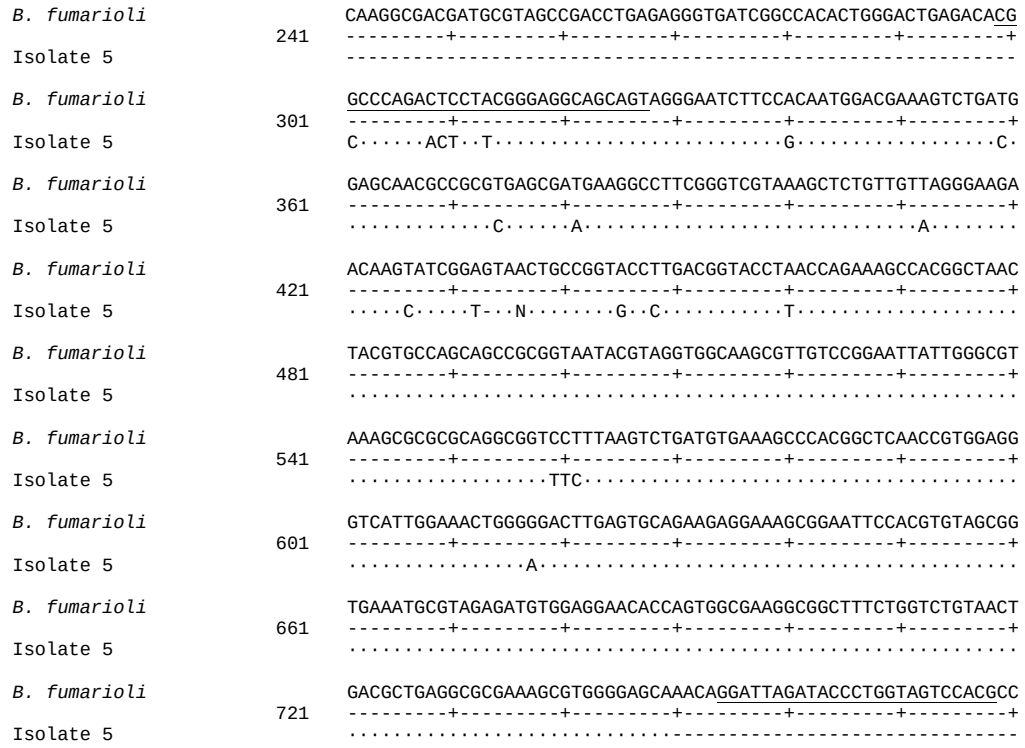

Figure 2.5b: Sequence alignment and comparison of the partial sequence of the *rrnA* gene of environmental isolate 5 with that of *B. fumarioli*.
Sequences underlined indicate universal primers.

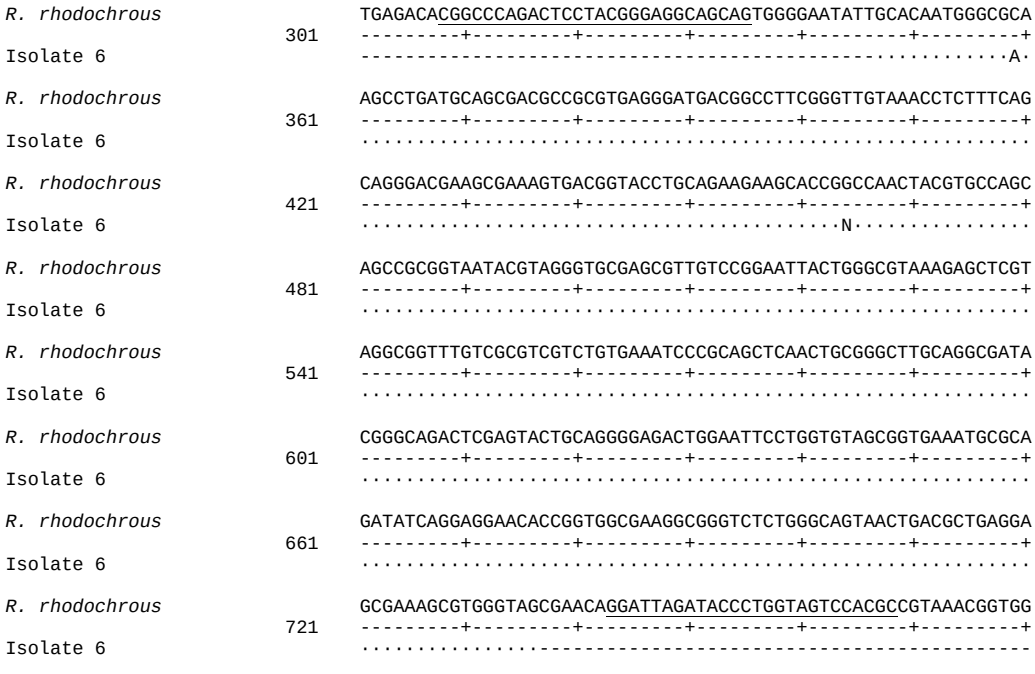

Figure 2.5c: Sequence alignment and comparison of the partial sequence of the *rrnA* gene of environmental isolate 6 with that of *R. rhodochrous*.
Sequences underlined indicate universal primers.

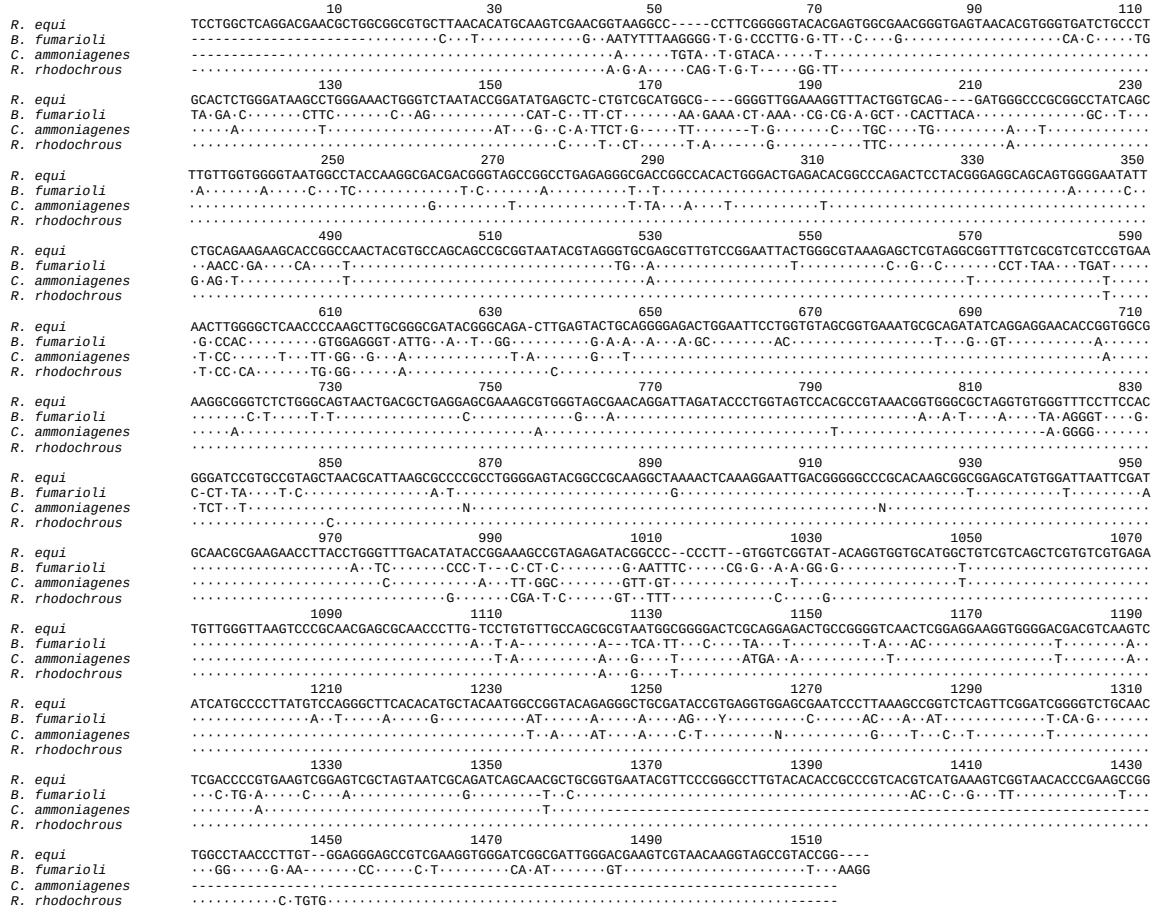

Figure 2.6: Sequence alignment and comparison of the *rrnA* gene of *R. equi* with those of the three cross-reactive species.

2.3.4. Modification of the DNA hybridisation technique to identify *R. equi*

When hybridisation of the *rrnA* probe to blotted colonies of the atypical isolates was performed at 68°C, with a final wash in 0.1 x SSC, 0.1 % SDS, at 68°C, the probe bound to the atypical isolates. When hybridisation was performed at 80°C and the final washing step was performed in 0.05 x SSC, 0.1% SDS, at 80°C, the binding to the atypical isolates, but not that to *R. equi*, was eliminated (Figure 2.7).

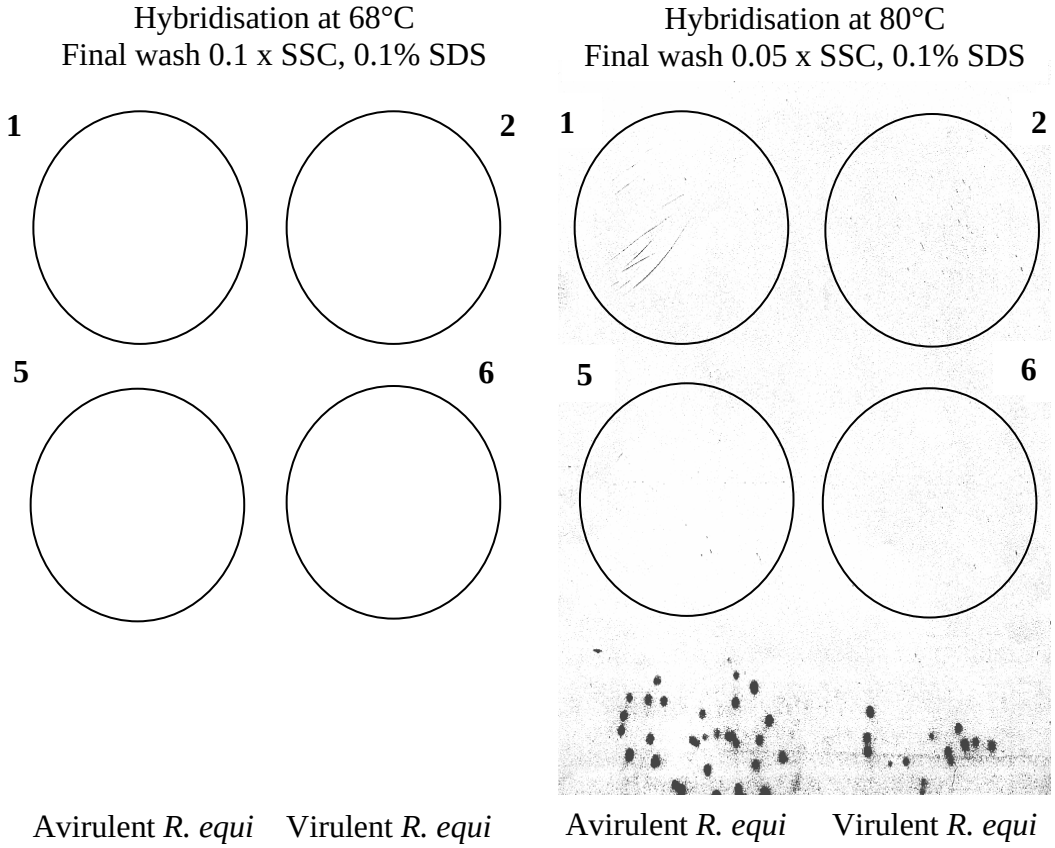

Figure 2.7: Optimisation of hybridisation to detect *R. equi.*
Hybridisation of *R. equi rrnA* probe with cross-reactive field isolates (isolates 1, 2, 5 and 6) was initially performed at 68°C with a final wash in 0.1 x SSC, 0.1% SDS. Subsequently hybridisation was performed at 80°C with a final wash in 0.05 x SSC, 0.1% SDS, resulting in the specific detection of only *R. equi*.

2.4. Discussion

This technique is an effective and efficient method for isolation, identification and differentiation of *R. equi*. It has been suggested that a high proportion of virulent *R. equi* in the soil on farms correlates with a higher incidence of bronchopneumonia in foals due to *R. equi* (Takai *et al.* 1991c, 1994a; Takai 1997). Application of this technique will not only enable identification of *R. equi* in environmental samples, but also quantification and differentiation of avirulent and virulent *R. equi* isolates, allowing assessment of the relative risk of *R. equi* pneumonia on a farm, and even of specific high risk times and areas on the farm, for foals.

The *rrnA* sequence of *R. equi* is more closely related to *Nocardia* species than other *Rhodococcus* species (Goodfellow *et al.* 1998). Thus, a radiolabelled *rrnA* probe was expected to bind with only *R. equi* colonies. Whereas this was shown with a selection of related species (*Corynebacterium* species and *N. asteroides*), some cross-reaction was observed with some environmental isolates (*Corynebacterium*, *Bacillus* and other *Rhodococcus* species), necessitating an increase in hybridisation temperature and washing stringency, which allowed specific identification of *R. equi* colonies. Partial sequence analysis of the *rrnA* genes of the cross-reactive species suggested that the hybridisation and washing protocol developed for the *R. equi rrnA* probe will allow the identification of colonies as *R. equi* only if the *rrnA* genes of the organism have greater than 95% sequence identity with the *rrnA* genes of *R. equi*.

There is no known homologue of the *vapA* gene in other bacterial species and there was no cross-reactivity between the *vapA* probe and avirulent *R. equi* or any other bacterial species. It may be interesting to assess whether the *vapA* probe would detect intermediately virulent *R. equi* strains isolated from pigs and humans, as there is 83.6% sequence identity between the *vapB* gene possessed by intermediately virulent *R. equi* and the *vapA* gene of virulent *R. equi* (Takai *et al.* 1994c, 1996a; Byrne *et al.* 2001). Higher hybridisation temperatures and washing stringency may be required to differentiate these two types. While *R. equi* infections in humans have been associated with both virulent and intermediately virulent strains (Takai *et al.* 1995a; Nicholson and Prescott 1997; Makrai *et*

al. 2002), intermediately virulent *R. equi* have not been isolated from the environment of horses, so it is unlikely that intermediately virulent strains will be confused with virulent strains using the method described here when assessing risk on horse farms (Takai 1997; Makrai *et al.* 2002).

This technique allows the quantitative screening of large mixed bacterial population samples for *R. equi* and virulent *R. equi* without the use of laborious testing of individual colonies. Correlating changes in concentration and proportion of virulent *R. equi* in an environment with climatic, geographic and management differences within and between farms may enable more effective strategies to be developed to reduce the environmental burden of virulent *R. equi* and hence the incidence of disease. The application of this technique in the following studies in this thesis illustrates its potential to improve understanding of the ecology of virulent *R. equi* on horse farms.

Chapter 3

Associations between Thoroughbred farm characteristics and management and the prevalence and severity of *R. equi* pneumonia

3.1. Introduction

Epidemiological studies of *R. equi* pneumonia have indicated that this disease occurs endemically and with high prevalence on some horse breeding farms and sporadically, if ever, on other farms (Prescott 1987).

The environmental and management conditions on a farm may contribute to the risk of disease due to *R. equi*. Recent studies in the USA have defined some farm and management characteristics associated with increased risk of *R. equi* pneumonia in foals (Chaffin *et al.* 2003a, b). Large acreage farms (>200 acres) with high numbers of horses, a high foal density and a high proportion of transient mares and foals were all identified as significant risk factors. Housing of foals in stalls with dirt floors was also suggested to increase the risk of *R. equi* pneumonia. Significantly, a lack of preventative health practices such as vaccination, anthelmintic administration and treatment of foals with hyperimmune serum, did not influence the risk of *R. equi* pneumonia. These findings contradicted earlier reports that suggested that good preventative health practices were associated with a reduced prevalence of disease due to *R. equi* (Bain 1963; Debey and Bailie 1987; Clarke 1989).

The work described in this chapter aimed to examine how the management of horses and land on farms influenced the prevalence of *R. equi* pneumonia and the risk of mortality from this disease.

3.2. Materials and methods

3.2.1. Survey of farm management and disease prevalence

Each participating farm completed a questionnaire (Appendix 1) at the end of both the 2000 and 2001 breeding seasons. The questionnaire required farm managers and veterinarians to supply information about farm characteristics, stock and land management practices and preventative health management programs. The final question of the survey asked the farm to report on cases of pneumonia caused by *R. equi* during the breeding season.

Only minor changes were made to the questionnaire between the two seasons (Appendix 1). In the 2001 season, additional questions on probiotic and hyperimmune serum usage were included and managers were asked to supply estimates of the frequency of other diseases affecting foals on the farm and more specific details about cases of *R. equi* pneumonia.

3.2.2. Farms

Managers on 10 Thoroughbred breeding farms in 4 geographically distinct areas of the state of Victoria, Australia, completed questionnaires in the 2001 season. Six of these 10 also participated in the 2000 season. Details of the geographic location of the farms and the reported past prevalence of *R. equi* pneumonia on the farm were recorded (Table 3.1).

Table 3.1: Location of, and past prevalence of *R. equi* pneumonia on Victorian farms involved in the study

Farm	Participation in:		Location	Past prevalence of *R. equi* pneumonia*
	2000	2001		
A	+	+	North Eastern Victoria	Low
B	+	+	North Eastern Victoria	Low
C	+	+	Southern Victoria	Low
D	+	+	Southern Victoria	Low
E	+	+	Central Victoria	Low
F	+	+	Central Victoria	High
G	-	+	North Eastern Victoria	Sporadic
H	-	+	South Eastern Victoria	Low
I	-	+	South Eastern Victoria	Low
J	-	+	Central Victoria	Sporadic

* Sporadic: a case every 2 to 5 years
Low: less than 5%
High: 10% or above

Managers on 12 Thoroughbred farms in 4 distinct geographical areas of the state of New South Wales (NSW), Australia, also completed questionnaires in the 2001 season and the geographical locations and the past prevalence of *R. equi* pneumonia on these farms are reported in Table 3.2.

Table 3.2: Location of, and past prevalence of *R. equi* pneumonia on NSW farms involved in the study

Farm	Location	Past prevalence of *R. equi* pneumonia*
1	Lower Hunter	High
2	Lower Hunter	Low
3	Lower Hunter	High
4	Lower Hunter	Low
5	Lower Hunter	High
6	South Western Slopes	Sporadic
7	Central Tablelands	Low
8	Upper Hunter	High
9	Upper Hunter	Low
10	Upper Hunter	Low
11	Upper Hunter	Low
12	Upper Hunter	Low

* Sporadic: a case every 2 to 5 years
Low: less than 5%
High: 10% or above

3.2.3. Data analysis

The data collected from the survey during the 2001 season was sorted to allow the analysis of categorical data. This was performed using Fisher's exact test to assess the association between farm characteristics, management variables (size of farm, foal numbers, stocking rate, land, feed and health management), and morbidity and mortality due to *R. equi* pneumonia.

Management and farm characteristics were compared between farm groups in the following categories: those farms that were classified as having a low disease prevalence or not, farms that were classified as having a high disease prevalence or not, and farms that did or did not report deaths as a consequence of *R. equi* pneumonia. Data from the 2000 season were used to examine the effect of any changes between the two seasons on these farms and compare these with associations between variables and morbidity and mortality due to *R. equi* on farms in the 2001 season.

3.3. Results

3.3.1. Prevalence of *R. equi* pneumonia

All farms involved in the 2000 season survey reported cases of *R. equi* pneumonia during that foaling season. In the 2001 season, 7/10 farms in Victoria reported cases of *R. equi* pneumonia. Eleven of the 12 NSW farms reported cases. Farms B, G, J and 7 did not report cases of *R. equi* pneumonia in the 2001 season (Table 3.3).

Table 3.3: Morbidity, mortality and prevalence of *R. equi* pneumonia on individual Thoroughbred farms

State	Location	Farm	*R. equi* pneumonia					
			No. Cases		Prevalence (%)		No. Deaths	
			2000	2001	2000	2001	2000	2001
Vic	North Eastern	A	3	4	3.1	4.7	0	0
		B	2	0	3.3	0	1	0
		G	NS	0	NS	0	NS	0
	Southern	C	6	8	4.2	5.8	0	0
		D	3	2	2.9	2.1	0	0
	Central	E	8	2	4.6	0.9	1	0
		F	23	22	11.0	9.2	2	1
		J	NS	0	NS	0	NS	0
	South Eastern	H	NS	7	NS	4.7	NS	1
		I	NS	4	NS	3.9	NS	0
NSW	Lower Hunter	1	NS	25	NS	20.8	NS	2
		2	NS	19	NS	8.2	NS	1
		3	NS	82	NS	16.8	NS	1
		4	NS	24	NS	6.2	NS	2
		5	NS	17	NS	9.6	NS	0
	Upper Hunter	8	NS	21	NS	9.1	NS	2
		9	NS	1	NS	2.1	NS	0
		10	NS	2	NS	1.1	NS	0
		11	NS	7	NS	7.0	NS	0
		12	NS	2	NS	3.7	NS	0
	South Western Slopes	6	NS	2	NS	1.0	NS	1
	Central Tablelands	7	NS	0	NS	0	NS	0

NS: Not surveyed

As these prevalence data were not normally distributed the upper and lower quartile figures were used to define high and low prevalence of *R. equi* pneumonia (Table 3.4). Farms with a prevalence of *R. equi* pneumonia <3% were grouped as farms with a low prevalence of *R. equi* pneumonia. This category included farms that did not report *R. equi* pneumonia. Farms with a prevalence of *R. equi* pneumonia >9% were grouped as farms with a high prevalence of *R. equi* pneumonia.

Table 3.4: Prevalence of *R. equi* pneumonia on Thoroughbred farms in the 2000 and 2001 seasons

	N	Mean	Median	Lower quartile	Upper quartile
Number of cases	28	10.6	4.0	2.0	18.5
Maximal number of foals	28	150.8	128.5	67.3	209.0
Prevalence of *R. equi* pneumonia*	28	7.0%	3.1%	3.0%	8.9%

N: number of farms surveyed. 6 Victorian farms in 2000 and the 22 Victorian and NSW farms in 2001
*Prevalence of *R. equi* pneumonia on the basis of the number of cases and the maximal number of foals on each farm in each year.

Cases of *R. equi* pneumonia were diagnosed using combinations of clinical signs, ultrasonographic examinations of the lungs, haematological examinations and cultures from tracheal lavages. In the 2000 season one farm (farm D) had a low disease prevalence and one farm (farm F) had a high disease prevalence. Three farms (B, E and F) reported mortalities due to *R. equi*. In the 2001 season 9 farms had a low disease prevalence (5 from Victoria - farms B, D, E, G and J and 4 from NSW - farms 6, 7, 9 and 10), whilst 5 farms had a high disease prevalence (1 from Victoria – farm F and 4 from NSW – farms 1, 3, 5 and 8). Eight farms reported mortalities due to *R. equi* (2 from Victoria – farms F and H, 6 from NSW – farms 1, 2, 3, 4, 6 and 8) (Table 3.3). Overall the mortality rates associated with *R. equi* pneumonia in this study was 0.4% (15/4221), with a case fatality rate of 5.1% (15/296).

In the 2001 season farms with a high prevalence of *R. equi* pneumonia (>9%) were more likely to report *R. equi* associated mortalities than other farms (4/5 farms vs. 4/17 farms with mortalities, respectively; P=0.04). Farms with a low prevalence of *R. equi* pneumonia (<3%) were not significantly less likely to report mortality due to *R. equi* pneumonia than other farms (8/9 farms vs. 6/13 farms with no mortalities, respectively; P=0.07). The mortality rate on farms with a high disease prevalence (8/1465 - 0.5%) was approximately five times that seen on the farms with a low disease prevalence (1/973 - 0.1%). However, the case fatality rate on farms with a low disease prevalence (1/12 - 8.3%), was not significantly different from that seen on farms with a higher prevalence of disease (14/282 - 4.9%). Two farms reported mortalities in the 2000 season but not in the 2001 season (farms B and E). On both of these farms the disease prevalence was lower in 2001 (<3%) than in 2000 (>3%).

3.3.2. Farm characteristics and management

a. Farm size and numbers of foals

The total area dedicated to horse husbandry on the Thoroughbred farms surveyed in Victoria during the 2000 and 2001 seasons ranged from 36 to 144 hectares (Ha)/farm. The maximal number of foals on these farms ranged from 42 to 238 during the breeding season. The total area dedicated to horse husbandry on the Thoroughbred farms surveyed in NSW during the 2001 season ranged from 49 to 182 Ha/farm. The maximal number of foals on these farms ranged from 47 to 488 during the breeding season (Table 3.5).

Table 3.5: Area dedicated to horse husbandry, maximal foal populations and stocking rates on Thoroughbred farms

State	Farm	Hectares (Ha)		Maximal number of foals		Stocking rate (foals/Ha)	
		2000	2001	2000	2001	2000	2001
Vic	A	61	61	97	86	1.6	1.4
	B	36	36	60	44	1.7	1.2
	C	85	85	144	137	1.7	1.6
	D	69	69	104	94	1.5	1.4
	E	101	101	174	219	1.7	2.2
	F	144	144	210	238	1.5	1.7
	G	NS	40	NS	42	NS	1.1
	H	NS	69	NS	150	NS	2.2
	I	NS	57	NS	102	NS	1.8
	J	NS	40	NS	43	NS	1.1
NSW	1	NS	81	NS	120	NS	1.5
	2	NS	162	NS	231	NS	1.4
	3	NS	182	NS	488	NS	2.7
	4	NS	162	NS	387	NS	2.4
	5	NS	101	NS	177	NS	1.8
	6	NS	182	NS	206	NS	1.1
	7	NS	81	NS	61	NS	0.8
	8	NS	57	NS	232	NS	4.1
	9	NS	49	NS	47	NS	1.0
	10	NS	101	NS	174	NS	1.7
	11	NS	65	NS	100	NS	1.5
	12	NS	51	NS	54	NS	1.1

NS: Not surveyed

On those farms surveyed in consecutive seasons, no change was observed in the total area dedicated to horse husbandry, whilst maximal foal numbers decreased on four farms, and increased on two, in the 2001 season compared the 2000 season.

Farms were divided into groups based on the total area devoted to horse husbandry, maximal stocking rate (foals/Ha) and on the maximal foal numbers. Using the upper and lower quartiles of the maximal foal numbers as the cut-off values (Table 3.4), farms were classified as having small (<70 foals), medium (70-210 foals) or large (>210 foals)

numbers of foals. Using the data on area dedicated to horse husbandry (median 75.0 Ha, mean 89.8 Ha, lower quartile 55.5 Ha, upper quartile 111.7 Ha in the 2001 season), farms were classified as small (<60 Ha), medium (60-110 Ha) or large (>110 Ha). Using the maximal stocking rate data for farms (median 1.5 foals/Ha, mean 1.7 foals/Ha, lower quartile 1.1 foals/Ha, upper quartile 1.9 foals/Ha in the 2001 season, farms were divided into those with a low (<1.1 foals/Ha), medium (1.1-1.9 foals/Ha) or high (>1.9 foals/Ha) stocking rate.

A low prevalence of *R. equi* pneumonia was associated with a small foal population in the 2001 season. Five of the 6 farms with a small foal population reported a disease prevalence below 3%, whilst only 4/16 farms with medium or large foal populations reported a low disease prevalence (P=0.02). Similarly the presence of mortality due to *R. equi* pneumonia was associated with medium or large foal populations. None of the 6 farms with a small foal population reported deaths from *R. equi* pneumonia, whilst 8/16 farms with medium or large foal populations reported deaths from *R. equi* pneumonia (P=0.04). No farm surveyed in consecutive seasons moved into or out of the small foal population category. On the four farms that decreased their maximal foal numbers in 2001, two reported reduced disease prevalence. The two farms that increased their maximum foal numbers, both from the medium to the large foal population category, also reported reduced disease prevalence. Of the two farms that reported mortality due to *R. equi* in the 2000 season, but not in the 2001 season, one reduced its maximal foal numbers, while the other increased its maximum foal numbers.

No significant associations were seen between the farm area used for horse husbandry or maximal stocking rate and morbidity or mortality due to *R. equi* pneumonia (Table 3.6).

Table 3.6: Associations between stocking rates, area used for horse husbandry, maximal foal numbers and morbidity and mortality due to *R. equi* pneumonia in the 2001 season

Farms with:	Maximal number of foals			Area used for horse husbandry (Ha)			Stocking rate (foals/Ha)		
	Small (<70)	Medium or large (>70)	P[a]	Small (<60)	Medium or large (>60)	P[b]	Low (<1.1)	Medium or high (>1.1)	P[c]
Low disease prevalence (<3%)	5/6	4/16	0.02*	4/7	5/15	0.28	4/5	5/17	0.07
High disease prevalence (>9%)	0/6	5/16	0.17	1/7	4/15	0.48	0/5	5/17	0.23
Mortality due to *R. equi* pneumonia	0/6	8/16	0.04*	1/7	7/15	0.16	0/5	8/17	0.08

P[a]: Probability that the proportion of farms in the category among those with a small foal population differed from the proportion of farms in the category among those with medium or large foal populations.
P[b]: Probability that the proportion of farms in the category among those small in area differed from the proportion of farms in the category among those medium or large in area.
P[c]: Probability that the proportion of farms in the category among those with low stocking rates differed from the proportion of farms in the category among those with medium or high stocking rate.

b. Housing and mob management

Mares on all farms foaled outdoors in small yards that were usually flood lit, allowing mares to be observed continuously. Mares and foals were then grouped onto paddocks or larger yards, generally at around 72 hours *post partum.*

Grouping of mare-foal pairs on paddocks was generally determined by the reproductive status of the mare and the age of the foal. All farms had a general strategy of grouping mares and foals according to age up until the mare's first oestrus after foaling (7-15 days after foaling). Once the mare had been mated and observed to be pregnant by ultrasonographic examination at 45 days after service, the mares and foals were less intensively handled and were moved to larger paddocks away from the main handling yards on the property. Those mares not owned by the farm, but that had foaled and been served by stallions on the farm (transient mares), were returned to the owner's property some time after pregnancy diagnosis at 45 days gestation. During the early stages of gestation (<45 days) the mares were checked periodically (routinely at 14 and 28 days after service) by ultrasonographic examination to assess the progression of the pregnancy.

All farms, with the exception of F, G and 10, stocked foals primarily according to the mare's reproductive status after her foal heat. These farms could have foals over a wide age range in the same paddock. For example, if a mare failed to become pregnant after the

2nd oestrus after foaling or experienced embryonic loss before 45 days of gestation, repeated service would be required. Once pregnant, this mare would have an older foal at foot than mares in the group that foaled later in the season but became pregnant at one of their first two oestruses after foaling. Farms F, G and 10 stocked foals in tight, age-specific groups, regardless of the mare's reproductive status. On these farms mares and foals were kept in groups of up to ten mare-foal pairs.

All farms, except farm 7, had both home mares (owned by the farm) and transient mares (owned by other parties) foaling and being served on the farm. Two farms (H and 3) had geographically separate areas for transient mares and foals, while on the remaining farms the transient mares shared the same paddocks as the home mares.

All farms had specific yards for sick or injured foals and for foals with poor conformation, and some had stables for these foals.

c. Land and feed management

The land and feed management strategies on the farms were evaluated. Aspects examined included use of creep feed, rotation of feeding bins and/or feeding stations, picking up of faecal material from yards, and irrigation and harrowing of paddocks (Table 3.7).

Table 3.7: Land and feed management strategies used on Thoroughbred farms

State	Farm	Creep feed		Rotation of feeding bins or stations		Faecal pick up		Irrigation		Harrowing	
		2000	2001	2000	2001	2000	2001	2000	2001	2000	2001
Vic	A	-	-	+	+	+	+	+	+	+	+
	B	-	-	+R	+R	-	-	-	-	-	+
	C	+	+	+R	+R	+	+	-	-	+	+
	D	-	-	+	+	-	-	-	+	+	+
	E	-	-	+	+R	+	-	+	+	+	+
	F	-	-	+	+	+	+	+	+	+R	+R
	G	NS	-	NS	+R	NS	+	NS	-	NS	+
	H	NS	-	NS	+	NS	-	NS	-	NS	+
	I	NS	+	NS	+	NS	+	NS	-	NS	+
	J	NS	-	NS	+	NS	+	NS	+	NS	+R
NSW	1	NS	-	NS	+R	NS	+	NS	+	NS	-
	2	NS	-	NS	+R	NS	-	NS	+	NS	+R
	3	NS	-	NS	+	NS	+	NS	+	NS	-
	4	NS	-	NS	-	NS	+	NS	+	NS	+
	5	NS	-	NS	+R	NS	+	NS	+	NS	-
	6	NS	+	NS	-	NS	+	NS	-	NS	+
	7	NS	-	NS	-	NS	-	NS	+	NS	+R
	8	NS	+	NS	ϕ	NS	+	NS	+	NS	+
	9	NS	+	NS	+	NS	+	NS	+	NS	-
	10	NS	+	NS	ϕ	NS	+	NS	+	NS	-
	11	NS	*	NS	+	NS	+	NS	+	NS	+
	12	NS	-	NS	+	NS	-	NS	+	NS	+

NS: Not Surveyed
* Farm 11 gave no information about creep feed
ϕ Farms 8 and 10 did not use feeding bins or feeding stations
R: Occurred regularly

Mares and foals on all farms were fed a mixture of roughage and concentrates. All farms, except B, included pelleted concentrate feed in the mixture. In the 2001 season creep feed was used on 6 farms. Creep feed was placed in small, partially confined feeding areas in the paddocks, away from the main feeding bins. Creep feed consisted of increasing quantities of concentrates and/or pelleted supplements. Farm I only gave creep feed to foals that were growing slowly.

The number of feeding bins in paddocks varied with the number of mare–foal pairs kept in the paddock. Bins were generally not evenly distributed in the paddocks. Bins were mostly situated in one quarter of the paddock. Large metallic feeding stations were used on farms 2, 4 and 7. Unlike the bins, which allowed a mare-foal pair to feed away from the rest of the mob, the feeding stations created a communal feeding environment, with all horses feeding from a central location. Farms 8 and 10 only used bins in small yards for

younger, sick or injured foals, and placed feed on the ground in larger paddocks. Farms 2 and 4 used bins and feeding stations on larger paddocks and bins only in small paddocks. Most farms rotated the position of bins when the ground became worn or muddy. Farms that used feeding stations usually had one per paddock and they were rarely moved. In the 2001 season 6 farms rotated bins on a regular basis, regardless of paddock condition, at a frequency ranging from every 5 weeks on farms E and G to daily on farms I and 2.

Faecal material in paddocks was picked up, harrowed or left. In the main, faeces was only picked up in the small yards used for foaling, which held foals under 14 days of age, and the small yards used for sick or injured foals, and the holding pens. Farm 2 harrowed the small yards daily. Faeces were removed daily on most farms and twice daily on some farms. Harrowing was not performed on 5 farms in the 2001 season. All other farms harrowed paddocks or yards at least once during the foaling season.

Mare and foal paddocks were irrigated on all but one NSW farm, but only 5 Victorian farms irrigated paddocks in the 2001 season. Farm A irrigated only foaling yards. Irrigation was performed daily on this farm. Irrigation frequency on other farms ranged from every 2 to every 12 weeks during the foaling season, depending on the prevailing weather conditions. Farm E irrigated each paddock for 3 days each between December and February. Four farms irrigated on a regular basis, irrespective of climatic conditions, with the frequency of irrigation ranging from every 2 to every 6 weeks. Farm F increased the irrigation frequency in the 2001 season to every 2 weeks, from every 3 weeks in the 2000 season. Other farms increased or decreased the frequency of irrigation depending on the weather conditions.

The irrigation and harrowing of large paddocks necessitated the movement of groups of horses to adjacent paddocks.

Herbicides were not used on the paddocks of 9 farms in the 2001 season (4 from Victoria, 5 from NSW). Either herbicides or fertilisers were applied to paddocks on all but 5 farms (2 from Victoria, 3 from NSW) in the 2001 season (Table 3.8). The most commonly used fertiliser was superphosphate, which was applied by 12 farms in the 2001 season. Lime was only used on 2 Victorian farms, whilst potash was used on one farm in

Victoria and a nitrogen/sulphur mixture (Grassboosta, Incitec Pivot, Southbank, Victoria) was used on one farm in NSW. Three of the farms surveyed in consecutive seasons (A, C and F) changed their herbicide and/or fertiliser usage between seasons. All of these farms applied lime to paddocks in the 2000 season, but did not do so in the following season.

Table 3.8: Chemicals used on paddocks on Thoroughbred farms

State	Farm	Herbicide		Lime		Superphosphate		Potash		Nitrogen/sulphur mixture	
		2000	2001	2000	2001	2000	2001	2000	2001	2000	2001
Vic	A	+	+	+	-	-	+	-	-	-	-
	B	+	+	-	-	-	-	-	-	-	-
	C	-	-	+	-	+	+	+	-	-	-
	D	+	+	-	-	-	-	-	-	-	-
	E	+	+	-	-	+	+	-	-	-	-
	F	+	-	+	-	-	-	-	-	-	-
	G	NS	+	NS	+	NS	-	NS	-	NS	-
	H	NS	+	NS	-	NS	+	NS	+	NS	-
	I	NS	-	NS	-	NS	+	NS	-	NS	-
	J	NS	-	NS	+	NS	-	NS	-	NS	-
NSW	1	NS	-	NS	-	NS	-	NS	-	NS	-
	2	NS	+	NS	-	NS	+	NS	-	NS	-
	3	NS	-	NS	-	NS	-	NS	-	NS	-
	4	NS	-	NS	-	NS	+	NS	-	NS	-
	5	NS	+	NS	-	NS	+	NS	-	NS	-
	6	NS	+	NS	-	NS	+	NS	-	NS	-
	7	NS	-	NS	-	NS	-	NS	-	NS	-
	8	NS	+	NS	-	NS	+	NS	-	NS	+
	9	NS	-	NS	-	NS	-	NS	-	NS	-
	10	NS	+	NS	-	NS	+	NS	-	NS	-
	11*	NS		NS		NS		NS		NS	
	12	NS	-	NS	-	NS	+	NS	-	NS	-
Total		4/6	11/21	3/6	2/21	2/6	12/21	1/6	1/21	0/6	1/21

*Farm did not provide information
NS: Not surveyed

There was no significant association between the different approaches to land management, the feeding of mares and foals, and the prevalence of *R. equi* pneumonia or the occurrence of mortalities due to *R. equi* pneumonia in the 2001 season (Table 3.9).

Table 3.9: Associations between land and feed management practices and the prevalence of *R. equi* pneumonia and the occurrence of mortalities due to *R. equi* pneumonia in the 2001 season

Land and feed management practices		Low disease prevalence (<3%)	P	High disease prevalence (>9%)	P	Mortalities due to *R. equi* pneumonia	P
Creep feed	+	3/6	1	1/6	1	2/6	1
	-	6/15		4/15		6/15	
Rotation of feeding areas	+	6/17	0.95	4/17	1	5/17	0.27
	-	2/3		0/3		2/3	
Faecal pick up	+	5/15	0.94	5/15	1	6/15	0.84
	-	4/7		0/7		2/7	
Harrowing	+	7/17	1	2/17	0.055	6/17	1
	-	2/5		3/5		2/5	
Irrigation	+	6/16	0.84	5/16	1	6/16	0.75
	-	3/6		0/6		2/6	
Herbicide or fertilizer	+	7/15	0.68	2/15	0.11	5/15	0.63
	-	2/6		3/6		3/6	
Herbicide	+	6/12	0.38	2/12	0.35	4/12	0.47
	-	3/9		3/9		4/9	
Superphosphate	+	3/12	0.07	2/12	0.92	5/12	0.53
	-	6/9		3/9		3/9	
Lime	+	2/2	0.17	0/2	0.57	0/2	0.37
	-	7/19		5/19		8/19	

P: Probability that the proportion of farms in the category among those performing one practice differs from the proportion of farms in the category among those farms not performing the practice.

3.3.3. Preventative health care

a. Vaccination

Foals were vaccinated on all farms. The different vaccination regimens are described in Table 3.10. The age of the foals when first inoculated ranged from 2 to 6 months. Seven farms vaccinated foals at a specific time of the year, regardless of the age of the foal. All farms vaccinated against *Clostridium tetani* (tetanus) and all but 3 farms vaccinated against *Streptococcus equi* subspecies *equi* (strangles). Seven farms vaccinated foals against equine herpesviruses 1 and 4 (EHV 1 and EHV 4). Farm D introduced the vaccination of foals against EHV 1 and EHV 4 in the 2001 season. Nine farms vaccinated foals against *Salmonella enterica* Typhimurium (salmonellosis). Six of these were located in NSW.

Mares were vaccinated on all farms. The different vaccination regimens are described in Table 3.11. Seventeen farms vaccinated mares against EHV 1 and EHV 4 and all but 3 of these farms vaccinated each mare 4-6 weeks before her due foaling date. All farms that vaccinated mares 4-6 weeks prior to foaling vaccinated against tetanus, with the exception of farm 4. All but 3 farms that vaccinated mares 4-6 weeks prior to foaling vaccinated against strangles. Vaccination of mares against salmonellosis was performed 4-8 weeks prior to foaling on 7 farms. Three farms vaccinated pregnant mares against rotavirus. All of these farms were located in NSW. The vaccination histories of transient mares coming onto the farm were reliably obtained on 6 farms (E, G, 5, 6, 9 and 12), while farm B required strangles vaccination of mares prior to arrival. Nine farms vaccinated some mares without a vaccination history on arrival.

Table 3.10: Vaccines and administration regimens used for foals on Thoroughbred farms in the 2001 season

State	Farm	Tetanus			Strangles			EHV 1 and EHV 4			Salmonellosis		
		Doses	1st dose	Interval	Doses	1st dose	Interval	Doses	1st dose	Interval	Doses	1st dose	Interval
Vic	A	2	January	4 w	3	January	2 w	-	-	-	-	-	-
	B	2	January	4 w	3	January	2 w	-	-	-	-	-	-
	C	2	4 m	4 w	3	4 m	2 w	-	-	-	-	-	-
	D*	3	3 m	1 and 6 m	3	3 m	1 and 6 m	2	3 m	6 w	-	-	-
	E	2	3 m	4 w	-	-	-	2	3 m	4 w	-	-	-
	F	2	4 m	4 w	-	-	-	-	-	-	2	4 m	4 w
	G	2	3 m	4 w	3	3 m	2 w	2	5 m	4 w	2	3 m	4 w
	H	2	3 m	4 w	3	3 m	2 w	-	-	-	2	3 m	4 w
	I	2	6 w	4 w	3	6 w	2 w	-	-	-	-	-	-
	J	2	5 m	4 w	3	5 m	2 w	2	5 m	4 w	-	-	-
NSW	1	2	6 m	4 w	3	6 m	2 w	-	-	-	2	6 m	4 w
	2	3	January	2 w	3	January	2 w	-	-	-	3	January	2 w
	3	2	12 w	4 w	3	12 w	2 w	2	12 w	2w	-	-	-
	4	2	February	4 w	3	February	2 w	-	-	-	-	-	-
	5	2	3 m	4 w	-	-	-	-	-	-	2	3 m	4 w
	6	2	3 m	4 w	3	3 m	2 w	-	-	-	-	-	-
	7	2	February	4 w	3	February	2 w	2	February	4 w	-	-	-
	8	2	January	4 w	2	January	4 w	-	-	-	2	January	4 w
	9	2	4 m	4 w	3	4 m	2 w	-	-	-	2	4 m	4 w
	10	2	10 w	4 w	3	10 w	4 w	2	14 w	4 w	-	-	-
	11	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	12	2	February	4 w	3	February	2 w	-	-	-	2	February	4 w

Vaccination regimens for foals on farms A, B, C, E and F in the 2000 season were as for the 2001 season.
* Farm D did not administer EHV 1 and EHV 4 vaccine to foals in the 2000 season
NA: Information not provided
w: weeks
m: months

Table 3.11: Vaccines and administration regimens used on Thoroughbred farms for mares in the 2001 season

State	Farm	Tetanus			Strangles			EHV 1 and EHV 4			Salmonellosis		
		Doses	1st dose	Interval	Doses	1st dose	Interval	Doses	1st dose	Interval	Doses	1st dose	Interval
Vic	A	1	4 w pp		1	4 w pp		3	6 m pp	2 m	-	-	-
	B	1		1 y	1[T]		1 y	-	-	-	-	-	-
	C	3[T]	February	6 m and 1 m pp	3[T]	February	6 m and 1 m pp	-	-	-	-	-	-
	D	1[T]	1 m pp		1[T]	1 m pp		3	6 m pp	2 m	-[T]	-	-
	E	1	1 m pp		-	-	-	3	6 m pp	2 m	-	-	-
	F	1[T]	1 m pp		-	-	-	2	January	6 m	1[T]	1 m pp	
	G	2	2 m pp	1 m	2	2 m pp	1 m	3	6 m pp	2 m	2	2 m pp	1 m
	H	1	3 w pp		-	-	-	3	6 m pp	2 m	-	-	-
	I	1[T]	4 w pp		1[T]	4 w pp		3	6 m pp	2 m	-	-	-
	J	1[T]	4 w pp		1[T]	4 w pp		3	6 m pp	2 m	-	-	-
NSW	1	1	6 w pp		1	6 w pp		-	-	-	1[T]	6 w pp	
	2	1[T]	6 w pp		1[T]	6 w pp		3	6 m pp	2 m	-	-	-
	3	1	4 w pp		1	4 w pp		3	6 m pp	2 m	-	-	-
	4[R]	1[T]	June		1[T]	June		3	6 m pp	2 m	1[T]	4 w pp	
	5	1	4 w pp		1	4 w pp		-	-	-	-	-	-
	6	1	6 w pp		1	6 w pp		3	6 m pp	2 m	-	-	-
	7	1	1 m pp		1	1 m pp		3	6 m pp	2 m	-	-	-
	8[R]	1[T]	1 m pp		1[T]	1 m pp		3	6 m pp	2 m	1[T]	4 w pp	
	9	1	6 w pp		1	6 w pp		3	6 m pp	2 m	-	-	-
	10[R]	1[T]	1 m pp		1[T]	1 m pp		3	6 m pp	2 m	1[T]	1 m pp	
	11	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	12	1	6 w pp		1	6 w pp		3	6 m pp	2 m	1	6 w pp	

Vaccination regimens for mares on farms A–F in the 2000 season were as for the 2001 season.
[R] Farms that administered rotavirus vaccine to mares; 3 doses one month apart commencing at 3 months *pre partum*
[T] Vaccine given to transient mares on or prior to arrival
NA: Information not provided
w: weeks
m: months
y: years
pp: *pre partum*

b. Anthelmintic treatment

All farms administered anthelmintics to their foals. The different anthelmintic treatment regimens are described in Table 3.12. The age at initial treatment ranged from 7 days to 8 weeks. Seven farms administered anthelmintics to foals at 4 weeks of age or younger. Six farms, all in NSW, rotated between 2 or more anthelmintics in a season. Seven farms used a single anthelmintic agent in a season. Of these, 4 farms in the 2001 season and 3/6 Victorian farms in the 2000 season used ivermectin as the single agent. Only one of the 6 farms (A) participating in consecutive seasons changed the anthelmintic used between seasons.

Mares were treated with anthelmintics on all farms. The anthelmintics used in home mares were the same as those used in foals. The period between treatment varied from 6 to 8 weeks. Ten farms (A, B, C, D, E, I, 1, 6, 9 and 10) treated mares within 48 hours of foaling, while 2 farms (2 and 3) treated mares at or around the time of the foal heat (7-15 days after foaling). Pregnant mares on 6 farms (A, B, D, I, 9 and 10) did not receive anthelmintic treatment within one month of their due foaling date. None of the 6 farms that did not administer anthelmintics to mares within one month of their due foaling date reported mortalities due to *R. equi* and all had a prevalence of *R. equi* pneumonia <9%. The anthelmintic treatment histories of transient mares were reliably obtained on 5 farms (B, E, J, 5 and 6). For these mares anthelmintic treatment continued for the duration of their stay on the farm using the anthelmintics previously used on them. Mares with an unknown anthelmintic treatment history arriving on all other farms, with the exception of D and 3, were treated on arrival or within the first month after arrival.

Table 3.12: Anthelmintics and administration regimens used to control parasites in foals on Thoroughbred farms in the 2001 season

State	Farm	Anthelmintic(s)	Rotation	Initial dose	Dose interval
Vic	A	Oxibendazole*	-	6 w	Single
	B	Abamectin	-	6 w	Single
	C	Abamectin and praziquantel (Equimax)	-	3 w	6 w
	D	Ivermectin	-	1 m	1 m
	E	Oxfendazole and pyrantel (Strategy-T)	-	4 w	6-8 w
	F	Ivermectin	-	7-15 d	8 w
	G	Equimax	-	1 m	6 w
	H	Equimax	-	6 w	6 w
	I	Equimax	-	4 w	5 w
	J	Equimax	-	1 m	8 w
NSW	1	Oxibendazole, Equimax	+	2 m	2 m
	2	Fenbendazole, Oxibendazole, Ivermectin, Equimax	+	7 d	4-6 w
	3	Ivermectin	-	6 w	6 w
	4	Equimax	-	1 m	4-6 w
	5	Ivermectin, Strategy-T	+	6 w	6 w
	6	Oxibendazole, Fenbendazole	+	10 d	30 d
	7	Ivermectin	-	4 w	4-6 w
	8	Fenbendazole	-	10 d	6-8 w
	9	Ivermectin and praziquantel (Genesis)	+	6 w	6 w
	10	Ivermectin, Equimax	-	7 d	4-6 w
	11	NA	NA	NA	NA
	12	Equimax , Genesis	+	6 w	6 w

Anthelmintic drug and dosage regimens for foals on farms B–F in the 2000 season were as for the 2001 season.
* Farm A used ivermectin to control parasites in foals in the 2000 season
NA: Information not provided
d: days
w: weeks
m: months

c. Prophylactic antibiotics, probiotics and hyperimmune serum

Eight farms (A, B, C, D, I, 2, 5 and 7) used prophylactic antibiotics on all neonatal foals. Neomycin, penicillin and/or oxytetracycline were used. The course of treatment ranged from a single dose within 24 hours of birth to a 3 day course of administration twice a day. Two farms (3 and 4) administered antibiotics to ‘at risk’ neonatal foals. An ‘at risk’ foal was defined as a foal with diarrhoea, a serum IgG concentration <800 mg/L or with a complicated parturition. Farms C and E administered probiotics (Protexin, International Animal Health Pty Ltd, Huntingwood, NSW) to foals. Farm C administered probiotics (Protexin) to foals suffering from diarrhoea in 2001 only, whilst farm E gave probiotics to all foals for the first 3-5 days of life.

Hyperimmune serum was administered to all foals on farm 1 at 48 hours and 14 days of age, but the volume administered was not specified. Six other farms (C and D in 2001 only and H, 4, 6 and 9) administered hyperimmune serum to foals with poor passive transfer of maternal antibody (serum IgG concentration <800 mg/L in the first 24 hours of life).

d. Associations between preventative health care practices and the prevalence of *R. equi* pneumonia and the occurrence of mortality due to *R. equi*

Farms that vaccinated foals against EHV 1 and EHV 4 were significantly more likely to have a low prevalence of *R. equi* pneumonia (P=0.009). Six of the 7 farms that vaccinated foals against EHV 1 and EHV 4 reported a prevalence of *R. equi* pneumonia <3%, whilst only 3/14 farms that did not vaccinate foals against EHV 1 and EHV 4 reported a prevalence <3%. Three farms (G, J and 7) which vaccinated foals against EHV 1 and EHV 4 were among the 6 farms (50%) that had a small foals population (maximum <70), whilst the other 4 farms made up only 25% (4/16) of the medium to large foal population farms. Farms that vaccinated foals against strangles, EHV 1 and EHV 4 were also significantly more likely to have a low prevalence of *R. equi* pneumonia (P=0.03), however no significant association was reported between farms that vaccinated foals against strangles and the prevalence of *R. equi* pneumonia.

Farms that administered anthelmintics to mares in the last month of pregnancy were more likely to report *R. equi* associated mortalities (P=0.03) than farms that did not administer anthelmintics to mare within one month of foaling. Eight of the 15 farms that treated mares with anthelmintic within one month of their due foaling date reported deaths due to *R. equi* pneumonia, whilst none of the 6 farms that did not administer anthelmenthics to mares within one month of their foaling date reported deaths due to *R. equi* pneumonia (Table 3.13).

Table 3.13: Associations between preventative health management practices and the prevalence of *R. equi* pneumonia and the occurrence of mortalities due to *R. equi* pneumonia in the 2001 season

Preventative health management practices		Low disease prevalence (<3%)	P	High disease prevalence (>9%)	P	Mortalities due to *R. equi* pneumonia	P
Foal vaccination							
Age specific	+	7/14	0.32	4/14	0.90	5/14	0.56
	-	2/7		1/7		3/7	
Strangles	+	8/18	0.61	3/18	0.13	6/18	0.32
	-	1/3		2/3		2/3	
EHV 1 and EHV 4	+	6/7	0.009**	1/7	0.44	1/7	0.13
	_	3/14		4/14		7/14	
Strangles and EHV 1 and EHV 4	+	5/6	0.03*	1/6	0.55	1/6	0.22
	-	4/15		4/15		7/15	
Salmonellosis	+	2/9	0.18	4/9	0.12	5/9	0.20
	-	7/12		1/12		3/12	
Age at vaccination$^{\Omega}$	≤ 4w	5/8	0.30	2/8	0.60	3/8	0.76
	> 4w	2/6		2/6		2/6	
Mare vaccination							
EHV 1 and EHV 4	+	8/18	0.61	4/18	0.58	8/18	1
	-	1/3		1/3		0/3	
Pre partum strangles	+	7/16	0.64	3/16	0.33	5/16	0.26
	-	2/5		2/5		3/5	
Salmonellosis	+	2/6	0.66	2/6	0.60	3/6	0.63
	-	7/15		3/15		5/15	
Anthelmintic treatment							
No. anthelminthics used in foals	1	3/7	1	3/7	0.28	3/7	1
	> 1	6/14		2/14		5/14	
Foal age at initial treatment	≤ 4w	4/9	1	2/9	1	4/9	0.67
	> 4w	5/12		3/12		4/12	
Mare treatment *pre partum*	< 1 m	5/15	0.18	5/15	0.15	8/15	0.03*
	≥ 1 m	4/6		0/6		0/6	
Mare treatment *post partum*	≤ 48 h	6/10	0.15	1/10	0.18	2/10	0.12
	> 48 h	3/11		4/11		6/11	
Other prophylaxis							
Routine antibiotics, serum or probiotics to foals	+	4/10	0.76	2/10	0.55	2/10	0.12
	-	5/11		3/11		6/11	
Routine antibiotics in foals	+	3/8	0.80	1/8	0.34	1/8	0.07
	-	6/13		4/13		7/13	

P: Probability that the proportion of farms in the category among those using a particular health management option differed from the proportion of farms in the category among those farms not using that option.
$^{\Omega}$ Only farms that vaccinated foals at a specific age were included

3.3.4. Diseases affecting foals

All farms in the 2001 survey were asked to provide information about the prevalence of diarrhoea, respiratory disease not associated with *R. equi*, conformational problems and traumatic injuries in foals during the season (Table 3.14).

Table 3.14: Number of cases and prevalence of four diseases affecting foals on Thoroughbred farms in the 2001 season

State	Farm	Diseases affecting foals			
		Diarrhoea	Respiratory disease	Conformational problems	Traumatic injuries
Vic	A	40 (46.5%)	0 (0%)	12 (14.0%)	5 (5.8%)
	B	8 (18.2%)	0 (0%)	2 (4.5%)	0 (0%)
	C	0 (0%)	5 (3.6%)	10 (7.3%)	5 (3.6%)
	D	4 (4.2%)	2 (2.1%)	9 (9.6%)	5 (1.0%)
	E	33 (15.1%)	66 (30.1%)	11 (5.0%)	11 (5.0%)
	F	11 (4.6%)	2 (0.8%)	47 (19.7%)	2 (0.8%)
	G	4 (9.3%)	1 (2.3%)	11 (25.6%)	8 (18.6%)
	H	30 (20.0%)	0 (0%)	45 (30.0%)	15 (10.0%)
	I	3 (2.9%)	0 (0%)	8 (7.8%)	3 (2.9%)
	J	15 (34.9%)	0 (0%)	0 (0%)	6 (14.0%)
NSW	1	36 (30.0%)	2 (1.7%)	48 (40.0%)	6 (5.0%)
	2	9 (3.9%)	3 (1.3%)	36 (15.6%)	30 (13.0%)
	3	244 (50.0%)	24 (4.9%)	146 (29.9%)	48 (9.8%)
	4	120 (31.0%)	4 (1.0%)	101 (26.0%)	41 (10.6%)
	5	5 (2.8%)	2 (1.1%)	25 (14.1%)	8 (4.5%)
	6	4 (1.9%)	1 (0.5%)	25 (12.1%)	18 (8.7 %)
	7	0 (0%)	2 (3.3%)	6 (9.8%)	3 (4.9%)
	8	28 (12.0%)	0 (0%)	17 (7.3%)	3 (1.3%)
	9	4 (8.5%)	9 (19.0%)	9 (19.0%)	7 (14.9%)
	10	17 (9.7%)	3 (1.7%)	121 (69.5%)	43 (24.7%)
	11	NA	NA	NA	NA
	12	3 (5.5%)	2 (3.6%)	8 (14.8%)	7 (13.0%)

NA: Information not provided

Nineteen farms reported the occurrence of diarrhoea. The prevalence of diarrhoea in foals on affected farms ranged from 1.9 to 50%. Eight farms (A, B, E, H, J, 1, 3 and 4) reported the prevalence of diarrhoea in foals to be >15% and on 5 of these farms (A, J, 1, 3 and 4) the prevalence was ≥30%.

Respiratory disease not associated with *R. equi* was reported on 15 farms. The prevalence of respiratory disease in foals on the affected farms ranged from 0.5 to 30.1%. Six farms (2 in Victoria – farms C and E, 4 in NSW – farms 3, 7, 9 and 12) reported a prevalence >3% and 2 (farms E and 9) reported a prevalence >15%. On both of these farms foals had upper respiratory tract disease (URTD) with primary clinical signs of nasal discharge and no evidence of lung pathology on auscultation or ultrasonographic examination. On farm E the majority of the cases of URTD in foals were associated with

the isolation of *Streptococcus equi* subspecies *zooepidemicus* and/or *Streptococcus equisimilis*. No attempt was made to isolate microorganisms associated with URTD on farm 9.

All farms, except J, reported the occurrence of conformational problems, mainly of the limbs, that required some form of correction. The prevalence of foals with conformational problems ranged from 4.5% to 69.5%. Nine farms (3 in Victoria – farms F, G and H, 6 in NSW – farms 1, 2, 3, 4, 9 and 10) reported conformational problems in >15% of foals and on 3 farms (H, 1 and 10) the prevalence of conformational problems was ≥30%. Six of the 9 farms (F, H, 1, 2, 3 and 4) with a prevalence of conformational problems of over 15% also reported mortalities associated with *R. equi* pneumonia.

All farms, except B, reported the occurrence of traumatic injuries requiring intervention. The prevalence of traumatic injuries ranged from 0.8 to 24.7%. Eight farms (G, H, J, 2, 4, 9, 10 and 12) reported traumatic injuries in ≥10% of their foals and 2 farms (G and 10) reported a prevalence >15%.

A significant association was observed between the prevalence of conformational problems and mortalities due to *R. equi* pneumonia. Farms with a high prevalence of conformational problems (>15%) were more likely to report *R. equi* associated mortalities (P=0.03), than farms with a lower prevalence of conformational problems. Six of the 9 farms that reported a prevalence of conformational problems of over 15% also reported mortalities due to *R. equi* pneumonia, while only 2/12 farms with <15% of their foals affected by conformational problems had mortalities due to *R. equi* pneumonia (Table 3.15).

Table 3.15: Associations between the prevalence of other diseases and the prevalence of *R. equi* pneumonia and the occurrence of mortalities due to *R. equi* pneumonia in the 2001 season

Prevalence of other diseases affecting foals		Low disease prevalence (<3%)	P	High disease prevalence (>9%)	P	Mortalities due to *R. equi* pneumonia	P
Diarrhoea	> 15%	3/8	1	2/8	1	4/8	0.65
	< 15%	6/13		3/13		4/13	
Respiratory disease (not associated with *R. equi*)	> 3%	3/6	0.82	1/6	0.85	1/6	0.97
	< 3%	6/15		4/15		7/15	
Conformational problems	> 15%	3/9	0.38	3/9	0.35	6/9	0.03*
	< 15%	6/12		2/12		2/12	
Traumatic injuries	≥ 10%	4/8	0.83	0/8	1	3/8	0.69
	< 10%	5/13		5/13		5/13	

P: Probability that the proportion of farms in the category among those with a high prevalence of one of the other diseases affecting foals differed from the proportion of farms in the category among those with a lower prevalence of the same disease.

3.4. Discussion

Few studies have investigated the associations between farm characteristics and management practices and the impact of *R. equi* pneumonia. This study described and categorised farm characteristics and management practices and related them to two indicators of the severity of *R. equi* pneumonia on farms, the prevalence of disease and the occurrence of mortalities due to this disease.

This study highlighted the associations between the prevalence of *R. equi* pneumonia and the occurrence of mortality due to *R. equi* pneumonia. Farms with high prevalence of *R. equi* pneumonia reported a high *R. equi* mortality rate. This may reflect an increased likelihood of mortality when *R. equi* pneumonia is very prevalent, or it may reflect the size of the dose of virulent *R. equi* a foal receives when it is exposed. An association between the prevalence of pneumonia due to *R. equi* and the prevalence and concentration of virulent *R. equi* in soil on farms has been suggested (Takai 1997). In an Argentinian study the prevalence of pneumonia due to *R. equi* and mortality due to *R. equi* appeared to be associated with an elevated prevalence of virulent *R. equi* in the soil (Becu *et al.* 2000).

Large acreage farms with large numbers of foals and high foal densities have been associated with an increased risk of *R. equi* pneumonia on farms (Chaffin *et al* 2003b). Farms on which ≥60 acres were used for horse husbandry, that kept >160 horses and/or >17 foals, and had foal densities of >0.25 foals/acre were more likely to have foals develop *R. equi* pneumonia than other farms (Chaffin *et al* 2003b). Some results described in this chapter confirm the findings of Chaffin *et al.* (2003b). While there was no association in this study between farm area or maximal stocking rate and the prevalence of disease or occurrence of mortalities due to *R. equi* pneumonia, there were associations between the maximal number of foals on farms and the prevalence of *R. equi* pneumonia and the occurrence of mortalities due to *R. equi*. In the 2001 season, farms with a small foal population were more likely to have a low prevalence of *R. equi* pneumonia and to not have mortalities due to *R. equi* than farms with medium to large foal populations. As the case fatality rates were no greater on the farms with a high disease prevalence than those with a low disease prevalence, the likelihood of mortality does not appear to be simply a consequence of the number of affected foals on the farm. The association between a small foal population and a low prevalence of *R. equi* pneumonia may reflect the communicable nature of the disease. Farms with a larger foal population may have a greater environmental burden of virulent *R. equi* due to increased contamination of soil with *R. equi* in foal faeces (Takai *et al.* 1994a) and an increased amount of equine faeces, which is utilised by *R. equi* for proliferation, in the environment (Hughes and Sulaiman 1987) than smaller farms. Other factors that might contribute to the association between the size of the foal population and the prevalence and severity of disease are explored in Chapters 4 and 6.

The lack of association between the farm area used for husbandry or the maximal stocking rate and the prevalence of *R. equi* pneumonia and mortality due to *R. equi* may be a consequence of the generally large size and high stocking rates of farms involved in this study compared to the study of Chaffin *et al.* (2003b). All farms in the study reported in this thesis used >24 hectares (or 60 acres) for horse husbandry purposes and had >0.6 foals/Ha (or 0.25 foals/acre) and would have been defined as large farms with high stocking rates in the study of Chaffin *et al.* (2003b). For this reason the small sizes of

farms and low stocking rate found to be significantly associated with reduced risk of *R. equi* on farms by Chaffin *et al.* (2003b) were not relevant to the study reported here.

As there was little variation in the study reported here between the farms in housing and mob management it was not possible to examine the effects these might have on the prevalence and severity of *R. equi* pneumonia. Most farms kept mare-foal pairs on paddocks in groups based on the reproductive status of the mare *post partum*. Farms that kept mare-foal pairs in groups based on the age of the foal did not have similar prevalences of *R. equi* pneumonia. Two of the three farms had a low prevalence of disease, while the other had a high prevalence of disease and had mortalities due to *R. equi*. An association was seen between the presence of a low proportion of transient mare-foal pairs (25% or fewer) and a reduced risk of *R. equi* pneumonia by Chaffin *et al.* (2003b). In the study reported here there was no *R. equi* pneumonia on the one farm with no transient mares (farm 7), but the farms with geographically separate areas for transient mares (farm H and 3) both reported at least a moderate prevalence of *R. equi* pneumonia (>3%) and mortalities due to *R. equi* pneumonia.

Management and feeding practices that may be thought to influence disease prevalence and severity were also investigated. Creep feed, usually high in concentrates, may alter the flora of the gastrointestinal tract of the foal and thus the replication of *R. equi* in the gastrointestinal tract. However, no association was seen between the use of creep feed and the prevalence of *R. equi* pneumonia or the occurrence of mortalities due to *R. equi*.

Feeding bins and stations promote congregation of mares and foals in paddocks and induce them to feed in areas where grass cover is sparse because of the amount of animal traffic. Inhalation of *R. equi* from the soil is likely in such areas. However, rotation of feeding bins and stations around the paddock to prevent excessive wear did not have any effect on the prevalence of *R. equi* pneumonia or the occurrence of mortalities due to *R. equi*, which suggests that inhalation of contaminated dust in paddocks may not totally explain the occurrence of *R. equi* pneumonia.

Young foals spent up to the first 2 weeks of their lives in small yards. Sick or injured foals were also kept in similar small yards. Mobs of mares and foals were mustered into holding pens to await farriery and/or veterinary treatment and these yards and pens were often worn and dusty. Soil contaminants may become airborne more easily in these environments and hence the dose of virulent *R. equi* inhaled may be greater than areas with more grass cover and lower stocking density. Removal of faeces from these areas may reduce the dose inhaled (Clarke 1989, Prescott and Yager 1991, Barton 1991, Giguere and Prescott 1997). However, no association was seen in this study between the routine removal of faecal material from small yards and holding pens and the prevalence of *R. equi* pneumonia or the occurrence of mortalities due to *R. equi*.

Harrowing, irrigation, herbicidal treatment of pasture and the use of fertilisers had no association with the prevalence of *R. equi* pneumonia or the occurrence of mortalities due to *R. equi*. However, irrigation of paddocks and yards and changes in soil pH may influence the prevalence of *R. equi* pneumonia by affecting replication in the soil and aerosolisation of the organism from the soil, affecting the concentration and proportion of virulent *R. equi* inhaled by foals (Hughes and Sulaiman 1987; Barton 1991; Giguere and Prescott 1997). As the discriminatory power of this study was limited, further studies were performed examining the influence of these factors on the ecology of virulent *R. equi* on farms and these are presented in the next chapter.

The accepted dogma on most horse breeding farms is that good preventative health practices are important in reducing the risk of *R. equi* pneumonia. This is based on observations that improvements in preventative health practices on single farms between seasons were associated with reductions in morbidity and mortality due to *R. equi* (Bain 1963; Debey and Bailie 1987; Clarke 1989). However, extensive studies of this issue have not been conducted. In the study described here some associations were seen between preventative health practices and the prevalence of *R. equi* pneumonia and the occurrence of mortality due to *R. equi* pneumonia. Associations were seen between the vaccination of foals against EHV 1 and EHV 4 and the prevalence of *R. equi* pneumonia, and between mortalities due to *R. equi* and pre-partum anthelmintic treatment. However, no association was seen between the timing of vaccination of foals or the vaccination of foals against

S. equi subspecies *equi* and disease due to *R. equi*. Similarly Chaffin *et al.* (2003a) found no association between vaccination of foals against *S. equi* subspecies *equi* and the occurrence of *R. equi* pneumonia.

Infection with other respiratory pathogens may predispose foals to *R. equi* pneumonia. Thus, immunisation against other respiratory pathogens may reduce this predisposition (Clarke 1989). Vaccination against EHV 2 multiple times within the first two months of life has been shown to reduce the prevalence of *R. equi* pneumonia (Nordengrahn *et al.* 1996; Varga *et al.* 1997), possibly by preventing EHV 2 suppression of the cell mediated immune response and hence decreasing the foal's susceptibility to infection. However, EHV 2 vaccination is not performed on commercial Thoroughbred farms in Australia.

The association between EHV 1 and EHV 4 vaccination of foals and a low prevalence of *R. equi* pneumonia is likely to be attributable to some other correlated environmental or management factor. The effect of vaccination initiated at approximately 3 months of age and *R. equi* pneumonia, which usually manifests itself before the foal reaches this age (see Chapter 4), is not likely to be causal. There was some correlation between farms with small foal populations and farms that vaccinate foals against EHV 1 and EHV 4 which may explain the apparent association between this vaccination strategy and low disease prevalence. The lack of association between vaccination of mares against EHV 1 and EHV 4 and low prevalence of disease also suggests that antibody passively acquired by the foal against EHV 1 and EHV 4 is not influencing the development of *R. equi* pneumonia. A cohort study examining the effects of earlier vaccination of foals against EHV 1 and EHV 4 on a farm with a high prevalence of *R. equi* pneumonia would be required to establish whether vaccination against EHV 1 and EHV 4 influences the risk of a foal developing *R. equi* pneumonia.

As most parasite control programs are initiated within the first two months of a foal’s life it is possible that effective parasite control could affect the predisposition of a foal to developing *R. equi* pneumonia. However, in this study the lack of association between anthelmintic programs for foals (timing and number of anthelmintics used) and

the prevalence of, or the occurrence of mortalities due to *R. equi* pneumonia inferred that the implementation of parasite control strategies in foals is not an effective strategy for reducing the prevalence or severity of *R. equi* pneumonia on farms. Other recent studies have also failed to find an association between anthelmintic regimens for foals and the occurrence of *R. equi* pneumonia on farms (Chaffin *et al.* 2003a).

A common predisposing factor for infectious disease in young foals is failure of passive transfer (FPT) of immunoglobulins. Numerous studies have associated FPT with septicaemia and other infectious disorders in foals (McGuire *et al.* 1975, 1977; Robinson *et al.* 1993; Raisis *et al.* 1996). Infectious disease and parasitism can compromise the mare's health in gestation and the foal's health and development *in utero* and when at foot (Brewer 1990; Paradis 2003). Pre-partum immunisation and, pre- and post-partum parasite control in mares, so as to reduce the risk of disease and parasitism and maximise the quality of colostrum and milk produced, may well influence the foal's chance of developing *R. equi* pneumonia and/or surviving infection. No associations were seen between immunisation strategies in mares nor the timing of the first anthelmintic treatment of mares *post partum*, and the prevalence of, or the occurrence of mortalities due to *R. equi* pneumonia. However, farms administering anthelmintics to pregnant mares within one month of their due foaling date were more likely to have mortalities due to *R. equi*. This association may be related to the effect of stresses caused by mustering and administration of a substance late in gestation on the quality of colostrum produced by the mare, and hence on the level of passive immunity against *R. equi* acquired by foals. The association between anthelmintic treatment of mares close to foaling and the occurrence of mortalities due to *R. equi* would benefit from a cohort study to evaluate the potential significance of late-term stresses on mares and the risk of foals subsequently dying from *R. equi* pneumonia. In general, parasite and immunisation programs appeared to have relatively little effect on the prevalence of, or the occurrence of mortalities due to, *R. equi* pneumonia on farms in the 2001 season.

Prophylactic therapy aimed at helping the neonate combat disease, or enhancing its immune system, did not reduce a foal's predispostion to *R. equi* pneumonia in this study. The inability to detect an association between the use of prophylactic therapies in foals and

the prevalence of, or the occurrence of mortalities due to, *R. equi* pneumonia in the study reported in this chapter confirmed the findings of another study (Chaffin *et al.* 2003c) that suggested that prophylactic therapy is not an effective strategy for reducing the prevalence or severity of *R. equi* pneumonia on farms.

In the study reported in this chapter only one farm (farm 1) used hyperimmune serum routinely on all foals. However, this farm still experienced a high prevalence of *R. equi* pneumonia and mortalities during the 2001 season. Administration of hyperimmune serum has been shown to be an effective prophylactic measure against *R. equi* pneumonia in some studies (Madigan *et al.* 1991; Higuchi *et al.* 1999), but has been ineffective in others (Chaffin *et al.* 1991, 2003a; Hurley and Begg 1995; Giguere *et al.* 2002). As the mechanisms by which hyperimmune serum might protect foals from *R. equi* pneumonia are unknown, and optimal timing and dosage regimens for administration are unknown (Muller and Madigan 1992; Giguere and Prescott 1997; Higuchi *et al.* 1999), it is not surprising that its efficacy on farms with a high prevalence of *R. equi* pneumonia may be unpredictable.

The prevalences of other diseases affecting foals were examined for associations with the prevalence of, or the occurrence of mortality due to, *R. equi* pneumonia on farms. Foal diarrhoea, respiratory disease not caused by *R. equi* and traumatic injuries were not associated with the prevalence of, or occurrence of mortalities due to, *R. equi* pneumonia. There was a significant association between a high prevalence of conformational problems in foals and the occurrence of mortalities due to *R. equi* pneumonia. No such association was observed between conformation and the prevalence of *R. equi* pneumonia. Foals with limb conformational problems are confined in small yards to facilitate assessment and correction of the fault. These yards are usually devoid of pasture cover, and thus are dry and dusty environments. Extended stays in such areas may result in a greater risk of inhalation of high concentrations of virulent *R. equi* and subsequent severe bronchopneumonia.

Two farms (B and E) had a substantial reduction in the prevalence of *R. equi* pneumonia between the 2000 and 2001 seasons. The only obvious change on farm B was a

reduction in maximal foal numbers. However, the prevalence of disease on farm E fell despite an increase in maximal foal numbers. The only management change observed between the two seasons on this farm was that feeding bins were moved every 5 weeks in the 2001 season, while bin rotation in the 2000 season was not performed on a routine basis. However, rotation of feeding bins was not associated with a difference in the prevalence of *R. equi* pneumonia, or the occurrence of mortalities due to, *R. equi* on farms in the 2001 season. Other farms reported minor changes in the prevalence of *R. equi* pneumonia or mortality due to *R. equi* between the breeding seasons, but none of the management changes on these farms involved variables found to be associated with the prevalence of, or the occurrence of mortalities due to, *R. equi* pneumonia in the 2001 season.

Even with the numerical limitations in this study, associations were seen between the prevalence of *R. equi* pneumonia or the occurrence of mortalities due to the disease and several farm characteristics and management strategies. Associations were seen between high disease prevalence and mortalities due to *R. equi* pneumonia, and a small foal population and a low prevalence of, and reduced risk of observed mortalities due to, *R. equi* pneumonia. There was no conclusive evidence that immunisation, parasite control or other health management practices in mares or foals reduced the prevalence or severity of *R. equi* pneumonia.

The effects of other variables on the prevalence and severity of *R. equi* pneumonia on farms, such as irrigation and use of fertilisers, may be better assessed by evaluating their potential to alter the levels of virulent *R. equi* in specific farm environments. Similarly, the association between a high prevalence of foals with limb conformational problems and an increased risk of mortality due to *R. equi* pneumonia may be better assessed by evaluating the levels of virulent *R. equi* in the farm environments in which these foals are kept.

Chapter 4

The ecology of virulent *R. equi* and epidemiology of *R. equi* pneumonia on Thoroughbred farms

4.1. Introduction

Serological testing of horses has indicated that there is a high prevalence of exposure to *R. equi* (Takai *et al.* 1985, 1995b). *R. equi* have been found in the soil on many horse farms and virulent *R. equi* have been found on endemically affected, sporadically affected, and unaffected farms (Takai *et al.* 1991c; Martens *et al.* 2000). The prevalence of virulent *R. equi* within the soil *R. equi* population and the concentration of virulent *R. equi* in soil have been previously shown to be important factors affecting the prevalence of pneumonia (Takai 1997). Factors that influence the survival and proliferation of virulent *R. equi* on a farm and its propensity to infect the lungs of foals are unknown. Assessment of the environmental virulent *R. equi* burden on farms has relied on isolation of bacteria from soil and subsequent assessment for *vapA* by PCR or VapA by immunoblotting (Takai *et al.* 1993b, 1995c). The primary route of infection is thought to be inhalation of aerosolised virulent *R. equi*, so measurement of airborne virulent *R. equi*, rather than those in soil, may yield a more meaningful assessment of the risk of disease on a farm.

A novel technique was devised to measure airborne and soil *R. equi* on farms in this study. This technique allowed the quantitative assessment of the environmental *R. equi* population on farms.

The work described in this chapter aimed to examine the relationships between the concentration of virulent *R. equi*, the proportion of *R. equi* that were virulent, environmental and management variables, and the prevalence of *R. equi* pneumonia on farms.

4.2. Materials and methods

4.2.1. Farms

This study was performed in conjunction with the questionnaire-based study described in Chapter 3. All farms participated in both studies. In the 2000 season, samples were collected from 6 Thoroughbred farms (A-F) in Victoria. In the 2001 season, samples were collected from 10 Thoroughbred farms (A-J) in Victoria and 12 Thoroughbred farms (1-12) in NSW. The prevalence of *R. equi* pneumonia on these farms is described in Table 3.3. Farm sizes (area used for horse husbandry), stocking rates and the maximal numbers of foals on the farms in the season in which samples were collected are described in Table 3.5. Samples were collected from farms A-F in both seasons, allowing comparisons to be made on these farms between seasons.

4.2.2. Sampling period

In Victoria during the 2000 season, monthly air and soil samples were collected on each farm, starting in September 2000 and ending in February 2001. Four mare and foal paddocks were randomly selected on each farm and these paddocks were sampled monthly throughout the season. Foals on these farms varied in age from newborn to 4-5 months at the time of sampling. Samples were generally taken from worn areas around feeding stations, as dust has been considered a major factor in infection by many investigators (Barton and Hughes 1984; Falcon *et al.* 1985; Takai *et al.* 1987; Prescott and Yager 1991; Giguere and Prescott 1997). Air samples were collected monthly from holding pens and lanes, starting in October. No soil samples were collected from these locations in 2000.

During the 2001 season, samples were collected every two weeks from Thoroughbred farms in Victoria. Samples were collected from 4 mare and foal paddocks, a holding pen and a lane on each farm throughout the season as in the previous season. On farms sampled in the 2000 season the same or similar paddocks were sampled in 2001, when possible. During the 2001 season samples were collected fortnightly from the middle of October 2001 to the end of January 2002, except during the end of December 2001. Soil

and air samples were collected from the holding pens and lanes from all farms during the 2001 season.

On farms in NSW, air and soil samples were collected in November and December 2001 from 4 mare and foal paddocks, a holding pen and a lane on each of the 12 farms. The paddocks were randomly selected in November and these paddocks were sampled again the following month.

4.2.3. Air samples

Air samples were collected using a portable air monitoring system (M Air T, Millipore, Saint-Quentin-Yveline, France) loaded with a cassette containing NANAT medium (Woolcock *et al.* 1979) as described in Section 2.2.2. The air monitoring system was placed approximately 5 cm above the soil surface and 2 or 3 samples of increasing volume were taken at each location (250 l, 500 l and 1000 l). The sieve of the air monitoring system is immediately adjacent to the agar and contains perforations that facilitate delivery of air onto the cassette. The sieve was disinfected with an isopropanol wipe (Isowipe, Kimberly-Clark, Milson's Point NSW) before the collection of each sample. After incubation at 37°C for 48 hours the plates that were derived from the largest volume of air sampled, still had well spaced colonies and did not have any fungal contamination were used for enumeration of environmental *R. equi* (representative cultures).

4.2.4. Soil samples

Adjacent to each air sampling site in the paddock, holding pen or lane, two superficial soil samples (down to a depth of 5 cm) were taken using a soil auger. The auger was disinfected with an isoproponal wipe (Isowipe) before each sample was collected. Samples were placed in sterile containers and sealed with an airtight lid for transfer to the laboratory. One sample was used for analysis of soil characteristics (pH, moisture and texture) whilst the other was used for microbiological analysis. The samples for microbiological testing were air dried and placed at -70°C for storage. Diluted soil samples were later cultured to quantify *R. equi* in soil by diluting 1 gram in 9 ml phosphate

buffered saline (PBS) and spreading 50 or 100 μl onto duplicate NANAT agar plates. After incubation at 37°C for 48 hours, representative cultures were selected for enumeration of environmental *R. equi*, as for air samples.

a. Soil moisture

At least 10 grams (wet weight) of soil from each sampling site was placed onto 3MM Whatman paper, weighed and then incubated at 200°C for approximately 2 hours. The samples were then re-weighed. The soil moisture was calculated according to the following formula:

$$\frac{\text{Wet weight} - \text{Dry weight}}{\text{Wet weight}} \times 100 = \%\ \text{soil moisture}\ (\%H_2O)$$

b. Soil pH

Approximately 10 grams of the oven dried soil was added to 25 ml of distilled H_2O. Samples were then mixed thoroughly by vortexing or placed in a shaker at 200 rpm for 15-30 minutes. The pH was then determined using a pH meter (Tan 1998).

c. Soil texture

Soil that was not used for measuring moisture or pH was used to assess soil texture. The soil was moistened, rolled into a ball and pressed. The manner in which the ball of soil broke up indicated the predominant soil type (either clay or sand). If the ball crumbled the soil was considered to have a predominantly sand base, whilst balls of soil that fissured were considered to have a predominantly clay base. In addition, general feel and malleability was considered, with gritty soil being considered sandy, and softer soils with the ability to hold form considered more clay-like (McLaren and Cameron 1996a).

4.2.5. Pasture height

At the time of air sample collection from each paddock the pasture height was measured at 20 randomly chosen sites within a 10 metre radius of the sampling site. This

was performed using a stick on which one centimetre intervals were marked. The mean of the 20 measurements was used as an estimate of pasture height in the sampled area of the paddock.

4.2.6. Foal numbers and cases of *R. equi* pneumonia

Foals were counted on sampled paddocks at the time of sampling as a measure of paddock group (or mob) size at the time of sampling. The number of foals on the farm and their ages at the time of each sample collection were derived from information given in the questionnaire (Appendix 1). The farm managers were asked to provide information on foaling dates and the number of foals on the property between prescribed dates, mostly coinciding with the time of sample collection. They were asked to report on cases of *R. equi* pneumonia, including the age of the foal at diagnosis, the date of diagnosis, the duration of therapy (2001 season only) and the outcome for each case.

4.2.7. Meteorological data

Mean temperature, humidity and wind speed at the time of sampling was obtained for the Australian Bureau of Meteorology weather station nearest to each farm (within 30 km) from www.bom.gov.au.

4.2.8. Colony blotting and DNA hybridisation

Single representative air and soil samples from each paddock, pen and lane on each farm at each time of sampling were selected for colony blotting and DNA hybridisation (Chapter 2). In total, 768 air samples and 708 soil samples were blotted and probed over the two years. Table 4.1 shows the number of air and soil samples blotted and probed each season and at each location (paddocks, pens and lanes).

Table 4.1: Number of samples used to enumerate environmental *R. equi* by colony blotting and DNA hybridisation

State	Season	Paddocks		Pens		Lanes		Total		Total
		Air	Soil	Air	Soil	Air	Soil	Air	Soil	
Vic	2000	144	144	30	-	30	-	204	144	**348**
Vic	2001	280	280	70	70	70	70	420	420	**840**
NSW	2001	96	96	24	24	24	24	144	144	**288**
Total	**Both**	**520**	**520**	**124**	**94**	**124**	**94**	**768**	**708**	**1476**

Four plates were blotted on each 16 x 21 cm sheet of nylon membrane along with half plates of a positive control culture (virulent *R. equi* – isolate number 7) and a negative control culture (*Corynebacterium ammoniagenes* – isolate number AP2). In the 2001 season, the negative control plate was divided in half to include an avirulent *R. equi* control (isolate number 128) so that, on blotting, one side of the half plate contained avirulent *R. equi* and the other the *C. ammoniagenes* negative control. This allowed confirmation that the *rrnA* probe identified both avirulent and virulent *R. equi*. Each control isolate was cultured in LB broth overnight and the culture diluted 10^4 fold. A 50 µl sample of the diluted culture was spread onto NANAT agar and the plates incubated at 37°C for 48 hours.

4.2.9. Statistical analysis

The number of colony forming units (cfu) of *R. equi*/1000 l were calculated for each air sample and the number of cfu/milligram (mg) were calculated for each soil sample. The geometric means of the concentrations of *R. equi* and virulent *R. equi* were determined for each farm, each location on the farm, each sampling time during the season (date) and each soil texture from which samples were taken, with 1 added to all values to allow inclusion of all data in the calculation of the geometric mean. The proportions of *R. equi* that were virulent were derived from these geometric means.

Fisher's exact test and the Mann-Whitney test were used to evaluate the significance of associations at the farm level. The farm level was defined as the 28 farm/year combinations. The associations evaluated were between categories of disease prevalence, presence or absence of mortalities as a result of *R. equi* pneumonia, categories of geometric mean concentration of *R. equi* and virulent *R. equi* and categories of proportions of *R. equi* in the soil or air that were virulent. The categories of the geometric mean concentration and proportion of *R. equi* that were virulent in air and soil samples were based on the approximate upper quartile points over the 28 farm/year units.

As the concentrations of *R. equi* and virulent *R. equi* in the soil and air samples were not normally distributed, Spearman's rank correlation coefficient (R_s) was used to investigate the strength of the correlation between paired soil and air samples for both the

concentration of *R. equi* and the concentration of virulent *R. equi*, with R_s >0.5 regarded as indicating a strong correlation. McNemar's test was used to compare the proportions of soil samples that were positive for *R. equi*, and virulent *R. equi*, to the proportions of air samples that were positive for *R. equi*, and virulent *R. equi*. McNemar's adjustment to the chi-squared test to account for clustering of observations within farm/year, was performed according to the method Eliasziw and Donner (1991) using the program "PAIRSetc" (Abramson 2004).

The concentrations of *R. equi* in air or soil were not normally distributed. The variance was greater than the mean, so the distribution was also overdispersed compared with a Poisson distribution, which has a variance equal to the mean. A visual comparison of the distribution of the counts with both a Poisson distribution and a negative binomial distribution was made using the nbvargr macro of STATA. The negative binomial distribution was a better fit to the data and this distribution was used in statistical models.

There were 28 farm/year combinations. Within each of these combinations there were between 12 and 42 observations, so the observations were clustered. To take account of this clustering a random effects negative binomial model was used (Hausman *et al.* 1984; Cameron and Trivedi 1998; StataCorp 2003a). The model building strategy involved two stages. All predictor variables were screened in a univariable random effects negative binomial regression. Four outcome variables were used individually when screening the predictor variables. These were the concentration of *R. equi* in air or soil, and the concentration of virulent *R. equi* in air or soil. Only predictor variables with a $P<0.25$ using a likelihood ratio test were included in the second stage. The second stage used a backward stepwise method to arrive at a final model that only included statistically significant variables. The criteria for exclusion of a variable was $P\geq0.05$ and for inclusion was $P<0.05$ using a likelihood ratio test.

Missing values for predictor variables were taken into account in the second stage by only including observations that did not have any missing values for the variables that entered the second stage. If only one variable remained in the final model and it had some missing values, the number of observations used for this final model was equal to the

number of observations at the beginning of the backward stepwise process when other variables were eligible for deletion. The majority of predictor variables did not have missing values, and the two variables (wind speed and soil moisture content) with the most missing values had less than 8% of observations missing.

The exponential of a coefficient from the random effects negative binomial regression model was interpreted as a count ratio (CR) for a unit change in the predictor variable (Dohoo *et al.* 2003). When the predictor variable was categorical, with two levels or groups, this represented the count ratio of being associated with the factor (the level that was coded as one) compared to the reference group (the level coded as zero). The count ratio was the ratio between the mean counts in the two groups. For example, if the count ratio were 1.5, this would indicate that the concentration of *R. equi* increased by 50% for a unit change in the predictor variable.

The likelihood ratio test (LRT) and the P value associated with a final model indicated the statistical significance of the predictor variables as a block compared with a model that did not contain this block of predictor variables. The P value associated with the "likelihood ratio test vs pooled" statistic compared the panel estimator with the pooled estimator (i.e., a negative binomial estimator with a constant dispersion). The P value that is displayed is set to half of the probability that a chi-squared statistic with 1 degree of freedom is greater than the calculated LRT statistic (StataCorp 2003a).

Two analyses were conducted because pasture height and the group size were not applicable to the pen and lane areas of the farm. One analysis included pasture height and the group size, as well as other variables, and so was only applicable to the paddocks. The other analysis excluded pasture height and numbers of foals on the paddock in the screening process and so was applicable to all locations (paddock, pen and lane) on the farm.

Collinearity between predictor variables (environmental and stocking (i.e. group size), excluding state/year and date) was assessed by calculating Spearman's rank correlation coefficient on binary categorical data. A coefficient of more than 0.8 was regarded as suggestive of collinearity (Dohoo *et al.* 2003).

The change in the estimate of the CR of the variables in the final model when a new variable was added to the final model was used to assess potential confounding variables. A change of over 10% in the CR was regarded as confounding. If the number of valid observations was decreased when adding the single variable, the assessment of confounding was done on a final model that only included observations where the single added variable was not missing. This only occurred when wind speed was the single variable that was added. The statistical significance of the added variable was also noted. This allowed assessment of variables that were not included in the final model.

Each environmental and stocking variable (excluding state/year and date) was assigned to one of two categories based on the median, lower or upper quartile, and biological relevance. Soil moisture, pasture height and soil pH cut-off values were based on the median. A pH of below 6 appears to favour the expression of virulence genes, so this cut-off has biological relevance (Takai *et al.* 1996b; Ren and Prescott 2003). The remaining environmental variables were group according to either the lower or upper quartile points of the data. The categories or cut-off values for the predictor variables are shown in Table 4.2.

A random effects logistic regression analysis was performed using the generalised linear latent and mixed model (gllamm) macro of STATA to assess predictor variables for the proportion of *R. equi* that were virulent in air and soil separately. The proportion of *R. equi* that were virulent in an air or soil sample was defined as the concentration of virulent *R equi* divided by the concentration of *R equi*. Only samples containing >4 *R. equi* cfu per unit of measurement in the denominator of the proportion were used in the regression analyses.

The univariable screening and multivariable model-building processes were performed as previously described, and the same predictor variables were used. The unit of analysis was the proportion, with a binomial denominator of one used in the gllamm command. The adapt option (adaptive quadrature) of gllamm was used, instead of ordinary quadrature, to fit the models.

McNemar's test, random effects negative binomial regression (xtnbreg, StataCorp 2003a) and random effects logistic regression (gllamm, Rabe-Hesketh *et al.* 2004) were performed using STATA 8.2 (StataCorp 2003b). All other statistical analyses were performed using MINITAB for Windows version 12.

Table 4.2: Categories used to assess the relationship between predictor variables and the concentration of environmental *R. equi*, the concentration of virulent *R. equi* and the proportion of *R. equi* that were virulent in the environment

Predictor variable	Categories or cut-off
Soil moisture (%H_2O)	≤10
	>10[a]
Pasture height (cm)	≤10
	>10[a]
Soil pH	≤6
	>6[a]
Group size (Number of foals on paddock)	>7
	≤7[a]
Wind speed (km/h)[*]	>10
	≤10[a]
Temperature (°C)[*]	>25
	≤25[a]
Humidity (%)[*]	≤70
	>70[a]
Texture of soil	Sand
	Clay[a]
Location on farm	Pen/lane
	Paddock[a]
State/year	Victoria 2000
	Victoria 2001
	New South Wales 2001[a]
Date of sampling	January-February (late)
	November-December (middle)
	September-October (early)[a]

[a] Reference category
[*] Mean reading for the duration of sampling on a given farm on a given day

4.3. Results

4.3.1. *R. equi* pneumonia on farms

The prevalences of *R. equi* pneumonia on farms, the mortalities associated with *R. equi* pneumonia and methods of diagnosis of *R. equi* pneumonia during the seasons in which samples were collected have been described previously (Section 3.3.1).

On the 6 Thoroughbred farms studied in the 2000 season, 45/789 foals were diagnosed with *R. equi* pneumonia. The first case was diagnosed in October on farm E, but large numbers of cases were first observed in November (7 cases), with most cases (32/45 - 71.1%) occurring in the December-January period. Death due to *R. equi* pneumonia was observed in 4 cases in the November-January period (Table 4.3).

Table 4.3: Distribution of cases of *R. equi* pneumonia on Victorian Thoroughbred farms during the 2000 season

Date of data collection	Number of cases of *R. equi* pneumonia on farm.						Total
	A	B	C	D	E	F	
Sept	0	0	0	0	0	0	0
Oct	0	0	0	0	1	0	1
Nov	0	0	2	1	1*	3	7*
Dec	2	0	2	1	3	8*	16*
Jan	1	1*	2	1	3	8*	16*
Feb	0	1	0	0	0	4	5
Total	3	2*	6	3	8*	23*	45*
Prevalence (%)	3.1	3.3	4.2	2.9	4.6	11.0	5.7

*Includes deaths from *R. equi* pneumonia
Approximate time between dates was 4 weeks

On the 10 Thoroughbred farms in Victoria studied in the 2001 season, 49/1155 foals were diagnosed with *R. equi* pneumonia. The first case was diagnosed in October on farm F, and the rate of diagnosis of new cases ranged from 4-7 per fortnight in the October-November period. Between the end of November and the middle of December 11 cases of *R. equi* were diagnosed and 15 cases were detected between the middle of December and the middle of January. In both of these periods deaths as a result of *R. equi* pneumonia were reported (Table 4.4).

Table 4.4: Distribution of cases of *R. equi* pneumonia on Victorian Thoroughbred farms during the 2001 season

Date of data collection	Number of cases of *R. equi* pneumonia on farm.										Total
	A	B	C	D	E	F	G	H	I	J	
Oct[1]	0	0	1	0	0	4	0	0	0	0	5
Oct[2]	0	0	1	0	0	3	0	0	0	0	4
Nov[1]	0	0	1	1	0	2	0	0	1	0	5
Nov[2]	0	0	1	1	0	4	0	0	1	0	7
Dec[1]	0	0	1	0	2	2	0	5*	1	0	11*
Jan[1]	4	0	2	0	0	7*	0	1	1	0	15*
Jan[2]	0	0	1	0	0	0	0	1	0	0	2
Total	4	0	8	2	2	22*	0	7*	4	0	49*
Prevalence (%)	4.7	0	5.8	2.1	0.9	9.2	0	4.7	3.9	0	4.2

*Includes deaths from *R. equi* pneumonia
[1]middle of the month
[2]end of the month
Approximate time between dates within the same month was 2 weeks

On the 12 Thoroughbred farms in NSW 202/2277 foals were diagnosed with *R. equi* pneumonia in the 2001 season. The first case was diagnosed in September on farm 3, and the rate of diagnosis of new cases peaked between the start and the middle of December, with 43 cases diagnosed in that period. Seven of the 9 foals that died as a result of *R. equi* pneumonia were diagnosed during the November-January period (Table 4.5).

Table 4.5: Distribution of cases of *R. equi* pneumonia on NSW Thoroughbred farms during the 2001 season

Date of data collection	Number of cases of *R. equi* pneumonia on farms.												Total
	1	2	3	4	5	6	7	8	9	10	11	12	
Sept[1]	0	0	0	0	0	0	0	0	0	0	0	0	0
Oct[1]	8*	0	7	1	0	0	0	0	0	0	1	0	17*
Nov[1]	1	5*	19	5	3	1	0	1	0	0	1	1	37*
Dec[1]	2	4	14*	9*	5	1*	0	9	0	1	1	1	47*
Dec[2]	1	3	25	5*	4	0	0	3	0	0	2	0	43*
Jan[2]	2	6	17	2	5	0	0	6*	0	0	1	0	39*
April[1]	11*	1	0	2	0	0	0	2	1	1	1	0	19*
Total	25*	19*	82*	24*	17	2*	0	21*	1	2	7	2	202*
Prevalence (%)	20.8	8.2	16.8	6.2	9.6	1.0	0	9.1	2.1	1.1	7.0	3.7	8.9

* Includes deaths from *R. equi* pneumonia
[1]start of the month
[2]middle of the month
Approximate time between dates within the same month was 2 weeks. Time between January and April was approximately 10 weeks.

The age at diagnosis of *R. equi* pneumonia and the duration of treatment were reported by all farms except farms C and 11 (Table 4.6).

Table 4.6: Age at diagnosis of, and duration of antimicrobial therapy for, *R. equi* pneumonia on Thoroughbred farms in the 2000 and 2001 seasons

State	Year	Farm	Age at diagnosis (days)					Duration of therapy (days)				Total
			≤30	31-60	61-90	91-120	121+	≤10	11-20	21-30	31+	
Vic	2000	A	0	1	0	0	0	NS	NS	NS	NS	1
		B	1	1*	0	0	0	NS	NS	NS	NS	2
		C	-	-	-	-	-	NS	NS	NS	NS	-
		D	0	0	3	0	0	NS	NS	NS	NS	3
		E	1	2*	5	0	0	NS	NS	NS	NS	8
		F	1	15*	7*	0	0	NS	NS	NS	NS	23
		Total	3	19	15	0	0	NS	NS	NS	NS	37
Vic	2001	A	0	3	1	0	0	3	1	0	0	4
		B	0	0	0	0	0	0	0	0	0	0
		C	-	-	-	-	-	-	-	-	-	-
		D	0	2	0	0	0	1	0	1	0	2
		E	0	1	1	0	0	1	1	0	0	2
		F	3	14*	5	0	0	8*	13	1	0	22
		G	0	0	0	0	0	0	0	0	0	0
		H	2*	5	0	0	0	3*	1	2	1	7
		I	1	3	0	0	0	0	1	3	0	4
		J	0	0	0	0	0	0	0	0	0	0
		Total	6	28	7	0	0	14	17	7	1	41
NSW	2001	1	2*	11*	3	1	8	17*	7	1	0	25
		2	0	15*	4	0	0	3	9	4*	3	19
		3	5	61	8	4	0	64*	16	2	0	82ϕ
		4	3	12*	5	4	0	10*	10	2	2	24
		5	0	16	1	0	0	4	13	0	0	17
		6	0	2*	0	0	0	1	0	0	1*	2
		7	0	0	0	0	0	0	0	0	0	0
		8	3	9*	8	1	0	3*	9	5	4	21
		9	0	0	0	0	1	0	1	0	0	1
		10	0	1	0	0	1	1	0	0	1	2
		11	-	-	-	-	-	-	-	-	-	-
		12	0	1	1	0	0	2	0	0	0	2
		Total	13	128	30	10	10	105	65	14	11	195
Both	Both	All	22	175	52	10	10	119	82	21	12	273

*Includes deaths from *R. equi* pneumonia
NS: farm not surveyed
ϕ Farm 3 did not provide the age at diagnosis of 4 cases

In the 2000 season the age at diagnosis was provided for 37 cases of *R. equi* pneumonia. All but 3 of these cases were seen in foals older than 30 days of age, the youngest being 25 days old. On farm F, 15/23 (65.2%) cases were seen in foals aged between 31 and 60 days. Three of the 4 foals that died as a consequence of *R. equi* pneumonia were also in the 31-60 days age group.

In the 2001 season on Victorian farms the age at diagnosis was provided for 41 cases of *R. equi* pneumonia. All but 6 of these cases were seen in foals older than 30 days of age, the youngest being 22 days old. Most cases (28/41 - 68.3%) were seen in foals between 31 and 60 days of age. On farm F, 14/22 (63.6%) cases were seen in foals aged between 31 and 60 days. Of the 2 deaths observed as a consequence of *R. equi* pneumonia, one foal was younger than 30 days of age at the time of diagnosis, whilst the other was aged between 31 and 60 days. Of the 39 foals that recovered fully from *R. equi* pneumonia, all but 8 were treated with antimicrobials for less than 21 days, with one case treated for more than 30 days. The 2 deaths reported occurred within the first week of antimicrobial therapy. On farm F, 13/21 (61.9%) cases that resolved were treated for between 11 and 20 days, with one foal treated for more than 20 days.

In the 2001 season on NSW farms the age at diagnosis was provided for 191 cases of *R. equi* pneumonia. Most cases (128/191 - 67%) were seen in foals between 31 and 60 days of age. The youngest a foal was diagnosed with *R. equi* pneumonia was at 16 days of age on farm 8, whilst the oldest was 158 days of age on farm 1. Farm 1 saw 32% (8/25) of their cases of *R. equi* pneumonia in foals older than 120 days of age. Of the 9 deaths observed as a consequence of *R. equi* pneumonia, the age of eight at the time of diagnosis was reported and all but one was between 31 and 60 days of age. The duration of treatment was reported for 195/202 cases, with 9 dying during treatment. Most cases (164/186 - 88.2%) were treated for less than 21 days. Seven of the 9 deaths occurred within the first 10 days of treatment. On farm 3, 63/81 (77.8%) cases that survived were treated for less than 10 days.

4.3.2. Environmental *R. equi*

a. General description

The concentration of *R. equi* in air samples ranged from 0 to 124 cfu/1000 l and in soil samples from 0 to 136 cfu/mg. The concentration of virulent *R. equi* in air samples ranged from 0 to 72 cfu/1000 l and in soil samples from 0 to 28 cfu/mg. The range and median of *R. equi* and virulent *R. equi* concentrations in environmental samples over the 2000 and 2001 seasons are summarised in Table 4.7.

Table 4.7: Concentrations of *R. equi* and virulent *R. equi* in air and soil samples collected from 22 Thoroughbred farms in the 2000 and 2001 seasons

Bacteria	Air (cfu/1000 l)					Soil (cfu/mg)				
	Range	Median	IQR	N[z]	N	Range	Median	IQR	N[z]	N
R. equi Total	0-124	2.0	0-6	237	768	0-136	4.0	1-9	99	708
Virulent *R. equi*	0-72	0.0	0-2	488	768	0-28	0.0	0-1	381	708

IQR: interquartile range (1st quartile-3rd quartile)
N[z]: number of samples from which *R. equi* or virulent *R. equi* were not recovered
N: number of samples

The correlation between the concentrations of *R. equi* in the paired soil and air samples was weakly positive (R_s=0.11). Similarly, the correlation between the concentrations of virulent *R. equi* in the paired soil and air samples was weakly positive (R_s=0.12).

A greater proportion of soil samples than air samples yielded both *R. equi* and virulent *R. equi*. *R. equi* were 2.8 times more likely to be recovered from soil samples than from air samples (P<0.001), but virulent *R. equi* were only 1.5 times more likely to be recovered from soil samples than from air samples (P=0.02) (Table 4.8).

Table 4.8: Proportion of soil and air samples positive for *R. equi* and virulent *R. equi*

R. equi

Air	Soil		Total (%)	Odds Ratio	95% CI	P
	Positive	Negative				
Positive	431	64	495 (70%)			
Negative	178	35	213 (30%)	2.8	1.7, 5.2	<0.001***
Total (%)	609 (86%)	99 (14%)	708			

Virulent *R. equi*

Air	Soil		Total (%)	Odds Ratio	95% CI	P
	Positive	Negative				
Positive	138	123	261 (37%)			
Negative	189	258	447 (63%)	1.5	1.1, 2.2	0.02*
Total (%)	327 (46%)	381 (54%)	708			

CI: confidence interval

The variances of the concentrations of *R. equi* and virulent *R. equi* in air samples were both about 25 times greater than the means. The variances of the concentrations of *R. equi* and virulent *R. equi* in soil were at least 5 times greater than the means. The concentrations of *R. equi* and the concentrations of virulent *R. equi* in air and soil both followed a negative binomial distribution with a degree of overrepresentation of even numbered concentrations, especially in the air samples, due to the effect of using 500 l and 250 l samples to estimate concentrations per 1000 l (Figure 4.1).

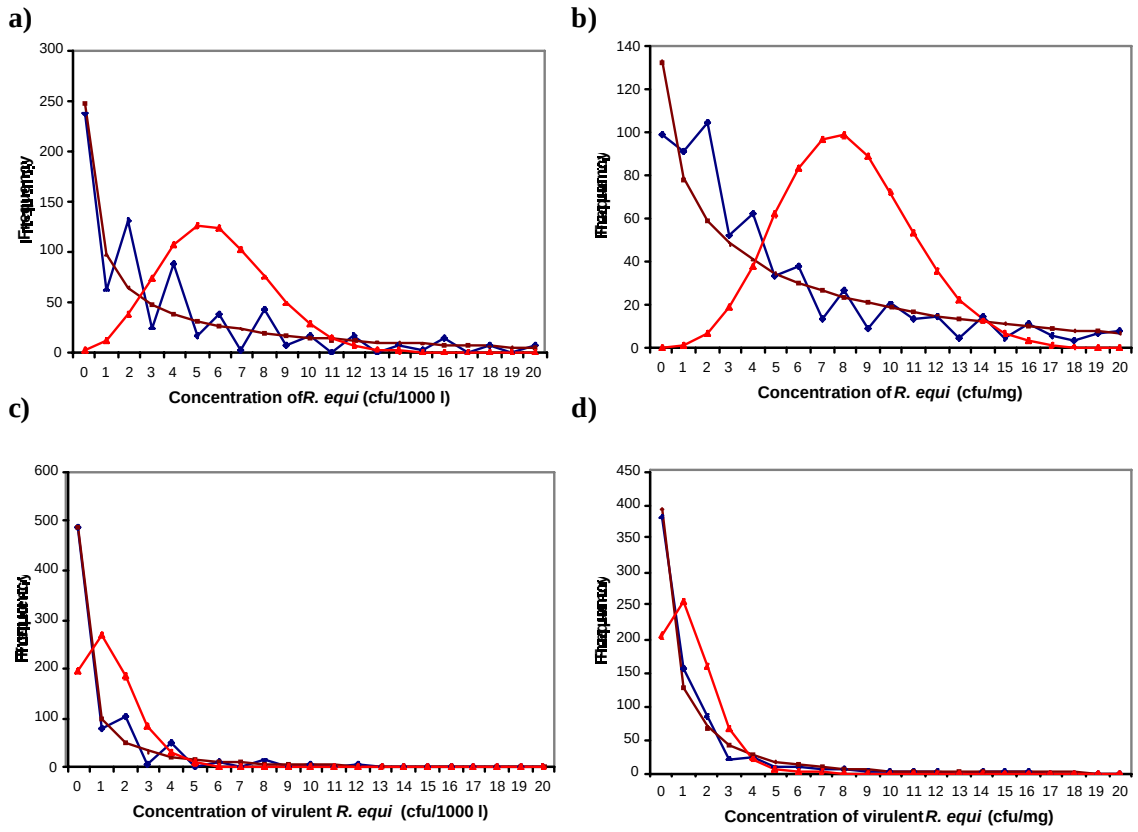

Figure 4.1: Distributions of concentrations of *R. equi* in air (a) and soil (b) and virulent *R. equi* in air (c) and soil (d).
Comparison of observed frequency of concentration (—υ—) with expected frequency for a negative binomial distribution (—ν—) or a Poisson distribution (—σ—) for 768 air samples and 708 soil samples on Thoroughbred farms in the 2000 and 2001 seasons.

Although logarithmic transformation did not normalise the data, it was deemed appropriate, because bacterial growth is exponential, to describe the concentrations of *R. equi* and virulent *R. equi* using the geometric means of the data, after 1 had been added to each value to allow inclusion of all data in the calculation.

Descriptive statistics for the geometric mean concentrations of *R. equi* and virulent *R. equi*, and the proportion of *R. equi* that were virulent, in environmental samples from each farm for each year are given in Table 4.9.

Table 4.9: Concentrations of *R. equi* and virulent *R. equi,* and proportion of *R. equi* that were virulent, in environmental samples on Thoroughbred farms in the 2000 and 2001 seasons

	Air (cfu/1000 l)					Soil (cfu/mg)			
	N	Mean	Median	Lower quartile	Upper quartile	Mean	Median	Lower quartile	Upper quartile
R. equi	28	3.1	2.5	1.6	4.2	4.9	4.1	2.7	7.0
Virulent *R. equi*	28	0.8	0.7	0.4	0.9	0.7	0.7	0.5	1.0
% virulent*	28	27.5	27.0	19.2	35.3	17.0	15.9	12.1	22.1

N: number of farm/year units
*Derived from the geometric mean concentration of virulent *R. equi* x 100/geometric mean concentration of *R. equi*

The means and medians of the geometric mean concentrations of virulent *R. equi* in soil and air were similar, but the mean and median of the geometric mean concentrations of total *R. equi* were greater in soil samples than in the air samples. This was reflected in the lower proportion of virulent *R. equi* in soil than in air samples.

b. Virulent *R. equi* on farms

Geometric mean concentrations of *R. equi* and virulent *R. equi* and proportions of *R. equi* that were virulent in air and soil samples from each Thoroughbred farm in the 2000 and 2001 seasons are shown in Table 4.10.

In the 2000 season, the proportion of airborne *R. equi* that were virulent was highest on farm F and the geometric mean concentrations of virulent *R. equi* in air and soil were highest on farm B (Table 4.10). The proportion of *R. equi* that were virulent in the soil samples was >16% on farms B and F. All three farms (B, E and F) that reported deaths due to *R. equi* pneumonia had higher geometric mean concentrations of virulent *R. equi* in both air and soil than farms that did not report deaths due to *R. equi*.

Table 4.10: Geometric means and interquartile ranges of concentrations of *R. equi* and virulent *R. equi*, and proportions of *R. equi* that were virulent, in air and soil samples from Thoroughbred farms in the 2000 and 2001 seasons

State	Farm	Year	Sample	N	Concentration of *R. equi*[a]			Concentration of virulent *R. equi*[a]			%*
					N[z]	Mean	IQR	N[z]	Mean	IQR	virulent
Vic	A	2000	Air	34	5	6.0	2.0-18.0	30	0.2	0.0-0.0	3.3
		2001	Air	42	7	3.7	2.0-8.2	25	0.7	0.0-2.0	18.9
	B	2000	Air	34	13	2.3	0.0-8.0	19	0.8	0.0-2.0	34.8
		2001	Air	42	13	2.2	0.0-6.0	23	0.7	0.0-2.0	31.8
	C	2000	Air	34	8	3.5	0.5-9.2	26	0.4	0.0-0.1	11.4
		2001	Air	42	16	2.0	0.0-4.2	33	0.3	0.0-0.0	15.0
	D	2000	Air	34	21	1.0	0.0-4.0	28	0.3	0.0-0.0	30.0
		2001	Air	42	22	1.0	0.0-2.0	31	0.3	0.0-1.0	30.0
	E	2000	Air	34	13	2.4	0.0-8.0	25	0.7	0.0-0.8	29.2
		2001	Air	42	3	3.9	2.0-6.5	27	0.6	0.0-2.0	15.4
	F	2000	Air	34	14	1.3	0.0-3.2	22	0.6	0.0-2.0	46.2
		2001	Air	42	10	3.1	0.7-8.0	18	1.1	0.0-2.2	35.5
	G	2001	Air	42	19	1.8	0.0-4.4	32	0.4	0.0-0.2	22.2
	H	2001	Air	42	18	1.4	0.0-4.0	32	0.3	0.0-0.2	21.4
	I	2001	Air	42	22	0.9	0.0-2.0	32	0.4	0.0-0.2	44.4
	J	2001	Air	42	12	1.5	0.0-4.0	27	0.4	0.0-1.0	26.7
NSW	1	2001	Air	12	0	7.6	2.2-25.1	0	3.3	1.0-7.5	43.4
	2	2001	Air	12	1	5.1	2.4-9.5	4	1.0	0.0-2.0	19.6
	3	2001	Air	12	2	4.2	3.0-7.5	2	2.2	1.2-3.7	52.4
	4	2001	Air	12	1	2.5	1.0-4.0	7	0.5	0.0-1.7	20.0
	5	2001	Air	12	4	2.2	0.0-6.8	8	0.6	0.0-1.0	27.3
	6	2001	Air	12	0	4.2	1.2-8.0	5	0.8	0.0-2.0	19.0
	7	2001	Air	12	4	1.5	0.0-4.0	8	0.3	0.0-1.0	20.0
	8	2001	Air	12	3	2.1	0.2-6.8	6	0.9	0.0-4.0	42.9
	9	2001	Air	12	1	4.7	1.2-10.2	7	0.7	0.0-1.7	14.9
	10	2001	Air	12	3	3.0	0.2-7.5	5	0.9	0.0-2.0	30.0
	11	2001	Air	12	1	6.1	1.2-22.9	2	2.4	1.0-6.8	39.4
	12	2001	Air	12	1	6.9	2.4-13.5	4	1.6	0.0-4.0	23.2
Vic	A	2000	Soil	24	2	7.0	2.0-17.5	14	0.6	0.0-2.0	8.6
		2001	Soil	42	3	3.2	1.7-7.0	22	0.5	0.0-1.0	15.6
	B	2000	Soil	24	1	7.8	4.0-13.5	13	1.3	0.0-4.0	16.7
		2001	Soil	42	7	2.8	1.0-8.0	20	0.8	0.0-2.0	28.6
	C	2000	Soil	24	4	4.6	2.0-30.0	17	0.2	0.0-0.0	4.3
		2001	Soil	42	10	2.5	0.7-5.4	24	0.6	0.0-1.0	24.0
	D	2000	Soil	24	7	3.2	0.0-8.0	11	0.5	0.0-1.3	15.6
		2001	Soil	42	11	2.6	0.0-5.2	28	0.5	0.0-1.0	19.2
	E	2000	Soil	24	3	9.7	4.0-23.5	12	0.7	0.0-2.0	7.2
		2001	Soil	42	1	6.0	2.0-14.2	17	1.0	0.0-2.0	16.6
	F	2000	Soil	24	3	6.0	2.0-15.5	11	1.0	0.0-2.0	16.6
		2001	Soil	42	6	3.0	1.0-6.0	22	0.7	0.0-2.0	23.3
	G	2001	Soil	42	5	3.1	2.0-4.2	17	0.7	0.0-1.2	22.6
	H	2001	Soil	42	8	2.9	1.0-8.0	21	0.6	0.0-1.0	20.7
	I	2001	Soil	42	5	3.7	1.0-10.0	22	0.6	0.0-1.0	16.2
	J	2001	Soil	42	8	2.1	1.0-4.0	29	0.3	0.0-1.0	14.3
NSW	1	2001	Soil	12	1	4.6	1.0-11.7	4	1.1	0.0-2.0	23.9
	2	2001	Soil	12	2	5.8	1.2-15.5	6	0.7	0.0-2.0	12.1
	3	2001	Soil	12	0	6.8	2.0-17.7	4	1.2	0.0-2.0	17.6
	4	2001	Soil	12	0	8.2	1.5-19.2	5	1.0	0.0-1.7	12.2
	5	2001	Soil	12	1	8.0	5.0-15.3	5	1.1	0.0-2.0	13.8
	6	2001	Soil	12	4	1.7	0.0-4.4	7	0.5	0.0-1.0	29.4
	7	2001	Soil	12	6	2.0	0.0-10.7	8	0.3	0.0-1.0	15.0
	8	2001	Soil	12	0	5.9	3.2-13.2	7	0.6	0.0-1.0	10.2
	9	2001	Soil	12	0	7.4	4.2-10.7	3	1.1	0.2-2.0	14.9
	10	2001	Soil	12	0	12.7	5.2-27.0	2	1.2	1.0-2.0	9.4
	11	2001	Soil	12	1	2.3	1.0-4.0	3	0.8	0.2-1.0	34.8
	12	2001	Soil	12	1	2.5	1.0-5.4	8	0.3	0.0-1.0	12.0

N: number of samples
N[z]: number of samples from which *R. equi* or virulent *R. equi* were not recovered
[a]cfu/1000 l for air samples; cfu/mg for soil samples
*Derived from the geometric mean concentration of virulent *R. equi* x 100/geometric mean concentration of *R. equi*

On Victorian farms in the 2001 season the proportion of airborne *R. equi* that were virulent was highest on farm I, whilst the geometric mean concentration of airborne virulent *R. equi* was highest on farm F (Table 4.10). The proportion of *R. equi* in soil samples that were virulent was greatest on farm B, whilst the geometric mean concentration of virulent *R. equi* in soil samples was greatest on farm E. Despite the high proportion of virulent *R. equi* in soil on farm B, this farm did not report cases of *R. equi* pneumonia in the 2001 season. Of these farms only farm F reported deaths due to *R. equi* in the 2001 season.

Comparisons of the geometric mean concentrations of virulent *R. equi* and the proportions of *R. equi* that were virulent between the 2000 and 2001 seasons on 6 Thoroughbred farms in Victoria showed that the geometric mean airborne concentration of virulent *R. equi* was 3.5 fold higher on farm A and nearly two fold higher on farm F in 2001 (Table 4.10). The geometric mean airborne concentrations of virulent *R. equi* on the remaining farms (B, C, D and E) were similar in 2000 and 2001.

The proportion of total *R. equi* that were virulent also varied between the 2000 and 2001 seasons (Table 4.10). The proportions of airborne *R. equi* that were virulent on farms E and F were approximately 50% and 20% lower, respectively, in the 2001 season. There was an approximately 6 fold increase in the proportion of airborne *R. equi* that were virulent on farm A and a lesser increase, of approximately 30%, on farm C. The proportions of airborne *R. equi* that were virulent were similar in both seasons on farms B and D.

The geometric mean concentrations of virulent *R. equi* in soil in both seasons on farms A, D, E and F were similar (Table 4.10). However, on farm B there was a 40% reduction in the geometric mean concentration of virulent *R. equi* in soil in the 2001 season, whilst a 3 fold increase was seen on farm C. The proportion of *R. equi* that were virulent in soil increased on all farms in the 2001 season compared to the 2000 season, with the greatest increase seen on farm C (4.5 fold). As only paddocks were sampled in the 2000 season, these increases in the proportion of *R. equi* that were virulent in the 2001 season reflect the inclusion of lanes and holding pens in the soil sampling procedure on

farms. The difference between seasons in the proportions of *R. equi* that were virulent in soil from paddocks was generally less (Table 4.14) than when pen and lane samples were included in the seasonal comparison (Table 4.10).

On NSW farms during the November-December period of the 2001 season, the proportions of airborne *R. equi* that were virulent were highest on farms 3, 1 and 8, with >40% of airborne *R. equi* on these farms being virulent (Table 4.10). All 3 farms reported deaths due to *R. equi*. The geometric mean concentration of airborne virulent *R. equi* was highest on farm 1. The proportion of *R. equi* that were virulent in soil was greatest on farm 11, whilst the geometric mean concentration of virulent *R. equi* in soil was greatest on farms 3, 10, 1, 9 and 5, all of which had a geometric mean concentration of virulent *R. equi* >1 cfu/mg.

The associations between the prevalence and severity of *R. equi* pneumonia and the geometric mean environmental burden of *R. equi* and virulent *R. equi* were examined using Fisher's exact test (Table 4.11) and the Mann-Whitney test (Table 4.12). Farms on which ≥35% of airborne *R. equi* were virulent were significantly less likely to have a low prevalence of *R. equi* pneumonia than farms on which <35% of airborne *R. equi* were virulent (P=0.03). Farms on which ≥35% of airborne *R. equi* were virulent were significantly more likely (P=0.001) to have a high prevalence of *R. equi* pneumonia than farms on which <35% of airborne *R. equi* were virulent (Table 4.11). A greater proportion of farms on which ≥35% of airborne *R. equi* were virulent reported mortalities associated with *R. equi* pneumonia (Table 4.11, 5/7 compared with 6/21), but this difference was not significant (P=0.06). A significantly greater proportion of the farms (10/22) on which the geometric mean airborne concentration of virulent *R. equi* was <1 cfu/1000 l reported a low prevalence (<3%) of *R. equi* pneumonia, than the farms (0/6) with a higher geometric mean airborne concentration of virulent *R. equi* (P=0.049). A greater proportion of farms on which the geometric mean airborne concentration of virulent *R. equi* was ≥1 cfu/1000 l had a high prevalence of *R. equi* pneumonia than farms on which the geometric mean airborne concentration of virulent *R. equi* was <1 cfu/1000 l (3/6 compared with 3/22), but this difference was not significant (P=0.09) (Table 4.11).

Differences in concentration of virulent *R. equi* in the soil were not associated with differences in prevalence or severity of *R. equi* pneumonia (Table 4.11). A greater proportion of farms with a geometric mean concentration of virulent *R. equi* of ≥1 cfu/mg in soil had a higher prevalence of *R. equi* pneumonia (4/9 compared with 2/19), but this difference was not significant (P=0.06).

Table 4.11: Associations between categories of geometric mean concentrations of *R. equi* and virulent *R. equi*, and proportions of *R. equi* that were virulent, in air and soil samples and categories of prevalence of *R. equi* pneumonia and mortality due to *R. equi* pneumonia on farms in the 2000 and 2001 seasons

		Low disease prevalence (<3%)	P	High disease prevalence (>9%)	P	Mortalities due to *R. equi* pneumonia	P
Airborne *R. equi*	≥5 cfu/1000 l	0/5		1/5		2/5	
	<5 cfu/1000 l	10/23	0.09	5/23	0.73	9/23	0.67
Airborne virulent *R. equi*	≥1 cfu/1000 l	0/6		3/6		4/6	
	<1 cfu/1000 l	10/22	0.049*	3/22	0.09	7/22	0.14
Proportion of airborne *R. equi* virulent	≥35%	0/7		5/7		5/7	
	<35%	10/21	0.03*	1/21	0.001**	6/21	0.06
R. equi in soil	≥5 cfu/mg	3/12		3/12		7/12	
	<5 cfu/mg	7/16	0.27	3/16	0.52	4/16	0.08
Virulent *R. equi* in soil	≥1 cfu/mg	3/9		4/9		5/9	
	<1 cfu/mg	7/19	0.60	2/19	0.06	6/19	0.21
Proportion of soil *R. equi* virulent	≥20%	3/8		2/8		4/8	
	<20%	7/20	0.72	4/20	0.57	7/20	0.38

P: probability that the proportions in each category differed using Fisher's exact test

The median prevalence of *R. equi* pneumonia on farms with a geometric mean airborne concentration of virulent *R. equi* ≥1 cfu/1000 l was significantly greater (P=0.008) than on farms with airborne concentration of virulent *R. equi* <1 cfu/1000 l (Table 4.12). Similarly, the median prevalence of disease due to *R. equi* on farms on which ≥35% of airborne *R. equi* were virulent was significantly greater (P=0.001) (Table 4.12) than on farms on which a lower proportion of airborne *R. equi* were virulent.

Table 4.12: Associations between categories of geometric mean concentrations of *R. equi* and virulent *R. equi*, and proportions of *R. equi* that were virulent, in air and soil samples and the median prevalence of *R. equi* pneumonia on farms in the 2000 and 2001 seasons

		N	Median disease prevalence (%)	P
Airborne *R. equi*	≥5 cfu/1000 l	5	7.0	
	<5 cfu/1000 l	23	4.0	0.19
Airborne virulent *R. equi*	≥1 cfu/1000 l	6	8.7	
	<1 cfu/1000 l	22	3.2	0.008**
Proportion of airborne *R. equi* virulent	≥35%	7	9.2	
	<35%	21	3.1	0.001**
R. equi in soil	≥5 cfu/mg	12	5.4	
	<5 cfu/mg	16	3.9	0.19
Virulent *R. equi* in soil	≥1 cfu/mg	9	6.2	
	<1 cfu/mg	19	4.0	0.18
Proportion of soil *R. equi* virulent	≥20%	8	5.2	
	<20%	20	3.9	0.96

N: number of farm/year units
P: probability that the median prevalence differed between the two categories

c. Virulent *R. equi* and locations on farms

The geometric mean concentrations of *R. equi* and virulent *R. equi*, and the proportions of *R. equi* that were virulent, in the air (Table 4.13) and soil (Table 4.14) samples from paddocks, holding pens and lanes were determined on all farms in the 2001 foaling season. As soil samples were not taken for microbiological analysis from the holding pens and lanes in the 2000 season, only airborne *R. equi* populations could be compared between seasons in these areas.

Table 4.13: Airborne concentrations of *R. equi* and virulent *R. equi*, and the proportion of airborne *R. equi* that were virulent, in paddocks and pens/lanes on each Thoroughbred farm in the 2000 and 2001 seasons

State	Farm	Year	Location	N	Concentration of *R. equi* (cfu/1000 l)			Concentration of virulent *R. equi* (cfu/1000 l)			%* virulent
					Nz	Geometric mean	IQR	Nz	Geometric mean	IQR	
Vic	A	2000	Paddock	24	3	5.9	2.0-16.0	22	0.1	0.0-0.0	1.7
		2001	Paddock	28	5	2.6	1.2-6.0	18	0.4	0.0-1.0	15.4
	B	2000	Paddock	24	8	3.0	0.0-9.5	11	1.1	0.0-3.4	36.7
		2001	Paddock	28	11	1.3	0.0-4.0	14	0.7	0.0-2.0	53.8
	C	2000	Paddock	24	5	4.0	0.7-9.7	20	0.2	0.0-0.0	5.0
		2001	Paddock	28	12	1.6	0.0-4.0	22	0.3	0.0-0.0	18.8
	D	2000	Paddock	24	14	1.3	0.0-4.0	19	0.4	0.0-0.0	30.8
		2001	Paddock	28	14	0.9	0.0-2.0	21	0.2	0.0-0.7	22.2
	E	2000	Paddock	24	12	1.0	0.0-2.0	21	0.2	0.0-0.0	20.0
		2001	Paddock	28	3	2.6	1.0-4.7	21	0.3	0.0-0.7	11.5
	F	2000	Paddock	24	8	1.6	0.0-3.7	16	0.6	0.0-1.5	37.5
		2001	Paddock	28	9	1.5	0.0-3.0	16	0.5	0.0-1.0	33.3
	G	2001	Paddock	28	16	1.1	0.0-3.0	24	0.1	0.0-0.0	9.1
	H	2001	Paddock	28	14	0.9	0.0-2.0	21	0.3	0.0-0.7	33.3
	I	2001	Paddock	28	17	0.5	0.0-2.0	23	0.2	0.0-0.0	40.0
	J	2001	Paddock	28	10	1.1	0.0-2.0	19	0.3	0.0-1.0	27.3
NSW	1	2001	Paddock	8	0	4.2	2.0-6.8	0	1.9	1.0-3.4	45.2
	2	2001	Paddock	8	1	3.7	2.0-8.0	3	0.7	0.0-1.7	18.9
	3	2001	Paddock	8	2	2.2	0.4-4.0	2	1.4	0.2-2.7	63.6
	4	2001	Paddock	8	0	2.6	1.0-4.0	5	0.4	0.0-1.0	15.4
	5	2001	Paddock	8	2	1.9	0.2-6.3	5	0.4	0.0-1.0	21.1
	6	2001	Paddock	8	0	3.3	1.0-7.5	4	0.5	0.0-1.0	15.2
	7	2001	Paddock	8	1	2.5	1.2-4.0	5	0.4	0.0-1.0	16.0
	8	2001	Paddock	8	3	0.9	0.0-2.0	6	0.2	0.0-0.7	22.2
	9	2001	Paddock	8	0	3.3	1.2-7.1	6	0.2	0.0-0.7	6.1
	10	2001	Paddock	8	1	4.5	1.2-11.5	3	0.9	0.0-3.0	20.0
	11	2001	Paddock	8	0	4.5	1.2-13.5	1	1.8	1.0-3.4	40.0
	12	2001	Paddock	8	1	4.9	1.2-12.2	3	1.3	0.0-3.4	26.5
Vic	A	2000	Pen/lane	10	4	6.1	1.3-41.6	8	0.2	0.0-0.3	3.3
		2001	Pen/lane	14	2	7.3	5.4-16.9	7	1.4	0.0-4.4	19.2
	B	2000	Pen/lane	10	1	1.1	0.0-4.0	8	0.3	0.0-0.3	27.3
		2001	Pen/lane	14	2	5.0	2.0-16.0	9	0.8	0.0-2.1	16.0
	C	2000	Pen/lane	10	3	2.6	0.0-6.8	6	0.8	0.0-2.4	30.8
		2001	Pen/lane	14	4	3.0	0.0-12.5	11	0.4	0.0-0.3	13.3
	D	2000	Pen/lane	10	7	0.6	0.0-2.4	9	0.1	0.0-0.0	16.7
		2001	Pen/lane	14	8	1.3	0.0-6.5	10	0.6	0.0-2.0	46.2
	E	2000	Pen/lane	10	1	10.3	5.4-24.1	4	2.7	0.0-9.3	26.2
		2001	Pen/lane	14	0	8.3	2.0-19.6	6	1.3	0.0-2.4	15.7
	F	2000	Pen/lane	10	6	0.8	0.0-2.4	6	0.6	0.0-2.0	75.0
		2001	Pen/lane	14	1	9.9	4.0-19.4	2	3.2	2.0-6.0	32.3
	G	2001	Pen/lane	14	3	4.0	1.3-8.9	8	1.2	0.0-4.0	30.0
	H	2001	Pen/lane	14	4	2.7	0.0-6.0	11	0.3	0.0-0.3	11.1
	I	2001	Pen/lane	14	5	1.7	0.0-4.0	9	0.8	0.0-4.0	47.1
	J	2001	Pen/lane	14	2	2.8	2.0-4.4	8	0.6	0.0-2.0	21.4
NSW	1	2001	Pen/lane	4	0	22.7	5.3-92.4	0	8.3	2.9-19.4	36.6
	2	2001	Pen/lane	4	0	9.4	8.0-11.5	1	1.9	0.3-4.0	20.2
	3	2001	Pen/lane	4	0	13.1	6.5-36.8	0	4.7	2.0-10.9	35.9
	4	2001	Pen/lane	4	1	2.3	0.5-4.0	2	1.0	0.0-3.4	43.5
	5	2001	Pen/lane	4	2	3.1	0.0-29.2	3	1.0	0.0-7.4	32.3
	6	2001	Pen/lane	4	0	6.5	2.7-16.0	1	1.6	0.3-3.4	24.6
	7	2001	Pen/lane	4	3	0.3	0.0-1.3	3	0.3	0.0-1.3	100
	8	2001	Pen/lane	4	0	8.0	4.8-11.5	0	4.0	4.0-4.0	50.0
	9	2001	Pen/lane	4	1	9.3	0.8-35.2	1	2.2	0.3-5.4	23.7
	10	2001	Pen/lane	4	2	1.1	0.0-4.7	2	0.7	0.0-2.0	63.6
	11	2001	Pen/lane	4	1	10.8	1.1-32.5	1	3.9	0.3-16.0	36.1
	12	2001	Pen/lane	4	0	13.0	4.8-42.5	1	2.4	0.3-6.8	18.5

N: number of samples
Nz: number of samples from which *R. equi* or virulent *R. equi* were not recovered
*Derived from the geometric mean concentration of virulent *R. equi* x 100/geometric mean concentration of *R. equi*

The airborne virulent *R. equi* burden (the proportion of *R. equi* that were virulent and/or the concentration of virulent *R. equi*) was generally higher in the pens and lanes than in the paddocks. All but 3 farm/year units (B 2000, D 2000 and H 2001) had greater airborne burdens of virulent *R. equi* in the combined pen/lane environments than in the paddocks (Table 4.13).

Of the 6 Thoroughbred farms surveyed in both the 2000 and 2001 seasons only farm A had an increase in the airborne virulent *R. equi* burden in both locations (paddocks and pens/lanes) between the 2000 and 2001 seasons. Farms E and F had decreases in the proportion of airborne *R. equi* that were virulent in both locations in the 2001 season. Farms C and E had a reduction in the geometric mean airborne concentration of virulent *R. equi* in the pens and lanes in the 2001 season (Table 4.13).

Twenty of the 22 farms sampled in 2001 had either a higher proportion of soil *R. equi* that were virulent or a higher geometric mean concentration of virulent *R. equi* in soil from the pens and lanes than in soil from the paddocks (Table 4.14). The two farms (G and 7) that did not have a greater burden of virulent *R. equi* in soil from the pens and lanes did not report any cases of *R. equi* pneumonia in the 2001 season.

All 6 of the Victorian farms surveyed in both the 2000 and 2001 seasons had an increase in the proportion of *R. equi* that were virulent in soil from the paddocks in the 2001 season (Table 4.14). Only 3 farms (C, D and E) had an increase in the geometric mean concentration of virulent *R. equi* in the soil from paddocks in the 2001 season.

Table 4.14: Soil concentrations of *R. equi* and virulent *R. equi*, and the proportion of soil *R. equi* that were virulent, in paddocks and pens/lanes on each Thoroughbred farm in the 2000 and 2001 seasons

State	Farm	Year	Location	N	Concentration of *R. equi* (cfu/mg)			Concentration of virulent *R. equi* (cfu/mg)			%* virulent
					N[z]	Geometric mean	IQR	N[z]	Geometric mean	IQR	
Vic	A	2000	Paddock	24	2	7.0	2.0-17.5	14	0.6	0.0-2.0	8.6
		2001	Paddock	28	2	2.6	1.0-4.7	17	0.4	0.0-1.0	15.4
	B	2000	Paddock	24	1	7.8	4.0-13.5	13	1.3	0.0-4.0	16.7
		2001	Paddock	28	6	2.3	1.0-5.7	15	0.7	0.0-1.0	30.4
	C	2000	Paddock	24	4	4.5	2.0-30.0	17	0.2	0.0-0.0	4.4
		2001	Paddock	28	10	2.0	0.0-4.0	19	0.4	0.0-1.0	20.0
	D	2000	Paddock	24	7	3.2	0.0-8.0	11	0.5	0.0-1.3	15.6
		2001	Paddock	28	6	3.6	1.0-9.0	18	0.6	0.0-1.0	16.7
	E	2000	Paddock	24	3	9.7	4.0-23.5	12	0.7	0.0-2.0	7.2
		2001	Paddock	28	1	6.8	2.2-15.7	12	1.1	0.0-2.7	16.2
	F	2000	Paddock	24	3	6.0	2.0-15.5	11	1.0	0.0-2.0	16.7
		2001	Paddock	28	5	2.9	1.0-5.7	15	0.6	0.0-1.7	20.7
	G	2001	Paddock	28	1	3.9	2.0-4.7	8	1.0	0.0-2.0	25.6
	H	2001	Paddock	28	4	3.9	1.2-11.0	13	0.8	0.0-2.0	20.5
	I	2001	Paddock	28	4	3.0	1.0-6.7	16	0.5	0.0-1.0	16.7
	J	2001	Paddock	28	4	2.8	1.0-5.7	18	0.4	0.0-1.0	14.3
NSW	1	2001	Paddock	8	0	3.4	1.0-7.7	3	0.7	0.0-1.7	20.6
	2	2001	Paddock	8	2	2.7	0.2-8.0	6	0.3	0.0-0.7	11.1
	3	2001	Paddock	8	0	6.6	2.2-15.6	3	1.0	0.0-2.0	15.2
	4	2001	Paddock	8	0	16.6	8.9-25.8	4	1.1	0.0-2.7	6.6
	5	2001	Paddock	8	0	8.5	5.0-15.3	4	0.9	0.0-2.0	10.6
	6	2001	Paddock	8	3	1.4	0.0-4.4	5	0.3	0.0 -1.0	21.4
	7	2001	Paddock	8	4	1.8	0.0-10.7	5	0.4	0.0-1.0	22.2
	8	2001	Paddock	8	0	4.8	1.5-8.7	5	0.5	0.0-1.0	10.4
	9	2001	Paddock	8	0	7.4	5.2-10.0	3	0.9	0.0-2.7	12.2
	10	2001	Paddock	8	0	16.9	6.5-28.5	1	1.1	1.0-2.0	6.5
	11	2001	Paddock	8	0	2.2	1.2-3.7	2	0.8	0.2-1.0	36.3
	12	2001	Paddock	8	0	3.3	1.2-7.4	6	0.3	0.0-0.7	9.1
Vic	A	2001	Pen/lane	14	1	4.7	2.0-10.9	5	0.8	0.0-1.0	17.0
	B	2001	Pen/lane	14	1	3.9	1.0-11.0	5	1.0	0.0-2.0	25.6
	C	2001	Pen/lane	14	0	3.8	1.0-8.2	5	1.0	0.0-1.4	26.3
	D	2001	Pen/lane	14	5	1.3	0.0-4.2	10	0.4	0.0-1.2	30.8
	E	2001	Pen/lane	14	0	4.5	1.7-7.5	5	0.9	0.0-2.0	20.0
	F	2001	Pen/lane	14	1	3.2	1.7-6.2	7	0.9	0.0-2.2	28.2
	G	2001	Pen/lane	14	4	2.0	0.0-4.6	9	0.3	0.0-1.0	15.0
	H	2001	Pen/lane	14	4	1.5	0.0-5.0	8	0.3	0.0-1.0	20.0
	I	2001	Pen/lane	14	1	5.5	2.0-16.7	6	1.0	0.0-2.4	18.2
	J	2001	Pen/lane	14	4	1.0	0.0-2.0	11	0.2	0.0-0.2	20.0
NSW	1	2001	Pen/lane	4	0	8.0	2.2-15.6	1	2.2	0.3-5.4	27.5
	2	2001	Pen/lane	4	0	22.5	14.5-39.8	0	1.9	1.2-2.7	8.4
	3	2001	Pen/lane	4	0	7.3	2.0-25.4	1	1.7	0.2-5.8	23.3
	4	2001	Pen/lane	4	0	1.5	1.0-3.0	1	0.7	0.2-1.0	46.7
	5	2001	Pen/lane	4	1	7.1	0.6-39.6	1	1.6	0.2- 6.2	22.5
	6	2001	Pen/lane	4	1	2.2	0.3-6.9	2	1.0	0.0-4.7	45.5
	7	2001	Pen/lane	4	2	2.4	0.0-14.4	3	0.2	0.0-0.7	8.3
	8	2001	Pen/lane	4	0	8.8	3.8-16.5	2	0.8	0.0-3.0	9.1
	9	2001	Pen/lane	4	0	7.4	2.2-21.0	0	1.4	1.0-2.0	18.9
	10	2001	Pen/lane	4	0	7.0	5.0-12.0	1	1.2	0.2-3.6	17.1
	11	2001	Pen/lane	4	1	2.4	0.2-9.2	1	0.9	0.2-1.7	37.5
	12	2001	Pen/lane	4	1	1.3	0.2-4.1	2	0.4	0.0-1.0	30.8

N: number of samples
N[z]: number of samples from which *R. equi* or virulent *R. equi* were not recovered
*Derived from the geometric mean concentration of virulent *R. equi* x 100/geometric mean concentration of *R. equi*

The geometric mean concentrations of virulent *R. equi* were higher in soil and air from the pens and lanes than in the soil and air from the paddocks. The geometric mean concentrations of virulent *R. equi* were greater in the soil and air samples collected from the pens than those collected from the lanes (Table 4.15). The proportions of *R. equi* that were virulent in the air and soil from the pens and lanes were generally higher than in the air and soil from the paddocks, with the exception of Victorian farms in the 2001 season, where the proportions of airborne *R. equi* that were virulent were similar in the pens and lanes to the paddocks. The differences in proportions of *R. equi* that were virulent between the pens/lanes and paddocks were greater in air samples than in the soil samples (Table 4.15).

Table 4.15: Concentrations of *R. equi* and virulent *R. equi*, and proportion of *R. equi* that were virulent, in paddocks, pens, lanes and pens/lanes on Thoroughbred farms in the 2000 and 2001 seasons

State	Sample	Year	Location	N	Concentration of *R. equi*[a]			Concentration of virulent *R. equi*[a]			%[*]
					N[z]	Geometric mean	IQR	N[z]	Geometric mean	IQR	virulent
Vic	Air	2000	Paddock	144	50	2.4	0.0-8.0	109	0.4	0.0-0.0	17.2
Vic	Air	2001	Paddock	280	111	1.3	0.0-3.0	199	0.3	0.0-1.0	25.0
NSW	Air	2001	Paddock	96	11	3.0	1.0-6.0	43	0.7	0.0-1.7	24.8
Vic	Air	2000	Pen	30	13	2.0	0.0-4.0	19	0.7	0.0-2.0	35.5
Vic	Air	2001	Pen	70	12	4.9	2.0-12.0	38	1.1	0.0-4.0	21.5
NSW	Air	2001	Pen	24	3	8.0	4.0-24.0	5	2.8	2.0-6.8	35.2
Vic	Air	2000	Lane	30	11	3.0	0.0-8.0	22	0.6	0.0-2.0	18.4
Vic	Air	2001	Lane	70	19	3.2	0.0-8.0	43	0.8	0.0-2.0	26.6
NSW	Air	2001	Lane	24	7	4.5	0.0-12.0	10	1.6	0.0-4.0	35.6
Vic	Air	2000	Pen/lane	60	24	2.5	0.0-7.5	41	0.6	0.0-2.0	25.5
Vic	Air	2001	Pen/lane	140	31	4.0	2.0-11.5	81	0.9	0.0-2.0	23.9
NSW	Air	2001	Pen/lane	48	10	6.1	2.0-12.0	15	2.2	0.0-4.0	35.6
Vic	Soil	2000	Paddock	144	20	6.0	2.0-15.5	97	0.7	0.0-2.0	11.7
Vic	Soil	2001	Paddock	280	43	3.2	1.0-7.0	151	0.6	0.0-1.0	19.2
NSW	Soil	2001	Paddock	96	9	4.9	2.0-10.0	47	0.7	0.0-1.0	13.4
Vic	Soil	2001	Pen	70	5	3.3	1.0-6.2	33	0.7	0.0-2.0	21.9
NSW	Soil	2001	Pen	24	2	7.1	2.0-17.0	6	1.5	0.2-2.7	20.6
Vic	Soil	2001	Lane	70	16	2.4	1.0-5.0	38	0.6	0.0-1.0	24.3
NSW	Soil	2001	Lane	24	4	3.4	1.0-10.5	9	0.8	0.0-1.0	22.5
Vic	Soil	2001	Pen/lane	140	21	2.8	1.0-5.7	71	0.7	0.0-1.0	23.1
NSW	Soil	2001	Pen/lane	48	6	5.0	1.0-15.0	15	1.1	0.0-2.0	21.8

N: number of samples
N[z]: number of samples from which *R. equi* or virulent *R. equi* were not recovered
[a]cfu/1000 l for air samples; cfu/mg for soil samples
[*]Derived from the geometric mean concentration of virulent *R. equi* x 100/geometric mean concentration of *R. equi*

d. Changes in virulent *R. equi* populations over time

There was considerable variation in the concentrations of *R. equi* and virulent *R. equi*, and the proportion of *R. equi* that were virulent, on farms throughout the foaling season (Table 4.16).

Table 4.16: Concentrations of *R. equi* and virulent *R. equi*, and the proportion of *R. equi* that were virulent, over time on Thoroughbred farms in the 2000 and 2001 seasons

State	Year	Date	Sample	N	Concentration of *R. equi*[a]			Concentration of virulent *R. equi*[a]			%*
					N[z]	Geometric mean	IQR	N[z]	Geometric mean	IQR	virulent
Vic	2000	Sept[1]	Air	24	10	0.9	0.0-2.3	20	0.1	0.0-0.0	11.1
		Oct[1]	Air	36	15	1.6	0.0-5.0	30	0.2	0.0-0.0	12.5
		Nov[1]	Air	36	9	4.6	0.3-14.0	26	0.7	0.0-1.7	15.2
		Dec[1]	Air	36	11	2.5	0.0-6.0	22	0.8	0.0-3.4	32.0
		Jan[1]	Air	36	15	2.0	0.0-5.4	26	0.4	0.0-1.0	20.0
		Feb[1]	Air	36	12	3.8	0.0-12.9	26	0.6	0.0-2.0	15.8
Vic	2001	Oct[1]	Air	60	26	1.5	0.0-4.0	49	0.3	0.0-0.0	20.0
		Oct[2]	Air	60	26	1.7	0.0-4.0	46	0.3	0.0-1.0	17.6
		Nov[1]	Air	60	20	2.0	0.0-6.0	41	0.4	0.0-1.0	20.0
		Nov[2]	Air	60	18	2.5	0.0-7.5	42	0.4	0.0-1.0	16.0
		Dec[1]	Air	60	17	2.8	0.0-6.0	37	0.7	0.0-2.0	25.0
		Jan[1]	Air	60	19	1.8	0.0-4.0	34	0.7	0.0-1.7	38.9
		Jan[2]	Air	60	16	1.9	0.0-4.0	31	0.8	0.0-2.0	42.1
NSW	2001	Nov[2]	Air	72	8	4.0	2.0-8.0	25	1.2	0.0-2.0	30.0
		Dec[1]	Air	72	13	3.7	1.0-8.0	33	1.0	0.0-2.0	27.0
Vic	2000	Sept[1]	Soil	24	4	3.1	2.0-6.0	18	0.2	0.0-0.0	6.5
		Oct[1]	Soil	24	3	4.9	2.0-12.0	15	0.3	0.0-1.3	6.1
		Nov[1]	Soil	24	2	10.2	2.4-27.8	13	0.8	0.0-2.0	7.8
		Dec[1]	Soil	24	4	5.9	2.0-22.0	12	0.7	0.0-2.0	11.9
		Jan[1]	Soil	24	3	6.2	2.0-18.0	13	0.8	0.0-2.0	12.9
		Feb[1]	Soil	24	4	7.9	4.4-19.5	7	0.7	0.0-2.0	8.9
Vic	2001	Oct[1]	Soil	60	12	2.8	1.0-9.0	27	0.8	0.0-1.0	28.6
		Oct[2]	Soil	60	9	2.5	1.0-5.0	32	0.7	0.0-1.0	28.0
		Nov[1]	Soil	60	11	2.8	1.0-5.0	41	0.4	0.0-1.0	14.3
		Nov[2]	Soil	60	8	3.2	2.0-6.0	31	0.7	0.0-1.0	21.9
		Dec[1]	Soil	60	9	3.1	1.0-5.7	33	0.5	0.0-1.0	16.1
		Jan[1]	Soil	60	8	3.0	1.0-7.1	32	0.6	0.0-1.0	20.0
		Jan[2]	Soil	60	7	4.3	1.0-9.7	26	0.9	0.0-2.0	20.9
NSW	2001	Nov[2]	Soil	72	9	5.0	2.0-11.7	33	0.7	0.0-1.7	14.0
		Dec[1]	Soil	72	6	4.8	2.0-11.0	29	0.8	0.0-2.0	16.7

[1]middle of the month
[2]end of the month
N: number of samples
N[z]: number of samples from which *R. equi* or virulent *R. equi* were not recovered
[a]cfu/1000 l for air samples; cfu/mg for soil samples
*Derived from the geometric mean concentration of virulent *R. equi* x 100/geometric mean concentration of *R. equi*

During the 2000 season the concentration of virulent *R. equi* on all 6 farms increased sharply in November (Table 4.16). The geometric mean concentration of virulent *R. equi* in air and soil increased approximately three fold from October to November. The geometric mean concentrations of virulent *R. equi* did not fall to the concentrations seen in October for the remainder of the season. The proportion of *R. equi* in soil that were

virulent increased steadily from September to January, although the proportion of airborne *R. equi* that were virulent peaked in December.

In the samples collected from farms in Victoria during the 2001 season the geometric mean concentration of virulent *R. equi* in the air was greatest in the period from the middle of December to the end of January, whilst the geometric mean concentration of virulent *R. equi* in soil varied throughout the season (Table 4.16). The proportion of *R. equi* that were virulent in the air or soil did not fall below 10% on Victorian farms during the 2001 season. The proportion of airborne *R. equi* that were virulent did not peak until January, whilst the proportion of *R. equi* that were virulent in the soil was greatest in October. The concentration of virulent *R. equi* and the proportion of *R. equi* that were virulent were lowest during November.

In samples collected from farms in NSW during the 2001 season the geometric mean concentrations of *R. equi* and virulent *R. equi*, and the proportion of *R. equi* that were virulent, in soil and air varied little between the two sampling times (November and December) (Table 4.16). The geometric mean airborne concentration of virulent *R. equi* on NSW farms was ≥1 cfu/1000 l in both November and December. This was higher than the concentrations seen on Victorian farms at comparable times, where the highest geometric mean airborne concentration of virulent *R. equi* was 0.8 cfu/1000 l.

4.3.3. Descriptive analysis of environmental and stocking variables and their relationship with virulent *R. equi*

a. Soil conditions

The soil conditions on Thoroughbred farms in Victoria and NSW during the 2000 and 2001 seasons are described in Appendix 2.

The holding pens and lanes had drier soil than the paddocks on most farms. The sandy soil in the pens and lanes and the lack of grass cover in these areas probably contributed to drier soil conditions there. Paddocks with sandy soils were drier than those with clay soils.

Locations with sandy soils generally had higher airborne concentrations of virulent *R. equi* and higher proportions of airborne *R. equi* that were virulent than locations with clay soils (Table 4.17). The higher geometric mean concentrations of airborne virulent *R. equi* in sandy areas were most evident amongst farms in Victoria (75% and 167% higher in the 2000 and 2001 seasons, respectively). On NSW farms the locations with sandy soils had a 30% higher geometric mean concentration of airborne virulent *R. equi*. The proportions of airborne *R. equi* that were virulent were between 21% and 61% higher in locations with sandy soil. The differences between locations with different soil types in the geometric mean concentrations of virulent *R. equi* and in the proportions of *R. equi* that were virulent were less pronounced in soil samples.

Table 4.17: Soil moisture and pH, geometric mean concentrations of *R. equi* and virulent *R. equi*, and the proportion of *R. equi* that were virulent, in areas with sandy or clay soils on Thoroughbred farms in the 2000 and 2001 seasons

State	Year	Texture	Soil moisture (%H_2O)	Soil pH	Air (cfu/1000 l)					Soil (cfu/mg)				
					N	Concentration of *R. equi*		Concentration of virulent *R. equi*		%* virulent	N	Concentration of *R. equi*		Concentration of virulent *R. equi*		%* virulent
			Median (Range)	Median (Range)		Nz	Geometric mean (IQR)	Nz	Geometric mean (IQR)			Nz	Geometric mean (IQR)	Nz	Geometric mean (IQR)	
Vic	2000	Sand	11.1 (1.7-24.3)	6.5 (5.4-7.4)	79	24	2.6 (0.0-8.0)	52	0.7 (0.0-2.0)	26.9	44	5	7.3 (4.0-19.5)	25	1.0 (0.0-2.0)	13.7
		Clay	11.7 (1.4-28.6)	6.0 (4.4-7.5)	125	50	2.4 (0.0-8.0)	98	0.4 (0.0-0.0)	16.7	100	15	5.5 (2.0-13.5)	75	0.6 (0.0-0.0)	10.9
Vic	2001	Sand	6.3 (0.8-24.0)	6.1 (4.5-8.0)	184	46	3.1 (0.2-6.0)	110	0.8 (0.0-2.0)	25.8	184	29	3.0 (1.0-5.7)	92	0.6 (0.0-1.0)	20.0
		Clay	13.8 (1.8-31.5)	5.6 (4.3-8.2)	236	96	1.4 (0.0-3.0)	170	0.3 (0.0-1.0)	21.4	236	35	3.2 (1.0-7.0)	130	0.6 (0.0-1.0)	18.8
NSW	2001	Sand	6.6 (1.2-22.3)	6.3 (4.9-8.9)	64	14	3.9 (1.0-8.0)	25	1.3 (0.0-2.0)	33.3	64	7	4.1 (1.0-11.0)	27	0.8 (0.0-1.0)	19.5
		Clay	18.0 (2.3-40.1)	6.3 (5.0-8.0)	80	7	3.8 (1.0-8.0)	33	1.0 (0.0-1.0)	26.3	80	8	5.7 (2.0-11.7)	35	0.8 (0.0-2.0)	14.0

N: number of samples
Nz: number of samples from which *R. equi* or virulent *R. equi* were not recovered
*Derived from the geometric mean concentration of virulent *R. equi* x 100/geometric mean concentration of total *R. equi*
Air samples taken in 2000 included those collected in holding pens and lanes, but soil samples were not taken. In the 2000 season the soil samples were only collected from paddocks.

b. Paddock and weather conditions

The pasture and weather conditions on Thoroughbred farms in Victoria and NSW during the 2000 and 2001 seasons are described in Appendix 3.

In the 2000 season the median group size (number of foals/paddock) reduced from 4-5 foals between October and December to ≤3 foals in January-February. A similar reduction in the median group size in January was observed in the 2001 season on Victorian farms. The soil moisture and pasture height were lowest in December and January in both seasons on farms in Victoria. The soil moisture was higher in the 2001 season on Victorian farms. The median pH of soil samples was slightly lower in the 2001 season on Victorian farms (Table 4.18).

Table 4.18: Soil pH and moisture, pasture height and group size on Thoroughbred farms in the 2000 and 2001 seasons

State	Year	Date	Soil pH		Soil moisture (%H_2O)		Pasture height (cm)		Group size (No. foals/paddock)	
			Median	Range	Median	Range	Median	Range	Median	Range
Vic	2000	Sept[1]	6.4	5.0-7.4	16.8	9.0-28.6	12.8	0.0-18.2	4.0	0.0-8.0
		Oct[1]	6.4	5.0-7.5	15.0	7.7-23.0	10.8	0.0-17.9	4.0	0.0-24.0
		Nov[1]	6.2	5.0-7.0	13.3	3.6-20.1	8.2	0.0-16.3	5.0	0.0-27.0
		Dec[1]	6.1	5.2-7.1	5.5	1.7-18.7	7.4	0.0-15.4	5.0	0.0-20.0
		Jan[1]	5.9	4.9-7.3	4.9	1.4-16.0	3.8	0.0-10.9	3.0	0.0-9.0
		Feb[1]	6.1	4.4-7.3	13.4	3.2-27.3	4.4	0.0-12.0	0.0	0.0-10.0
Vic	2001	Oct[1]	5.4	4.3-7.0	17.9	7.4-30.5	14.1	4.6-26.2	5.0	0.0-20.0
		Oct[2]	5.7	4.3-6.9	14.6	5.0-29.3	15.0	2.7-27.7	6.0	0.0-30.0
		Nov[1]	5.7	4.5-6.8	17.5	7.0-30.2	14.4	3.6-28.1	6.0	0.0-23.0
		Nov[2]	5.9	4.8-6.9	12.8	3.5-31.5	11.7	2.1-29.9	6.0	0.0-24.0
		Dec[1]	5.8	5.0-7.2	4.9	2.1-26.9	8.0	3.1-23.8	5.0	0.0-17.0
		Jan[1]	5.5	4.4-6.8	3.8	1.7-20.2	6.1	2.4-21.4	4.0	0.0-9.0
		Jan[2]	5.3	4.7-6.9	12.1	3.5-21.0	7.2	1.6-19.1	3.0	0.0-13.0
NSW	2001	Nov[2]	6.3	5.2-8.5	11.0	1.6-40.1	7.9	1.3-19.6	5.0	0.0-13.0
		Dec[1]	6.4	4.9-8.9	13.3	1.2-29.8	6.2	2.1-18.4	5.5	0.0-14.0

[1]middle of the month
[2]end of the month

The average ambient temperatures at the time of sampling in the 2000 season were approximately 10°C higher in November-February than in September-October. In the 2001 season the median average ambient temperature at the time of sampling was within a 10°C range throughout the sampling period. The median average ambient temperature on farms at the commencement of sampling in the middle of October was less than 1°C different from the median average ambient temperature when samples were taken at the end of November. The median average ambient temperature at the time of sampling on NSW

farms in December was approximately 12°C higher than in November (Table 4.19). Median group size were similar in both sampling periods, but the median pasture height and soil moisture were approximately 20% lower and 20% higher, respectively, in December than in November (Tables 4.18). Generally high average ambient temperatures at the time of sampling were associated with lower humidity. Median wind speeds were within a 10 km/h range throughout the sampling periods over the two seasons in Victoria and varied little between sampling periods in NSW (Table 4.19).

Table 4.19: Environmental conditions at the time of sampling on Thoroughbred farms in the 2000 and 2001 seasons

State	Year	Date	Humidity (%)		Wind speed (km/h)		Temperature (ºC)	
			Median	Range	Median	Range	Median	Range
Vic	2000	Sept[1]	79.4	65.4-88.2	-	-	15.2	6.8-18.7
		Oct[1]	71.1	60.4-81.4	27.0	4.0-32.0	15.7	9.4-20.2
		Nov[1]	66.5	58.9-83.5	19.0	13.0-39.0	27.1	17.7-30.9
		Dec[1]	67.2	55.7-78.1	18.0	13.0-24.0	19.7	16.0-28.6
		Jan[1]	68.0	59.4-76.3	18.0	4.0-24.0	22.9	15.9-35.7
		Feb[1]	72.1	68.1-96.1	19.5	4.0-43.0	27.7	20.4-34.8
Vic	2001	Oct[1]	70.5	65.5-94.9	17.1	8.3-35.7	15.6	11.9-20.8
		Oct[2]	80.2	68.3-97.5	19.7	8.3-28.8	11.5	7.6-18.9
		Nov[1]	80.7	74.9-97.9	15.3	5.0-28.8	16.2	6.8-19.6
		Nov[2]	77.2	66.5-98.2	13.5	2.7-25.0	16.4	10.0-24.1
		Dec[1]	71.7	50.2-84.9	21.2	8.0-29.8	18.3	11.4-28.3
		Jan[1]	75.6	58.8-98.7	19.7	14.7-28.8	16.2	10.3-25.8
		Jan[2]	77.9	65.6-94.9	20.2	8.0-32.3	20.8	14.9-30.9
NSW	2001	Nov[2]	76.2	67.8-87.0	23.0	6.8-28.0	14.8	11.0-21.8
		Dec[1]	62.4	44.8-88.4	19.0	7.0-33.0	26.9	18.1-32.0

[1]middle of the month
[2]end of the month
Wind speed data for September 2000 was not recorded

c. Foal population dynamics

In the 2000 season, most of the 6 farms had the maximal number of foals <10 weeks of age and <16 weeks of age in November (Appendix 4). In the 2001 season, most of the 10 farms in Victoria had the maximal number of foals <16 weeks of age in November (Appendix 5). The proportions of foals between the ages of 4 and 12 weeks were high on most Victorian farms in the October-January period of the 2001 season, and then dropped rapidly by the end of January. The 3 farms in Victoria that did not report cases of *R. equi* pneumonia in the 2001 season (B, G and J) were all farms that had less than 50 foals <16 weeks of age, and less than 30 foals between 4 and 12 weeks of age, throughout the season. In NSW all farms had the maximal number of foals <16 weeks of

age by the start of December (Appendix 6). The number of foals aged between 4 and 12 weeks peaked during the sampling period (end of November to the middle of December) on most NSW farms. The number of foals between 4 and 12 weeks of age peaked on NSW farms in December, with the exception of farm 7, which was the only NSW farm in the survey that did not report any cases of *R. equi* pneumonia during the season. Farm 7 had peak numbers of foals in the 4 and 12 week age group at least a month earlier than other farms. Farms in NSW that reported deaths from *R. equi* pneumonia during the season (1, 2, 3, 4, 6 and 8) all had more than 100 foals <16 weeks of age and more than 60 foals between the ages of 4 and 12 weeks at times within the October-January period of the season.

4.3.4. Univariable and multivariable analysis of environmental and stocking factors

The mean, median, and upper and lower quartiles of the environmental and stocking variables on Thoroughbred farms sampled in the 2000 and 2001 seasons are given in Table 4.20.

Table 4.20: Descriptive statistics for environmental and stocking variables on Thoroughbred farms in the 2000 and 2001 seasons

Variable	N	Mean	Median	Lower quartile	Upper quartile
Soil moisture (% H_2O)	708	11.8	11.1	5.4	16.6
Soil pH	708	6.0	6.0	5.4	6.5
Pasture height (cm)*	505	9.8	8.5	4.9	14.0
Group size (No. foals/paddock)*	520	5.0	5.0	1.0	7.0
Humidity (%)	762	74.4	74.9	67.3	81.6
Temperature (°C)	762	18.6	18.0	14.2	22.4
Wind speed (km/h)	726	18.8	19.0	12.3	24.7

N: number of measurements taken
* Variables that are only relevant to the paddock environment

Each of these variables was assigned to one of two categories as described in Section 4.2.8. This facilitated univariable and multivariable regression analysis to determine the best predictors for inclusion in a model to explain the environmental burdens of *R. equi* and virulent *R. equi* on Thoroughbred farms.

a. Univariable analyses

Different variables were associated with the concentration of *R. equi* and virulent *R. equi* in air and in soil in univariable analyses, as shown in Tables 4.21 and 4.22 respectively.

The airborne concentration of *R. equi* was significantly associated (P<0.05) with state/year, date, location within the farm, soil texture, soil moisture, soil pH and temperature (Table 4.21). The greatest deviations of the count ratios (CR) from 1.0 were for associations between airborne concentrations of *R. equi* and different categories of state/year, date and location within the farm. The CR for concentrations of airborne *R. equi* were 43% lower on Victorian farms in the 2000 season and 32% lower in the 2001 season than on NSW farms in the 2001 season. The CR for airborne *R. equi* concentrations were 67% greater in the middle of the season and 34% greater late in the season than early in the season and the CR for airborne concentrations of *R. equi* in pens and lanes was 62% greater than in the paddocks. Other variables associated (P<0.25) with the airborne concentration of *R. equi* included pasture height and group size.

The airborne concentration of virulent *R. equi* was significantly associated (P<0.05) with state/year, date, location within the farm, soil texture, soil moisture, pasture height and temperature (Table 4.21). The greatest deviations of the CR from 1.0 were for associations between airborne concentrations of virulent *R. equi* and different categories of state/year, date and pasture height. The CR for airborne concentrations of virulent *R. equi* were 70% lower on Victorian farms in 2000 and 54% lower on Victorian farms in 2001 than on NSW farms in the 2001 season. The CR for airborne concentrations of virulent *R. equi* were 151% greater in the middle of the season and 129% greater late in the season than early in the season. The CR for airborne concentrations of virulent *R. equi* on paddocks with a low pasture height (≤10 cm) was 106% greater than on paddocks with a greater pasture height. Other variables associated (P<0.25) with the airborne concentration of virulent *R. equi* included soil pH and humidity.

Table 4.21: Univariable analyses, using random effects negative binomial regression, of the associations between environmental and stocking variables and the concentrations of airborne *R. equi* and virulent *R. equi* on farms

Variables		N	Concentration of *R. equi* (cfu/1000 l)						Concentration of virulent *R. equi* (cfu/1000 l)						%[ϕ] virulent
			N[z]	Geometric mean	IQR	CR	95% CI	P	N[z]	Geometric mean	IQR	CR	95% CI	P	
State/year		768	237					<0.001***	488					<0.001***	
	NSW 2001[a]	144	21	3.8	1.0-8.0	1			58	1.1	0.0-2.0	1			28.9
	Vic 2000	204	74	2.5	0.0-8.0	0.57	0.42, 0.77	<0.001***	150	0.5	0.0-0.6	0.30	0.19, 0.46	<0.001***	20.0
	Vic 2001	420	142	2.0	0.0-4.0	0.68	0.52, 0.88	0.004**	280	0.5	0.0-1.0	0.46	0.33, 0.64	<0.001***	25.0
Date		768	237					<0.001***	488					<0.001***	
	Sept–Oct[a]	180	79	1.5	0.0-4.0	1			145	0.3	0.0-0.0	1			20.0
	Nov–Dec	396	96	3.1	1.0-8.0	1.67	1.34, 2.08	<0.001***	226	0.8	0.0-2.0	2.51	1.74, 3.61	<0.001***	25.8
	Jan–Feb	192	62	2.2	0.0-4.8	1.34	1.04, 1.71	0.02*	117	0.6	0.0-2.0	2.29	1.54, 3.41	<0.001***	27.3
Location	Paddock[a]	520	172	1.9	0.0-4.0	1			351	0.4	0.0-1.0	1			21.1
	Pen/lane	248	65	3.8	0.0-10.0	1.62	1.37, 1.90	<0.001***	137	1.0	0.0-2.0	1.87	1.48, 2.36	<0.001***	26.3
Soil texture	Clay[a]	441	153	2.0	0.0-4.0	1			301	0.4	0.0-1.0	1			20.0
	Sand	327	84	3.1	0.0-8.0	1.38	1.16, 1.64	<0.001***	187	0.8	0.0-2.0	1.50	1.17, 1.91	0.001**	25.8
Soil moisture (% H_2O)	>10[a]	392	136	1.9	0.0-4.0	1			272	0.4	0.0-1.0	1			21.1
	≤10	316	77	3.2	0.7-8.0	1.44	1.22, 1.70	<0.001***	175	0.9	0.0-2.0	1.83	1.44, 2.34	<0.001***	28.1
Soil pH	>6[a]	336	87	2.9	0.0-8.0	1			203	0.7	0.0-2.0	1			24.1
	≤6	372	126	2.0	0.0-4.0	0.77	0.65, 0.92	0.003**	244	0.5	0.0-1.0	0.79	0.62, 1.01	0.06	25.0
Pasture height (cm)	>10[a]	209	85	1.4	0.0-3.2	1			159	0.2	0.0-0.0	1			14.3
	≤10	299	79	2.4	0.0-5.0	1.23	0.98, 1.55	0.08	178	0.6	0.0-1.0	2.06	1.45, 2.92	<0.001***	25.0
Temperature (°C)	≤25[a]	618	206	2.2	0.0-5.2	1			406	0.5	0.0-1.0	1			22.7
	>25	144	30	3.2	1.0-8.0	1.32	1.07, 1.63	0.01*	80	0.8	0.0-2.0	1.44	1.08, 1.92	0.02*	25.0
Humidity (%)	>70[a]	488	155	2.2	0.0-5.0	1			165	0.5	0.0-1.0	1			22.7
	≤70	274	81	2.7	0.0-8.0	1.06	0.89, 1.27	0.49	321	0.7	0.0-2.0	1.19	0.93, 1.53	0.17	25.9
Wind speed (km/h)	≤10[a]	120	42	2.5	0.0-6.0	1			81	0.5	0.0-1.0	1			20.0
	>10	606	183	2.4	0.0-6.0	1.14	0.90, 1.45	0.28	377	0.6	0.0-2.0	1.22	0.86, 1.73	0.26	25.0
Group size (No. foals/paddock)	≤7[a]	401	137	1.8	0.0-4.0	1			275	0.4	0.0-1.0	1			22.2
	>7	119	35	2.3	0.0-6.0	1.17	0.92, 1.49	0.20	76	0.5	0.0-1.0	1.20	0.84, 1.71	0.32	21.7

[a]reference category
N: number of samples
IQR: interquartile range
CI: confidence interval for the CR
N[z]: number of samples from which *R. equi* or virulent *R. equi* were not recovered
CR: count ratio
[ϕ]Derived from the geometric mean concentration of virulent *R. equi* x 100/geometric mean concentration of total *R. equi*

The concentration of *R. equi* in soil was significantly associated (P<0.05) with date, temperature, humidity and group size (Table 4.22). The greatest deviation of the CR from 1.0 was for the association between concentration of *R. equi* in soil and different categories of temperature. The CR for *R. equi* in soil samples collected when there was a high average ambient temperature (>25°C) was 38% greater than for those taken when there was a lower average ambient temperature (≤25°C). Other variables associated (P<0.25) with the concentration of *R. equi* in soil included state/year, soil moisture and pasture height.

The concentration of virulent *R. equi* in soil was significantly associated (P<0.05) with state/year and location within the farm (Table 4.22). The CRs for the concentrations of virulent *R. equi* in the soil on Victorian farms in the 2000 and 2001 seasons were 55% and 19% lower, respectively, than those for the concentration of virulent *R. equi* in the soil on the NSW farms in the 2001 season. The CR for the concentration of virulent *R. equi* in soil samples taken from pens and lanes was 27% greater than that for the concentration of virulent *R. equi* in soil from paddocks. Other variables associated (P<0.25) with the concentration of virulent *R. equi* in soil included date, soil texture, pasture height, temperature and humidity.

Table 4.22: Univariable analyses, using random effects negative binomial regression, of the associations between environmental and stocking variables and the concentrations of soil *R. equi* and virulent *R. equi* on farms

Variables		N	Concentration of *R. equi* (cfu/mg)						Concentration of virulent *R. equi* (cfu/mg)						%[ϕ] virulent
			N[z]	Geometric mean	IQR	CR	95% CI	P	N[z]	Geometric mean	IQR	CR	95% CI	P	
State/year		708	99					0.10	381					0.002**	
	NSW 2001[a]	144	15	4.9	2.0-11.0	1			62	0.8	0.0-2.0	1			16.3
	Vic 2000	144	20	6.0	2.0-15.5	0.85	0.62, 1.16	0.30	97	0.7	0.0-2.0	0.45	0.28, 0.74	<0.001***	11.7
	Vic 2001	420	64	3.1	1.0-6.8	0.77	0.60, 0.98	0.03*	222	0.6	0.0-1.0	0.81	0.58, 1.13	0.21	19.4
Date		708	99					0.03*	381					0.21	
	Sept–Oct[a]	168	28	3.0	1.0-6.8	1			98	0.6	0.0-1.0	1			20.0
	Nov–Dec	372	49	4.2	1.0-10.0	1.24	1.03, 1.49	0.02*	198	0.6	0.0-1.0	1.12	0.85, 1.48	0.41	14.3
	Jan–Feb	168	22	4.4	2.0-10.0	1.28	1.04, 1.57	0.02*	85	0.8	0.0-2.0	1.31	0.97, 1.78	0.08	18.2
Location	Paddock[a]	520	72	4.2	2.0-10.0	1			295	0.6	0.0-1.0	1			14.3
	Pen/lane	188	27	3.3	1.0-7.0	0.94	0.80, 1.11	0.47	86	0.8	0.0-1.0	1.27	1.01, 1.60	0.04*	24.2
Soil texture	Clay[a]	416	58	4.1	1.0-10.0	1			237	0.6	0.0-1.0	1			14.6
	Sand	292	41	3.7	1.0-8.0	0.92	0.79, 1.08	0.33	144	0.7	0.0-1.0	1.15	0.92, 1.44	0.22	18.9
Soil moisture (% H_2O)	>10[a]	392	46	4.1	2.0-10.0	1			211	0.7	0.0-2.0	1			17.1
	≤10	316	53	3.5	1.0-8.0	0.89	0.76, 1.03	0.10	170	0.7	0.0-1.0	1.01	0.82, 1.25	0.92	20.0
Soil pH	>6[a]	336	48	4.1	1.0-10.0	1			184	0.6	0.0-1.0	1			14.6
	≤6	372	51	3.7	1.0-8.7	0.96	0.82, 1.11	0.57	197	0.7	0.0-1.0	1.06	0.85, 1.32	0.60	18.9
Pasture height (cm)	>10[a]	206	32	3.8	1.0-10.0	1			124	0.6	0.0-1.0	1			15.8
	≤10	299	37	4.5	2.0-10.0	1.14	0.95, 1.38	0.16	159	0.7	0.0-2.0	1.29	0.97, 1.71	0.07	15.6
Temperature (°C)	≤25[a]	584	90	3.5	1.0-8.0	1			320	0.6	0.0-1.0	1			17.1
	>25	118	8	6.7	2.0-17.2	1.38	1.14, 1.68	0.001**	59	1.0	0.0-2.0	1.28	0.96, 1.70	0.10	14.9
Humidity (%)	>70[a]	462	77	3.5	1.0-8.0	1			259	0.6	0.0-1.0	1			17.1
	≤70	240	21	4.9	2.0-11.0	1.28	1.10, 1.50	0.002**	120	0.8	0.0-2.0	1.19	0.95, 1.50	0.13	16.3
Wind speed (km/h)	≤10[a]	110	17	4.0	1.0-9.2	1			55	0.7	0.0-2.0	1			17.5
	>10	556	77	4.0	1.0-10.0	1.02	0.83, 1.26	0.84	299	0.7	0.0-1.0	0.88	0.66, 1.19	0.41	17.5
Group size (No. foals/paddock)	≤7[a]	401	47	4.5	2.0-10.0	1			229	0.6	0.0-2.0	1			13.3
	>7	119	25	3.3	1.0-8.0	0.80	0.64, 0.99	0.04*	66	0.7	0.0-1.0	1.03	0.75, 1.40	0.87	21.2

[a]reference category
N: number of samples
IQR: interquartile range
CI: confidence interval for the CR
[z]: number of samples from which *R. equi* or virulent *R. equi* were not recovered
CR: count ratio
[ϕ]Derived from the geometric mean concentration of virulent *R. equi* x 100/geometric mean concentration of total *R. equi*

No significant (P<0.05) associations were found between any variable and the proportion of *R. equi* that were virulent in air (Table 4.23) or in soil samples (Table 4.24).

Table 4.23: Univariable analyses, using random effects logistic regression on samples with >4 cfu *R. equi*/1000 l, of the associations between environmental and stocking variables and the proportion of airborne *R. equi* that were virulent on farms

Variables		N	Mean % virulent	IQR	OR	95% CI	P
State/year		224					0.66
	NSW 2001[a]	55	23.7	8.3-33.3	1		
	Vic 2000	65	18.6	0.0-29.2	0.74	0.31, 1.79	0.51
	Vic 2001	104	24.7	0.0-47.5	1.05	0.49, 2.26	0.90
Date		224					0.27
	Sept–Oct[a]	36	14.9	0.0-25.0	1		
	Nov–Dec	140	22.2	0.0-33.3	1.60	0.59, 4.32	0.35
	Jan–Feb	48	29.9	0.0-57.5	2.38	0.79, 7.16	0.12
Location	Paddock[a]	118	19.7	0.0-33.3	1		
	Pen/lane	106	26.0	0.0-50.0	1.42	0.76, 2.66	0.27
Soil texture	Clay[a]	104	18.3	0.0-32.1	1		
	Sand	120	26.5	0.0-50.0	1.61	0.85, 3.05	0.14
Soil moisture (% H_2O)	>10[a]	85	16.8	0.0-26.8	1		
	≤10	121	26.6	0.0-50.0	1.77	0.88, 3.56	0.10
Soil pH	>6[a]	114	22.9	0.0-33.3	1		
	≤6	92	22.1	0.0-33.3	0.95	0.49, 1.84	0.88
Pasture height (cm)	>10[a]	31	15.7	0.0-33.3	1		
	≤10	85	21.5	0.0-22.2	1.48	0.48, 4.55	0.49
Temperature (°C)	≤25[a]	169	21.3	0.0-33.3	1		
	>25	53	26.9	0.0-50.0	1.36	0.67, 2.76	0.40
Humidity (%)	>70[a]	128	20.2	0.0-33.3	1		
	≤70	94	26.0	0.0-50.0	1.39	0.74, 2.61	0.31
Wind speed (km/h)	≤10[a]	37	16.5	0.0-33.3	1		
	>10	175	24.6	0.0-40.0	1.65	0.65, 4.20	0.27
Group size (No. foals/paddock)	≤7[a]	86	19.4	0.0-33.3	1		
	>7	32	20.6	0.0-33.3	1.07	0.39, 2.95	0.90

[a]reference category
N: number of samples with >4 cfu *R. equi*/1000 l
IQR: interquartile range
OR: odds ratios
CI: confidence interval

Table 4.24: Univariable analyses, using random effects logistic regression on samples with >4 cfu *R. equi*/mg, of the associations between environmental and stocking variables and the proportion of *R. equi* in the soil that were virulent on farms

Variables		N	Mean % virulent	IQR	OR	95% CI	P
State/year		298					0.71
	NSW 2001[a]	79	14.7	0.0-20.0	1		
	Vic 2000	80	14.4	0.0-21.9	0.97	0.40, 2.35	0.95
	Vic 2001	139	18.1	0.0-28.6	1.28	0.60, 2.73	0.52
Date		298					0.47
	Sept–Oct[a]	56	19.2	0.0-30.2	1		
	Nov–Dec	163	13.8	0.0-20.0	0.68	0.30, 1.50	0.34
	Jan–Feb	79	19.0	0.0-28.6	0.99	0.41, 2.36	0.98
Location	Paddock[a]	228	14.9	0.0-21.3	1		
	Pen/lane	70	20.5	5.0-30.9	1.47	0.74, 2.91	0.28
Soil texture	Clay[a]	182	16.0	0.0-20.3	1		
	Sand	116	16.5	0.0-23.2	1.04	0.55, 1.95	0.91
Soil moisture (% H_2O)	>10[a]	173	16.1	0.0-20.5	1		
	≤10	125	16.4	0.0-25.0	1.02	0.55, 1.91	0.94
Soil pH	>6[a]	148	13.2	0.0-20.0	1		
	≤6	150	19.2	0.0-28.6	1.57	0.84, 2.94	0.15
Pasture height (cm)	>10[a]	77	12.3	0.0-16.7	1		
	≤10	146	16.6	0.0-25.0	1.42	0.63, 3.18	0.40
Temperature (°C)	≤25[a]	227	16.1	0.0-21.4	1		
	>25	70	16.7	0.0-24.6	1.05	0.51, 2.15	0.90
Humidity (%)	>70[a]	181	17.2	0.0-21.2	1		
	≤70	116	15.7	0.0-24.9	1.12	0.60, 2.09	0.73
Wind speed (km/h)	≤10[a]	49	19.0	0.0-26.8	1		
	>10	235	15.9	0.0-21.4	0.81	0.36, 1.79	0.60
Group size (No. foals/paddock)	≤7[a]	182	14.3	0.0-20.0	1		
	>7	46	17.2	0.0-25.0	1.24	0.52, 2.97	0.62

[a]reference category
N: number of samples with >4 cfu *R. equi*/mg
IQR: interquartile range
OR: odds ratio
CI: confidence interval

Some weak associations (P<0.25) were seen between soil texture and soil moisture and the proportion of airborne *R. equi* that were virulent. The greatest odds ratio (OR) was for the association with soil moisture, with the proportion of *R. equi* that were virulent 1.77 times greater in drier soil samples (≤10% H_2O) than in more moist soil samples (>10% H_2O). The association between lower soil moisture and the increased proportion of virulent *R. equi* among airborne *R. equi* can be illustrated graphically using locally weighted scatterplot smoothing (LOWESS) of the continuous variables (Figure 4.2). A

weak association (P<0.25) was also seen between soil pH and the proportion of *R. equi* in soil that were virulent. The proportion of *R. equi* that were virulent in soil was 1.57 times greater in more acidic soil (pH≤6) than in more alkaline soil (pH>6). This association is illustrated graphically using LOWESS in Figure 4.3.

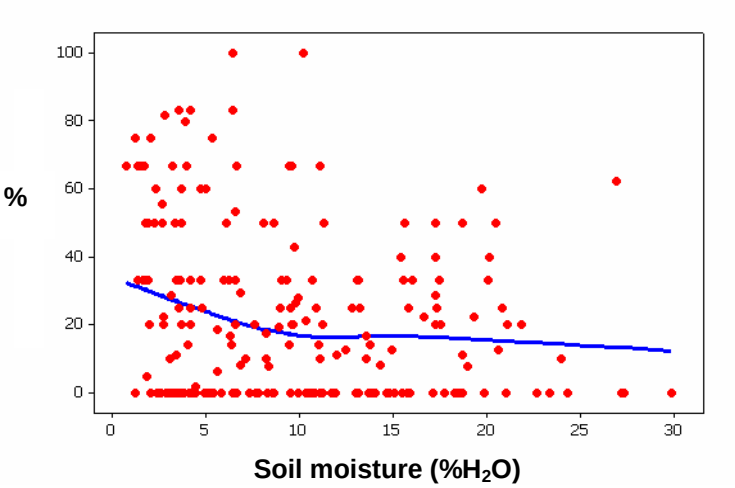

Figure 4.2: Locally weighted scatterplot smoothing (LOWESS) of proportion of airborne *R. equi* that were virulent (%) versus soil moisture

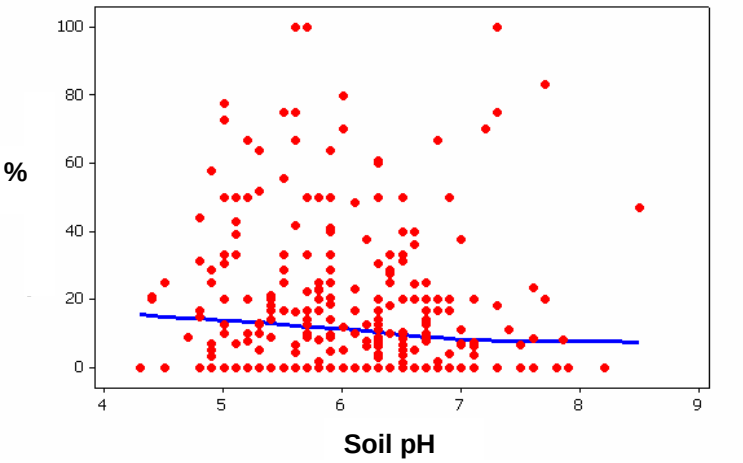

Figure 4.3: Locally weighted scatterplot smoothing (LOWESS) of proportion of *R. equi* in soil that were virulent (%) versus soil pH

b. Correlations between predictor variables

To evaluate the relationships and potential interactions between binary categorical environmental and stocking predictor data used in the regression analyses, Spearman's correlation coefficients were calculated (Tables 4.25 and 4.26).

There was no significant collinearity (R_s>0.8) between any of the predictor variables tested. However, a strong positive relationship was seen between temperature and humidity (R_s>0.5) when assessing the binary relationship between high temperature (>25°C) and low humidity (≤70%). Other variables with positive relationships (0.3<R_s<0.4) were sandy soils and pen/lane locations, low soil moisture (≤10% H_2O) and sandy soil, and low soil moisture (≤10% H_2O) and pen/lane locations.

Table 4.25a: Spearman's correlation coefficients (R_s) between variables used in the models describing the airborne concentrations of *R. equi* and virulent *R. equi*

Variable	Pen/lane	Sand	Soil moisture (≤10% H_2O)	Soil pH (≤6)	Pasture height (≤10 cm)	Group size (>7)	Temperature (>25°C)	Humidity (≤70%)
Sand	0.40 (n=768)	-						
Soil moisture (≤10% H_2O)	0.34 (n=708)	0.33 (n=708)	-					
Soil pH (≤6)	-0.22 (n=708)	-0.21 (n=708)	-0.10 (n=708)	-				
Pasture height (≤10 cm)	-	-0.02 (n=505)	0.26 (n=505)	-0.04 (n=505)	-			
Group size (>7)	-	-0.08 (n=520)	-0.05 (n=520)	0.16 (n=520)	0.04 (n=505)	-		
Temperature (>25°C)	0.01 (n=762)	-0.03 (n=762)	0.14 (n=702)	-0.01 (n=702)	0.23 (n=501)	0.01 (n=516)	-	
Humidity (≤70%)	0.01 (n=762)	0.07 (n=762)	0.19 (n=702)	-0.06 (n=702)	0.13 (n=501)	0.06 (n=516)	0.52 (n=762)	-
Wind speed (>10 km/h)	0 (n=762)	0.05 (n=726)	0.15 (n=666)	-0.07 (n=666)	-0.06 (n=484)	0.06 (n=516)	0.16 (n=726)	0.24 (n=726)

Table 4.25b: Spearman's correlation coefficients (R_s) between variables used in the models describing the concentrations of *R. equi* and virulent *R. equi* in soil samples

Variable	Pen/lane	Sand	Soil moisture (≤10% H_2O)	Soil pH (≤6)	Pasture height (≤10 cm)	Group size (>7)	Temperature (°C)	Humidity (≤70%)
Sand	0.42 (n=708)	-						
Soil moisture (≤10% H_2O)	0.34 (n=708)	0.33 (n=708)	-					
Soil pH (≤6)	-0.22 (n=708)	-0.21 (n=708)	-0.10 (n=708)	-				
Pasture height (≤10 cm)	-	-0.02 (n=505)	0.26 (n=505)	-0.04 (n=505)	-			
Group size (>7)	-	-0.08 (n=520)	-0.05 (n=520)	0.16 (n=520)	0.04 (n=505)	-		
Temperature (>25°C)	-0.08 (n=702)	-0.03 (n=702)	0.14 (n=702)	-0.01 (n=702)	0.23 (n=501)	0.01 (n=516)	-	
Humidity (≤70%)	-0.05 (n=702)	0.07 (n=702)	0.19 (n=702)	-0.06 (n=702)	0.13 (n=501)	0.06 (n=516)	0.51 (n=702)	-
Wind speed (>10 km/hr)	0 (n=666)	0.07 (n=666)	0.15 (n=666)	-0.07 (n=666)	-0.06 (n=484)	0 (n=484)	0.16 (n=666)	0.23 (n=666)

Table 4.26a: Spearman's correlation coefficients (R_s) between variables used in the models describing the proportion of airborne *R. equi* that were virulent

Variable	Pen/lane	Sand	Soil moisture (≤10% H_2O)	Soil pH (≤6)	Pasture height (≤10 cm)	Group size (>7)	Temperature (>25°C)	Humidity (≤70%)
Sand	0.47 (n=224)	-						
Soil moisture (≤10% H_2O)	0.29 (n=206)	0.38 (n=708)	-					
Soil pH (≤6)	-0.30 (n=206)	-0.21 (n=206)	-0.14 (n=206)	-				
Pasture height (≤10 cm.)	-	-0.05 (n=116)	0.26 (n=116)	0.01 (n=116)	-			
Group size (>7)	-	-0.08 (n=118)	-0.07 (n=118)	0.29 (n=118)	-0.06 (n=116)	-		
Temperature (>25°C)	-0.21 (n=222)	-0.11 (n=222)	0.10 (n=204)	0.05 (n=204)	0.22 (n=115)	0 (n=117)	-	
Humidity (≤70%)	-.12 (n=222)	-.04 (n=222)	0.17 (n=204)	-0.07 (n=204)	0.16 (n=115)	0.01 (n=117)	0.57 (n=222)	-
Wind speed (>10 km/h)	-0.03 (n=212)	0.01 (n=212)	0.26 (n=194)	0 (n=194)	-0.16 (n=115)	-.05 (n=110)	0.17 (n=212)	0.23 (n=212)

Table 4.26b: Spearman's correlation coefficients (R_s) between variables used in the models describing the proportion of soil *R. equi* that were virulent

Variable	Pen/lane	Sand	Soil moisture (≤10% H_2O)	Soil pH (≤6)	Pasture height (≤10 cm)	Group size (>7)	Temperature (>25°C)	Humidity (≤70%)
Sand	0.39 (n=298)	-						
Soil moisture (≤10% H_2O)	0.27 (n=298)	0.37 (n=298)	-					
Soil pH (≤6)	-0.25 (n=298)	-0.28 (n=298)	-0.09 (n=298)	-				
Pasture height (≤ 10 cm)	-	-0.05 (n=223)	0.18 (n=223)	-0.04 (n=223)	-			
Group size (>7)	-	-0.08 (n=228)	-0.14 (n=228)	0.26 (n=228)	-0.10 (n=223)	-		
Temperature (>25°C)	-0.12 (n=297)	0.01 (n=297)	0.17 (n=297)	0.01 (n=297)	0.29 (n=223)	0.02 (n=228)	-	
Humidity (≤70%)	-0.05 (n=297)	0.09 (n=297)	0.18 (n=297)	0.01 (n=297)	0.01 (n=223)	0.08 (n=228)	0.56 (n=297)	-
Wind speed (>10 km/h)	0.04 (n=284)	0.04 (n=284)	0.21 (n=284)	-0.03 (n=284)	-0.11 (n=216)	-0.08 (n=216)	0.19 (n=284)	0.26 (n=284)

c. Multivariable analyses

The outcome variables concentration of *R. equi* and virulent *R. equi* in both air and soil had multiple variables associated with them (P<0.25) in the univariable analyses, so these were used in the multivariable analyses.

Date of sample collection, location within the farm and temperature were significantly associated (P<0.05) with the airborne concentration of *R. equi* in both paddocks and pens and lanes (Table 4.27). The greatest deviation of the CR from 1.0 was for the association between airborne concentrations of *R. equi* and the location within the farm. The airborne concentration of *R. equi* was almost doubled (CR=1.93) in the pens and lanes compared to the paddocks.

Table 4.27: Variables identified, using backwards stepwise random effects negative binomial regression analyses, as significantly affecting the airborne concentration of *R. equi* and virulent *R. equi* on farms

	Location	N	Variable	Coefficient	SE	CR	95% CI	P
R. equi	All[ϕ]		Intercept	-1.11	0.11			
		702	Pen/lane	0.66	0.09	1.93	1.61, 2.30	<0.001***
		702	Temperature >25°C	0.28	0.12	1.32	1.05, 1.65	0.02*
		702	Date					<0.001***
		168	Sept–Oct[a]			1		
		366	Nov–Dec	0.48	0.12	1.61	1.28, 2.03	<0.001***
		168	Jan–Feb	0.20	0.14	1.23	0.94, 1.60	0.13

Log likelihood = -1815 LRT (4 df) = 79.5 (P<0.001***) LRT vs pooled = 56.1 (P<0.001***)

	Location	N	Variable	Coefficient	SE	CR	95% CI	P
R. equi	Paddock		Intercept	-1.00	0.14			
		501	Temperature >25°C	0.33	0.13	1.39	1.07, 1.80	0.017*
		501	Date					<0.001***
		92	Sept.–Oct[a]			1		
		129	Nov.–Dec	0.68	0.15	1.97	1.46, 2.64	<0.001***
		280	Jan.–Feb	0.29	0.17	1.34	0.96, 1.87	0.085

Log likelihood = -1176 LRT (3 df) = 35.8 (P<0.001***) LRT vs pooled = 48.4 (P<0.001***)

	Location	N	Variable	Coefficient	SE	CR	95% CI	P
Virulent *R. equi*	All[ϕ]		Intercept	-1.35	0.27			
		702	Pen/lane	0.65	0.14	1.91	1.44, 2.52	<0.001***
		702	Soil moisture ≤10% H_2O	0.30	0.14	1.35	1.02, 1.78	0.02*
		702	State/year					<0.001***
		138	NSW 2001[a]			1		
		144	Vic 2000	-0.96	0.27	0.38	0.22, 0.65	<0.001***
		420	Vic 2001	-0.78	0.20	0.46	0.31, 0.67	<0.001***
		702	Date					0.002**
		168	Sept–Oct[a]			1		
		366	Nov–Dec	0.56	0.21	1.75	1.16, 2.64	0.006**
		168	Jan–Feb	0.75	0.22	2.12	1.38, 3.26	<0.001***

Log likelihood = -937.3 LRT (6 df) = 89.6 (P<0.001***) LRT vs pooled = 12.0 (P<0.001***)

	Location	N	Variable	Coefficient	SE	CR	95% CI	P
Virulent *R. equi*	Paddock		Intercept	-0.86	0.29			
		501	Pasture height ≤10 cm	0.49	0.19	1.63	1.12, 2.36	0.009**
		501	Temperature > 25°C	0.49	0.20	1.64	1.11, 2.41	0.01*
		501	Soil moisture ≤10 % H_2O	0.42	0.17	1.52	1.09, 2.12	0.02*
		501	State/year					<0.001***
		92	NSW 2001[a]			1		
		129	Vic 2000	-1.34	0.36	0.26	0.13, 0.53	<0.001***
		280	Vic 2001	-0.61	0.25	0.54	0.33, 0.89	0.02*

Log likelihood = -561 LRT (5 df) = 44.7 (P<0.001***) LRT vs pooled = 8.8 (P=0.002**)

[a]reference category
SE: standard error
LRT: likelihood ratio test
[ϕ] Variables measured only in the paddocks (pasture height and group size) were excluded

Temperature and date of sample collection were the only variables significantly associated with the airborne concentrations of *R. equi* in paddocks (Table 4.27). The airborne concentrations of *R. equi* in paddocks in the middle of the season were almost double (CR=1.97) that seen in the earlier part of the season.

Variables that were significantly associated with the airborne concentrations of virulent *R. equi* on both paddocks and pens and lanes were the state/year, date, location within the farm and soil moisture (Table 4.27). The greatest deviations of the CR from 1.0 were for the associations between the airborne concentrations of virulent *R. equi* and state/year, date and location within farm. The airborne concentration of virulent *R. equi* was 62% lower on Victorian farms in the 2000 season and 54% lower in the 2001 season than on NSW farms in 2001. The airborne concentration of virulent *R. equi* was 75% greater in the middle of the season and 112% greater late in the season than early in the season, and in the pens and lanes the airborne concentration of virulent *R. equi* was 91% greater than in the paddocks.

The state/year, pasture height, soil moisture and average ambient temperature were significantly associated with the airborne concentrations of virulent *R. equi* in paddocks (Table 4.27). The airborne concentration of virulent *R. equi* was 63% greater in paddocks with a low pasture height (≤10 cm) than in paddocks with a greater pasture height, 52% greater in paddocks with low soil moisture (≤10% H_2O) than in those with higher soil moisture, and 64% greater in paddocks when the average ambient temperature at sampling was high (>25°C) than when the average ambient temperature was lower (≤25°C). The paddocks on Victorian farms in the 2000 and 2001 seasons had a 74% and 46% lower concentration of airborne virulent *R. equi*, respectively, than those on NSW farms in 2001 (Table 4.27).

Table 4.28: Variables identified, using backwards stepwise random effects negative binomial regression analyses, as significantly affecting the concentration of *R. equi* and virulent *R. equi* in soil on farms

	Location	N	Variable	Coefficient	SE	CR	95% CI	P
R. equi	All$^{\phi}$		Intercept	-0.48	0.09			
		702	Soil moisture ≤10% H_2O	-0.26	0.08	0.77	0.66, 0.91	0.002**
		702	Humidity ≤70%	0.27	0.08	1.31	1.12, 1.53	<0.001***
		702	Date					0.006**
		168	Sept – Oct[a]			1		
		366	Nov– Dec	0.25	0.10	1.29	1.07, 1.56	0.008**
		168	Jan – Feb	0.34	0.11	1.40	1.12, 1.74	0.003**
Log likelihood = -2145			LRT (4 df) = 24.9 (P<0.001***)			LRT vs pooled = 50.8 (P<0.001***)		
	Location	**N**	**Variable**	**Coefficient**	**SE**	**CR**	**95% CI**	**P**
R. equi	Paddock		Intercept	-0.31	0.08			
		501	Group size >7 foals/paddock	-0.26	0.11	0.77	0.62, 0.96	0.02*
		501	Humidity ≤70%	0.24	0.09	1.27	1.06, 1.53	0.01*
Log likelihood = -1569			LRT (2 df) = 11.5 (P=0.003**)			LRT vs pooled = 42.9 (P<0.001***)		
	Location	**N**	**Variable**	**Coefficient**	**SE**	**CR**	**95% CI**	**P**
Virulent *R. equi*	All$^{\phi}$		Intercept	-0.47	0.17			
		702	State/year					0.003**
		138	NSW 2001[a]			1		
		144	Vic 2000	-0.77	0.25	0.46	0.28, 0.75	0.008**
		420	Vic 2001	-0.20	0.17	0.82	0.58, 1.14	0.24
Log likelihood = -1038			LRT (2 df) = 11.6 (P=0.003**)			LRT vs pooled = 14.1 (P<0.001***)		

[a]reference category
SE: standard error
LRT: likelihood ratio test
$^{\phi}$ Variables measured only in the paddocks (pasture height and group size) were excluded

Soil moisture, humidity and date were significantly associated with the concentrations of *R. equi* in soil on both paddocks and pens and lanes (Table 4.28). Dry soils (≤10%) had a 23% lower concentration of *R. equi* than moist soils (>10%), whilst a low average air humidity at sampling (≤70%) was associated with 31% higher concentrations of *R. equi* in soil than in samples collected in more humid conditions. Soil samples collected in the middle and late parts of the season had higher concentrations of *R. equi* than those collected early in the season.

The foal group size on a paddock and air humidity were significantly associated with the concentrations of *R. equi* in soil on paddocks (Table 4.28). Soil samples collected when the average air humidity was low (≤70%) had concentrations of *R. equi* 30% higher than soil samples collected in more humid conditions, whilst the concentration of *R. equi*

in soil samples collected from paddocks with a greater foal group size (containing >7 foals/paddock) was 23% lower than in samples from paddocks with fewer foals (≤7).

State/year was the only variable significantly associated with the concentration of virulent *R. equi* in soil on both paddocks and pens and lanes (Table 4.28). The concentration of virulent *R. equi* was 54% lower in soil samples from Victorian farms in the 2000 season than in those from NSW farms in the 2001 season.

No variables were significantly associated with the concentration of virulent *R. equi* in soil from paddocks alone (Table 4.28).

All but one of the CRs for variables in the final models changed by less than 10% when different variables were added, suggesting that there were no confounding interactions. The exception was the final model for airborne concentrations of virulent *R. equi*, where the CR for the average ambient temperature at the time of sampling increased from 1.64 to 1.92 when humidity was added to the model. There were no occasions when the added variable was significant (P≥0.05).

4.4. Discussion

The amount of contamination of soil with virulent *R. equi* has been regarded as the explanation for differences between farms in the prevalence of *R. equi* pneumonia. It has been suggested that on farms with high concentrations of virulent *R. equi* in soil and on which a high proportion of *R. equi* in soil were virulent, *R. equi* pneumonia is likely to be endemic (Takai *et al.* 1991c; Takai 1997). However, a more recent study has challenged this by reporting that the concentration of virulent *R. equi* in soil on farms with endemic *R. equi* pneumonia was not significantly different from that on those farms that did not experience the disease (Martens *et al.* 2000). In the study reported in this chapter, the differences between farms with varying *R. equi* disease prevalence were more likely to be associated with the proportion of *R. equi* that were virulent and the concentrations of virulent *R. equi* in air than in the soil. These findings concord with the current understanding of the route of infection, the inhalation of contaminated dust, and support the suggestion that the level of exposure to aerosolised virulent *R. equi* affects the relative

prevalence of *R. equi* pneumonia on farms (Prescott and Yager 1991; Giguere and Prescott 1997).

A number of environmental variables influenced the concentration of both *R. equi* and virulent *R. equi,* and the proportion of *R. equi* that were virulent, in both air and soil. Low humidity, high average ambient temperature and high soil moisture were significantly associated with an increased concentration of *R. equi* in the air or soil in multivariable analyses. However, the significant associations of low soil moisture and low pasture height with elevated concentrations of airborne virulent *R. equi* in multivariable analyses, but not with elevated concentrations of total *R. equi*, and associations of low soil moisture and acidic soil with elevated proportions of *R. equi* that were virulent in air and soil, respectively, suggest that virulent *R. equi* may have a different ecological niche from avirulent *R. equi*. Environmental conditions such as dry and acidic soils and poor pasture cover appear to favour virulent *R. equi* in the environment.

As the airborne *R. equi* population is influenced by environmental variables (i.e. soil moisture, pasture height, temperature), management strategies that affect these environmental variables are likely to influence disease prevalence on a farm. In the 2001 season two farms experienced substantial changes in the airborne concentrations of virulent *R. equi* and the proportion of airborne *R. equi* that were virulent. These changes were associated with changes in disease prevalence and changes in soil moisture on farms. One farm (farm A) experiencing drier conditions had elevated airborne virulent *R. equi* burdens and increased disease prevalence, whilst the more moist soil conditions on the other farm (farm F) were associated with reduced airborne virulent *R. equi* burdens and reduced disease prevalence, despite an increased foal population. These observations support the earlier finding linking airborne concentrations of virulent *R. equi* and proportions of airborne *R. equi* that were virulent with disease prevalence and support the link between the prevalence of disease and the airborne virulent *R. equi* burden on the farm.

The concentrations of virulent *R. equi* were highest in the holding pens, with lesser burdens in the lanes. Both pens and lanes were significantly more heavily contaminated

with virulent *R. equi* than the paddocks on most farms. Dry areas with low pasture cover also had significantly higher airborne concentrations of virulent *R. equi.* The dry soils and lack of grass in the holding pens and lanes were probably partially responsible for the greater airborne concentrations in these areas. These findings showed that holding pens and lanes were dangerous areas for foals to occupy for prolonged periods of time. Prolonged exposure to elevated airborne concentrations of virulent *R. equi* is likely to increase the risk of developing *R. equi* pneumonia. Many of the farms with a low to average prevalence of disease (<9%) had substantially higher airborne concentrations of virulent *R. equi* in the pens and lanes than the paddocks. On these farms the pens and lanes were the most likely sources of infection. The fall in disease prevalence from 4.6% to 0.9% on farm E in the 2001 season illustrates this point, as it was associated with a fall in the geometric mean airborne concentration of virulent *R. equi* and the proportion of *R. equi* that were virulent in the air in the pens and lanes.

Aerosolisation of virulent *R. equi* appeared to be influenced by the soil texture. The most prominent environmental factor influencing airborne concentrations of virulent *R. equi* and the proportions of airborne *R. equi* that were virulent was soil moisture and this variable was correlated with soil texture. Sandy soils tended to be drier than clay soils. The soils in holding pens and lanes were sandy on many farms. The reduced capacity of sandy soils to hold water and their generally lower concentrations of nutrients (Laegreid *et al.*1999) may favour survival, replication and aerosolisation of virulent *R. equi*. *In vitro* studies have found that expression of some *vap* genes is upregulated when concentrations of micronutrients are restricted (Ren and Prescott 2003). Upregulation of *vap* genes in harsh environmental conditions may enhance the survival and replication of virulent *R. equi* outside the host.

R. equi have been shown to be acid tolerant (Benoit *et al.* 2000) and acidic conditions have been shown to be a positive regulator of the expression of *vap* genes, with *vapA* optimally expressed at 38°C in a mildly acidic (pH=6.5) environment (Takei *et al.* 1996b; Benoit *et al.* 2001; Ren and Prescott 2003). The association between acidic soils and elevated proportions of virulent *R. equi* in soil was not statistically significant, but did suggest that the soil pH may have some influence on the survival of virulent *R. equi*

outside the host. However, the lack of statistically significant associations between environmental variables and the concentration of virulent *R. equi* in soil suggests that key environmental factors, other than faecal contamination, influencing the proliferation of virulent *R. equi* in the soil may still need to be identified.

The differing strengths of association between predictor variables and the concentration of virulent *R. equi* in air compared to soil may reflect the habitat of virulent *R. equi* in the surface soil. As the soil samples that were analysed were core samples taken down to a depth of 5 cm, they were unlikely to fully reflect the population in the most superficial soil, which was more likely to be represented in air samples. The air samples were also likely to reflect a relatively greater area than the soil samples. Thus the greater relative concentrations of virulent *R. equi* in air, compared to other *R. equi*, might suggest that virulent *R. equi* may predominate in the most superficial soil layers.

Previous studies have noted that the number of foals on farms influences the chance of the farm reporting cases of *R. equi* pneumonia (Chaffin *et al.* 2003b). These findings were supported by the association noted earlier in this thesis between small foal populations and a lower prevalence of disease on farms. These associations also appear to be affected by the age dynamics of the foal population. The 3 Victorian farms that did not report *R. equi* pneumonia in the 2001 all had low numbers of foals <16 weeks of age and between 4 and 12 weeks of age throughout the season. The NSW farm that reported no *R. equi* pneumonia cases managed their breeding season so that maximal numbers of foals in these age groups occurred earlier in the season. Farm B reported cases of, and a death from, *R. equi* pneumonia in the 2000 season but no cases in the 2001 season, without an overt change in environmental virulent *R. equi* burdens. However the farm reduced foal numbers by 25% in the 2001 season. Reducing the number of foals under 3-4 months of age throughout the season, but especially when environmental conditions become warmer and drier, appears to reduce the risk of *R. equi* pneumonia.

The tightly regulated Thoroughbred breeding season results in a close relationship between the age of foals and calendar month of the year on most farms, so variations in environmental factors between and within years may affect the prevalence of disease. The

majority of cases occurred in the middle of the season, in association with significant increases in the airborne concentrations of virulent *R. equi* and the peak in the number of foals <4 months of age. In the 2001 season Victorian farms experienced milder conditions during October and November than in the previous season, and the incidence of *R. equi* pneumonia increased considerably in December, a month later than in the previous season. Elevated airborne concentrations of virulent *R. equi* and proportions of airborne *R. equi* that were virulent were also noted later in the 2001 season (December-January), but this was not associated with a concurrent rise in prevalence of *R. equi* pneumonia as the number of foals aged between 4 and 12 weeks had begun to fall on most farms.

Lengthy antimicrobial therapy has been traditionally recommended for the successful treatment of *R. equi* pneumonia (Hillidge 1987). The majority of cases of *R. equi* pneumonia reported in the 2001 season received less than 20 days of treatment and many received less than 10 days of treatment. This may be a consequence of the increased vigilance of staff and clinicians on Australian Thoroughbred farms and the increasing use of ultrasonographic examination to diagnose cases earlier in the course of disesae. The low case fatality rate in NSW during the 2001 season may also be a reflection of the increased awareness of the disease, with early diagnosis and treatment leading to a higher survival rate and more rapid recovery.

This work highlights the likely importance of the airborne *R. equi* population in determining the prevalence of *R. equi* pneumonia. Ecological factors (i.e. soil moisture, sandy soils, poor pasture cover) affecting the concentration of airborne virulent *R. equi* and the proportion of airborne *R. equi* that were virulent in the environment of susceptible foals appear to influence the prevalence of *R. equi* pneumonia and contribute to the severity of disease. Environmental management strategies focusing on reducing the level of aerosol challenge to susceptible foals, together with early diagnosis and treatment of cases, seems to be the best way forward in reducing the impact of *R. equi* pneumonia on the Australian Thoroughbred breeding industry.

Chapter 5

Comparison of two *R. equi* selective agar media

5.1. Introduction

R. equi competes with a variety of bacteria and fungi in the soil and the mammalian host. Studies of the ecology of *R. equi* and the epidemiology of disease caused by *R. equi* in horses, pigs and humans were enhanced by the development of a selective medium by Woolcock *et al.* (1979) that prevents the growth of many of these competing organisms. This first selective medium for *R. equi* consisted of a trypticase soy agar base supplemented with nalidixic acid (20 μg/ml), novobiocin (25 μg/ml), actidione (cycloheximide) (40 μg/ml) and potassium tellurite (0.005%) and was named NANAT. This medium has been used routinely in ecological and epidemiological studies of *R. equi* for the past 25 years (Woolcock *et al.* 1980; Takai *et al.* 1986a, 1991c, 2001a).

An alternative selective medium for the isolation of *R. equi* was developed by von Graeventiz and Punter-Streit (1995). This medium consisted of a Mueller-Hinton agar base supplemented with ceftazidime (20 μg/ml) and novobiocin (25 μg/ml) and was named CAZ-NB. Eleven *R. equi* strains unable to be cultured on NANAT medium were culturable on CAZ-NB. The reduced inhibition of *R. equi* growth and the inhibition of the growth of many related actinomycetes and Gram-negative contaminants suggested that CAZ-NB would be a superior selective medium for *R. equi* (von Graevenitz and Punter-Streit 1995). Fungal growth was found to be a problem with this medium, and supplementation with anisomycin was suggested as a measure to prevent this.

Only one study (Martens *et al.* 2000) has compared the two media for their ability to isolate *R. equi* from soil samples and found that samples that yielded *R. equi* on one medium often failed to yield *R. equi* on the other. *R. equi* were isolated from significantly greater numbers of samples (from either *R. equi* affected or control farms) when using

NANAT medium than when using CAZ-NB medium. Of the 10 virulent *R. equi* cultured from soil isolates 9 were obtained from soil inoculated onto NANAT medium.

The aim of the work described in this chapter was to compare the use of these two selective media (with modification of the CAZ-NB media to inhibit fungal growth) for isolation and enumeration of *R. equi* and virulent *R. equi* in soil samples and to examine their ability to support the growth of different strains of *R. equi*.

5.2. Materials and methods

5.2.1. Recoverability of defined strains of *R. equi* on different selective media

Seven *R. equi* isolates obtained from foals were used. The set of 7 isolates included 6 different genomic strain types based on analysis of restriction endonuclease digests by pulsed field gel electrophoresis (PFGE) (Morton *et al.* 2001). Six of the 7 isolates were defined as virulent on the basis of *vapA* PCR assays (Haites et al. 1997) (Table 5.1).

Table 5.1: Media inoculated and *R. equi* isolates used in comparing recoverability of *R. equi* on selective media

Isolate No.	Pathogenicity	Genotype*	Medium inoculated		
			TSA	NANAT	mCAZ-NB
7	Virulent	T	+	+	+
14	Virulent	G	+	+	+
21	Virulent	J	+	+	+
38	Virulent	B	+	+	+
35	Virulent	A	NI	+	+
42	Virulent	A	+	+	+
128	Avirulent	L	+	+	+

TSA: Trypticase soy agar (non-selective medium)
*Genotype of isolate as described by Morton *et al.* (2001)
NI: Isolate 35 not inoculated onto TSA medium

The ability of the media to support the growth of six genomically distinct strains was examined by inoculation of the strains into 10 ml LB and incubation for 24 to 48 hours in a shaking incubator at 37°C. The resulting cultures were serially diluted in PBS. One hundred microlitres of the 10^{-4} and 10^{-5} dilutions of each culture were spread on duplicate plates of the two selective agar media (NANAT and modified CAZ-NB) and non-selective trypticase soy agar (TSA). Addition of cycloheximide (40 μg/ml) to media has been shown to inhibit fungal growth in environmental samples (Lacey and Dutkiewicz 1976), so this antifungal agent was added to CAZ-NB to produce a modified CAZ-NB medium (mCAZ-

NB) in this study. The plates were incubated for 48 hours at 37°C and the number of colonies on each plate counted.

Two isolates from the same genomic group (isolates 35 and 42) were similarly grown in LB broth, serially diluted and plated onto the two selective agar media to investigate the possibility of variation between the two media in their ability to support growth of isolates from the same genomic group.

5.2.2. Isolation and enumeration of *R. equi* in soil

a. Soil samples

Sixty soil samples collected during the 2001 Thoroughbred breeding season from 9 farms in Victoria and NSW were used for microbiological analysis (Table 5.2). One gram of soil was diluted in 9 ml PBS, mixed vigorously and 100 µl inoculated onto duplicate NANAT and mCAZ-NB agar plates. After incubation at 37°C for 48 hours the number of colonies on one plate of each medium was determined and the number of bacteria per milligram of soil calculated.

Table 5.2: Soil samples used to compare NANAT and mCAZ-NB selective media

Farm[‡]	Period	No. samples[*]
A	Dec[1]	6
B	Nov[2]	6
B	Jan[1]	6
C	Oct[1]	6
E	Jan[2]	6
F	Oct[2]	6
H	Nov[1]	6
I	Nov[2]	6
9	Nov[2]	6
10	Dec[1]	6

[‡] Farms are as described in Chapter 3
[1] middle of the month
[2] end of the month
[*] Samples collected from 4 paddocks, a holding pen and a lane

Soil samples used in this study were used in the ecological study of *R. equi* on Thoroughbred farms described in Chapter 4. Results obtained in that study using NANAT medium are used in this chapter.

b. Colony blotting and DNA hybridisation

One plate of each of the selective medium for each of the 60 soil samples was selected for colony blotting and DNA hybridisation (Chapter 2).

Colony blotting was performed as described in Section 4.2.8, with the exception that *B. fumarioli* (isolate number BP1) was used as a negative control for blots of mCAZ-NB medium as *C. ammoniagenes* could not be cultured on mCAZ-NB.

5.2.3. Data and statistical analysis

To assess the ability of the two selective media to support the growth of *R. equi*, the numbers of colonies of each isolate of *R. equi* on the two selective media were compared to the numbers on the non-selective medium (TSA). An intra-strain comparison of the two selective media was performed using isolates 35 and 42 (both strain A).

The mean numbers of *R. equi* colonies on each medium were compared to those on the non-selective medium using paired t-tests.

Comparisons of the efficacy of the two selective media in the isolation and enumeration of *R. equi* and virulent *R. equi* from soil were also performed. An analysis of the proportion of plates yielding positive culture results with each of the two media was performed using McNemar's test. A Wilcoxon matched-pair signed-rank test was performed to compare the differences in the number of colonies isolated (all bacteria, *R. equi* and virulent *R. equi*) between paired samples as a measure of the efficacy of the two media.

5.3. Results

5.3.1. Growth of defined strains on selective media

There was variation in the ability of different isolates to grow on the two selective media (Table 5.3). The selective media inhibited the avirulent strain to a lesser extent than the virulent strains. Five isolates had higher yields on NANAT than on mCAZ-NB, but one isolate had an almost 5 fold higher recovery on mCAZ-NB than on NANAT. An isolate from the same genomic group as that which yielded higher numbers of colonies on mCAZ-NB was plated onto NANAT and mCAZ-NB and was found to yield similar numbers of colonies on both media (6.8×10^6 on NANAT compared to 6.5×10^6 on mCAZ-NB).

Table 5.3: Recoverability of different *R. equi* isolates on selective and non-selective media

Isolate No.	No. colonies / ml inoculum			Recoverability (%)	
	TSA	NANAT	mCAZ-NB	NANAT	mCAZ-NB
7	7.7×10^6	5.1×10^6	3.8×10^6	66	49
14	7.8×10^6	4.2×10^6	3.5×10^6	54	45
21	7.1×10^6	5.7×10^6	3.6×10^6	80	51
28	5.9×10^6	2.5×10^6	7.3×10^5	42	12
42	6.3×10^6	1.2×10^6	5.6×10^6	19	89
128	5.5×10^6	7.2×10^6	4.9×10^6	131	89

Even though all 6 isolates of *R. equi* grew on all 3 media, on average 35.8 % fewer colonies were obtained on NANAT and 44.8 % fewer on mCAZ-NB than on TSA (Table 5.4).

Table 5.4: Mean number of *R. equi* colonies on 3 media

Medium	N	Number of *R. equi* colonies (/ml)	
		Mean	95% CI
TSA	6	6.7×10^6	5.7×10^6, 7.7×10^6
NANAT	6	4.3×10^6	2.0×10^6, 6.6×10^6
mCAZ-NB	6	3.7×10^6	2.0×10^6, 5.4×10^6

N: Number of isolates used

There was a significant difference in the mean number of *R. equi* colonies obtained on mCAZ-NB compared to TSA (P=0.01), but no significant difference when comparing CAZ-NB to NANAT medium (P=0.6). The mean number of *R. equi* colonies obtained on NANAT medium was not significantly lower than the number obtained on TSA (P=0.06) (Table 5.5).

Table 5.5: Comparison between media of the difference in mean numbers of *R. equi* colonies

Media	N	Number of *R. equi* colonies (/ml)	
		Δ Mean	95% CI Δ Mean
TSA vs NANAT	6	2.4×10^6	-9.7×10^4, 4.8×10^6
TSA vs mCAZ-NB	6	3.0×10^6	9.6×10^5, 5.0×10^6
NANAT vs mCAZ-NB	6	6.1×10^5	-2.1×10^6, 3.3×10^6

N: Number of isolates used
Δ : Difference

5.3.2. Isolation of *R. equi* from soil samples

R. equi were detected in 48/60 (80%) soil samples cultured on NANAT media and 52/60 (87%) samples cultured on mCAZ-NB media. Virulent *R. equi* were detected in 31/60 (52%) samples cultured on NANAT media and 38/60 (63%) samples cultured on mCAZ-NB. There appeared to be a limited association between the isolation of *R. equi* and virulent *R. equi* from paired samples on the two selective media. The results were in agreement for 46/60 (77%) samples for the culture of *R. equi* and for 31/60 (52%) samples for the culture of virulent *R. equi*. There was no significant difference between the proportion of cultures positive for *R. equi* (Table 5.6) or the proportion positive for virulent *R. equi* (Table 5.7) on NANAT and on mCAZ-NB media.

Table 5.6: Detection of *R. equi* in 60 paired soil samples using NANAT and mCAZ-NB media

	mCAZ-NB		Total	P
NANAT	Positive	Negative		
Positive	43	5	48	
Negative	9	3	12	0.4
Total	52	8	60	

Table 5.7: Detection of virulent *R. equi* in 60 paired soil samples using NANAT and mCAZ-NB media

	mCAZ-NB		Total	P
NANAT	Positive	Negative		
Positive	20	11	31	
Negative	18	11	29	0.3
Total	38	22	60	

5.3.3. Enumeration of *R. equi* in soil samples

NANAT medium yielded 6,966 colonies from the 60 soil samples, of which 557 (8%) were *R. equi*. Of these 50 (9%) were virulent. The mCAZ-NB medium yielded 3,082 colonies from the same soil samples, of which 321 (10%) were *R. equi*. Of these 102 (32%) were virulent. There were significantly more colonies obtained from soil samples when they were cultured on NANAT media (P=0.001, Wilcoxon matched-pairs signed-rank test). However there were significantly more colonies of virulent *R. equi* obtained from these samples when they were cultured on mCAZ-NB (P=0.03, Wilcoxon matched-pairs signed-rank test). There was no significant difference in the number of *R. equi* cultured from soil samples on each of the two media (P=0.10) (Table 5.8).

Table 5.8: Culture of 60 paired soil samples on NANAT and mCAZ-NB media

	NANAT			mCAZ-NB			Δ (NANAT – mCAZ-NB)		
	Median	Range	Total	Median	Range	Total	Median	95% CI	P
No. bacteria (cfu/mg)	59.0	0-780	6,966	37.5	0-202	3,082	34.0	1.9, 51.5	0.001**
No. *R. equi* (cfu/mg)	4.0	0-104	557	3.0	0-48	321	0.0	-1.0, 1.1	0.10
No. virulent *R. equi* (cfu/mg)	1.0	0-5	50	1.0	0-27	102	0.0	-1.0, 0.0	0.03*

P: Probability that the number of bacteria, *R. equi* or virulent *R. equi* recovered on each of the two media differ, Wilcoxon matched-pairs ranked-sign test.

5.4. Discussion

The two common selective media used for *R. equi* have been shown to differ in their ability to suppress the growth of other microbial species and some strains of *R. equi* (Woolcock *et al.* 1979; Barton and Hughes 1981; von Graventinz and Punter-Streit 1995). The use of enrichment media consisting of a trypticase soy broth supplemented with cycloheximide (50 μg/ml), nalidixic acid (20 μg/ml), penicillin (10 IU/ml) and potassium tellurite (0.005%) (TANP) for soil, faecal or large bowel samples, with subculture onto M3 agar (Rowbotham and Cross 1977) supplemented with potassium tellurite (0.005%) successfully increased the isolation rate of *R. equi* from samples (Barton and Hughes 1981). The development of CAZ-NB medium promised an agar medium with reduced inhibition of *R. equi* than NANAT medium, but differences have been noted in the ability of these media to support growth of *R. equi* and virulent *R. equi*. In one study analysing paired soil samples NANAT medium was found to support isolation of *R. equi* from more samples than CAZ-NB (Martens *et al.* 2000).

The study described in this chapter also found differences between the two selective media in their inhibition of *R. equi* and other competing environmental microorganisms. Results from paired *R. equi* cultures showed that mCAZ-NB media significantly inhibited the recovery of *R. equi* compared to the non-selective agar, but that there was no difference in inhibition of *R. equi* growth between the two selective media. However, when comparing the recovery rate of individual *R. equi* strains, differences were seen between the two selective media in their ability to support the growth of some strains. A difference was also seen between the recoverability of two isolates of the same genotype on the two media. In addition studies on soil samples showed that the two selective media differed in their ability to inhibit the growth of competing environmental organisms.

The decision to add cycloheximide to the CAZ-NB media was based on its inclusion in NANAT medium as an inhibitor of fungal growth. This modified CAZ-NB medium was not compared to the CAZ-NB medium used in previous studies. This may explain the differences between the results from this study and that of Martens *et al.* (2000), who found that NANAT medium was significantly better as a selective medium for isolating *R. equi* from soil. The study described in this chapter found no evidence of a difference between mCAZ-NB and NANAT in detection of *R. equi* or virulent *R. equi* in paired soil samples.

Although there was no significant difference between the NANAT and mCAZ-NB media in their ability to detect *R. equi* or virulent *R. equi*, more samples were positive for *R. equi* and virulent *R. equi* when inoculated onto mCAZ-NB. Furthermore, the number of *R. equi* and virulent *R. equi* colonies isolated on the two media from soil samples differed. The mCAZ-NB medium yielded significantly more colonies of virulent *R. equi* than NANAT media. Thus, mCAZ-NB medium seemed to favour the recovery of virulent *R. equi* from soil samples. NANAT media was found to be less inhibitory of other bacteria, with significantly higher numbers of all bacteria isolated from soil samples inoculated onto NANAT. This did not affect the recovery of high numbers of *R. equi* from soil samples, but may have contributed to the reduced recovery of virulent *R. equi*. The reduced inhibition of other bacteria by the NANAT medium may have allowed growth of organisms that competed with virulent *R. equi*, or inhibited them. The more restrictive

mCAZ-NB medium may have restricted the growth of these competing organisms allowing more virulent *R. equi* to grow.

The better recovery of virulent *R. equi* when using mCAZ-NB and the better inhibition of other bacteria suggests that this medium is superior to NANAT medium. However, the variation seen between different isolates and samples suggests that investigations into the ecology and epidemiology of *R. equi* may benefit from the development of more effective selective agar media.

The use of mCAZ-NB might be best where high rates of recovery of virulent *R. equi* from samples is essential, such as in investigations of the efficacy of antimicrobial therapy in *R. equi* cases, the prevalence of carriage of respiratory tract infection in foals that are clinically or sub-clinically affected and the prevalence of carriage of virulent *R. equi* in the gastrointestinal tract of mares.

Chapter 6

Detection of virulent *R. equi* in expired air samples from foals

6.1. Introduction

The accepted pathogenesis of *R. equi* pneumonia is that the virulent *R. equi* in the soil on horse farms are aerosolised, inhaled and cause pneumonic disease. This generally accepted hypothesis has been based on the fact that the organism is a soil saprophyte and part of the normal gastrointestinal flora of horses. *R. equi* are able to flourish in soil on horse farms, especially within equine faeces, by utilising VFAs to multiply up to 10,000 fold (Barton and Hughes 1984; Hughes and Sulaiman 1987). Intestinal carriage has been demonstrated in adults, but foals up to 12 weeks of age have considerably higher concentrations of *R. equi* in their faeces than adults. Faeces from healthy foals <12 weeks of age and from clinically affected foals are thought to be the main source of environmental contamination (Takai *et al.* 1986a, 1991c, 1994a). Thus, inhalation of dust generated from dry soil contaminated with faecal matter containing virulent *R. equi* is accepted as the main route of transmission (Barton and Embury 1987; Giguere and Prescott 1997; Hondalus 1997). An increased concentration and proportion of the virulent organisms in the environmental *R. equi* population is associated with an increased risk of inhalation of virulent *R. equi* from the soil (Takai 1997), and thus an increase in prevalence of *R. equi* disease (Takai *et al.* 1991c, 1994a). Work described previously in this thesis also showed that higher concentrations of airborne virulent *R. equi* and proportions of *R. equi* that were virulent were associated with a higher prevalence of *R. equi* pneumonia (Chapter 4).

The faeces of young foals (<12 weeks of age) and foals with *R. equi* pneumonia not only have high concentrations of *R. equi*, but also a higher proportion of *R. equi* that are virulent than the faeces of older healthy foals and adults (Takai *et al.* 1994a). Areas frequented by foals and environmental conditions that favour multiplication in the soil and

aerosolisation of *R. equi* from the soil should be considered high risk areas for foals. The air in lanes and holding pen areas was shown previously to have high concentrations of virulent *R. equi* and high proportions of *R. equi* that were virulent (Chapter 4). These areas tended to be dry and sandy, and were frequented by many different mobs of foals. Many of these factors will contribute to increased aerosolisation of soil *R. equi* and thus increase transmission of the pathogen.

R. equi pneumonia has always been considered to be a non-contagious respiratory disease. Due to the intracellular nature of the infectious agent, the sometimes problematic recovery of *R. equi* from tracheal lavages from foals with clinical *R. equi* pneumonia and the production of abscesses in the lungs of the infected foal, direct animal to animal transmission has generally been considered an unlikely alternative route of transmission (Martens *et al.* 1982; Hillidge 1987; Barton and Embury 1987; Hondalus 1997). However, if foal-to-foal transmission by the aerosol route is possible, the 'host adapted' virulent *R. equi* may spread more effectively as a contagious agent within a mob rather than be acquired by inhalation of aerosols from the soils.

The aim of this study was, firstly, to establish whether virulent *R. equi* could be detected in the exhaled air from foals with or without *R. equi* pneumonia. If virulent *R. equi* were detected in exhaled air samples then a second aim was to compare the concentration of airborne virulent *R. equi* in high risk environmental areas with that detected in the exhaled air of foals. In this way the possibility of direct spread of virulent *R. equi* between foals and its significance in comparison to the currently accepted horse-environment cycle of infection could be evaluated.

6.2. Materials and methods

6.2.1. Farms and foals

Exhaled air samples were collected from 55 foals on 8 Thoroughbred farms in Victoria and NSW during the 2000 and 2001 breeding seasons. Environmental air samples were collected monthly between November 2000 and February 2001 from the lanes and

holding pens on 6 Thoroughbred farms. These 6 farms had participated in the ecological and epidemiological studies described previously in this thesis (Chapters 3 and 4).

All 6 farms from which environmental air samples were collected reported cases of *R. equi* pneumonia during the sampling period.

There was no formal, randomised sampling frame used to determine which foals were sampled. Initially, foals with *R. equi* pneumonia were selected to test the hypothesis that *R. equi* could be detected in exhaled air. Forty five of the 55 foals were being treated for *R. equi* pneumonia when sampled. Eleven foals were sampled when they were restrained for routine thoracic ultrasonographic examination. Additional samples were collected from these foals. Blood samples were taken from 10 of the 11 foals for haematological and serological analyses. Faecal samples were collected from 9 of these foals for culture for *R. equi*. One of these foals had been diagnosed with *R. equi* pneumonia prior to breath sampling.

6.2.2. Collection and analysis of samples

a. Air samples

Air samples were collected using a portable air monitoring system (M Air T, Millipore) onto mCAZ-NB agar (Section 5.2.1), as described previously (Section 2.2.2). A single 500 l air sample was taken in each of the pens and lanes. Exhaled air samples from foals were collected by holding the air monitoring device up to the muzzle of a manually restrained foal. Either 100 or 250 l samples were collected. The procedure took between 1 and 2 minutes, depending on the volume of air sampled. The sieve of the air monitoring device was disinfected prior to the collection of each sample, as described previously (Section 4.2.3). Colony blotting and DNA hybridisation using the radiolabelled *vapA* PCR product were performed as previously described (Chapter 2).

b. Faecal samples

The concentrations of virulent *R. equi* in faecal samples from 9 foals were determined using a method similar to that described for the quantitative analysis of *R. equi*

in soil samples (Section 4.2.4). One gram of faeces was serially diluted in PBS in 10-fold steps. Each dilution was inoculated onto NANAT agar to facilitate the growth of *R. equi* (Woolcock *et al.* 1979). The plates were incubated at 37°C for 48 hours and a plate containing between 10 and 100 colonies was used for quantitative analysis of virulent *R. equi* by colony blotting and DNA hybridisation using the radiolabelled *vapA* probe, as previously described (Chapter 2).

c. Haematological and serological analyses

Whole blood and serum samples were collected from 10 foals at the time of breath sampling on one farm. Total white cell and fibrinogen concentrations were determined for each sample.

An ELISA was used to detect IgG against VapA in serum samples. All the VapA ELISAs were performed at the Institute of Medical and Veterinary Science Infectious Disease Laboratories by Tongted Phumoonna under the supervision of Dr. Michael W. Heuzenroeder and Prof. Mary D. Barton. A 20 amino acid biotinylated peptide (biotin-SGSGTSLNLQKDEPNGRASDTAGQ, Mimotopes, Victoria, Australia) that defines a linear B-cell epitope at amino acid positions 62-81 of VapA was used as the ELISA antigen (Vanniasinkam *et al.* 2001). Each well of the 96-well microtitre plates (Maxisorp, Nunc, Rockilde, Denmark) was coated with 100 μl/well of NeutrAvidin (Pierce, Rockford, IL, USA) at a concentration of 0.33 μg/well. The plates were then blocked with 1% casein in PBS / 0.05% Tween 20 (PBST) for one hour at room temperature and then washed with PBST. A 100 μl volume of the peptide at a concentration of 0.286 μg/ml was added to each well. The plates were then washed with PBST and serial twofold dilutions of the sera in PBST added. A positive control serum and a reference serum (diluted 1:250) were included in each plate. After overnight incubation at 4°C, a secondary antibody conjugate (goat anti-horse IgG horseradish peroxidase, Bethyl Laboratories, Montgomery, TX, USA) was added at a 1:25,000 dilution in 1% casein in PBST. Tetramethylbenzidine (TMB) (Sigma, St.Louis, MO, USA) was used as a chromogenic substrate. Optical densities (OD) were read on a MR7000 ELISA plate reader (Dynatech Laboratories, Chantilly, VA, USA) using a test wavelength of 450 nm and a reference wavelength of 630 nm. The endpoint of

the titration was defined as the last dilution in which the mean OD reading was at least twice that of the negative reference serum well. The reference serum was obtained from a healthy foal and a 1:250 dilution of this serum yielded an OD of between 0.06 and 0.10 in the assay.

6.2.3. Data and statistical analysis

The concentrations of virulent *R. equi* in environmental and exhaled air samples were scaled to a common volume to facilitate comparative analyses. The Mann-Whitney test was used to compare and evaluate the significance of differences in concentration between the environmental and exhaled air samples. All statistical analyses were performed using MINITAB for Windows version 12.

6.3. Results

6.3.1. Concentrations of airborne virulent *R. equi*

All 6 farms reported cases of *R. equi* pneumonia during the 2000 foaling season. All but one farm had detectable airborne virulent *R. equi* in the lanes and pens during the sampling period. The range of the median concentrations of airborne virulent *R. equi* recovered from these farms was between 0 and 2 cfu/1000 l. The highest median concentration of airborne virulent *R. equi* over the sampling period were on farms C and F, with a median of 2 cfu/1000 l of air in the lanes and pens. The median airborne concentration of virulent *R. equi* in positive environmental air samples was 2 cfu/1000 l, with 19/48 (40%) environmental air samples positive for airborne virulent *R. equi*. Farms C and F had the greatest number of positive samples (Table 6.1).

Table 6.1: Airborne virulent *R. equi* in the pens and lanes of 6 farms during the 2000 foaling season

Farm	Total Samples	Positive samples	Total volume sampled (l)	Number of colonies of virulent *R. equi*	Virulent *R. equi* (cfu/1000 l)		
					Median	Range	IQR
A	8	2	4000	2	0.0	0.0-2.0	0.0-1.5
B	8	0	4000	0	0.0	0.0-0.0	0.0-0.0
C	8	5	4000	21	2.0	0.0-24.0	0.0-9.5
D	8	4	4000	7	1.0	0.0-8.0	0.0-2.0
E	8	3	4000	6	0.0	0.0-6.0	0.0-3.5
F	8	5	4000	5	2.0	0.0-2.0	0.0-2.0
Total	48	19	24000	41	0.0	0.0-24.0	0.0-2.0

IQR: interquartile range (1^{st} quartile – 3^{rd} quartile)

Virulent *R. equi* were detected in the lanes and pens during each monthly sampling period. The frequency of detection and concentration of airborne virulent *R. equi* were lower in November than later in the season (Table 6.2).

Table 6.2: Airborne virulent *R. equi* in the pens and lanes each month during the 2000 foaling season

Month	Total Samples	Positive samples	Total volume sampled (l)	Number of colonies of virulent *R. equi*	Virulent *R. equi* (cfu/1000 l)		
					Median	Range	IQR
Nov	12	2	6000	2	0.0	0.0-2.0	0.0-0.0
Dec	12	5	6000	10	0.0	0.0-12.0	0.0-2.0
Jan	12	6	6000	7	1.0	0.0-4.0	0.0-2.0
Feb	12	6	6000	22	1.0	0.0-24.0	0.0-5.0

IQR: interquartile range

6.3.2. Concentrations of virulent *R. equi* in exhaled air from foals

Exhaled air samples were collected from 55 foals on 8 Thoroughbred farms in the 2000 and 2001 seasons (farms A, E, F, H, I, 1, 3 and 5). A proportion of these foals (35/55 (64%)) were on farms F and 3. These farms had a prevalence of *R. equi* pneumonia of >9% in the 2000 and 2001 seasons (Table 3.3). Of the 55 foals sampled, 48 were diagnosed with *R. equi* pneumonia at, or prior to, sample collection. Seven of 11 foals sampled on farm 3 during routine ultrasonographic lung examination were considered to be healthy, with no obvious lung lesions suggestive of *R. equi* pneumonia.

Virulent *R. equi* were detected in exhaled air samples from 37/55 (67.3%) foals. The median concentration of virulent *R. equi* in exhaled air was 8 cfu/1000 l (Table 6.3).

Table 6.3: Virulent *R. equi* in exhaled air from foals on 8 Thoroughbred farms

Farm	Foals tested	Virulent *R. equi* positive foals	Diagnosed and treated foals	Total volume sampled (l)	Number of colonies of virulent *R. equi*	Virulent *R. equi* (cfu/1000 l)		
						Median	Range	IQR
A	1	1	3	100	4	40.0	NA	NA
E	6	3	6	600	21	10.0	0.0-160.0	0.0-62.5
F	24	12	24	4650	59	2.0	0.0-120.0	0.0-12.0
H	2	1	2	500	1	2.0	0.0-4.0	NA
I	2	2	2	500	4	8.0	8.0-8.0	NA
1	3	3	3	750	27	48.0	8.0-52.0	8.0-52.0
3	11	10	4	2750	37	8.0	0.0-60.0	8.0-16.0
5	6	5	6	1500	20	8.0	0.0-44.0	3.0-23.0
Total	55	37	48	11350	173	8.0	0.0-160.0	0.0-16.0

IQR: interquartile range
NA: not applicable

The median concentration of virulent *R. equi* detected in exhaled air from clinically normal foals was the same as that detected in exhaled air from diseased foals, but positive samples from diseased foals had slightly higher concentrations than healthy foals (Table 6.4). The volume of exhaled air sampled appeared to influence the likelihood of detection of virulent *R. equi*. When a greater volume of air was sampled a higher proportion of samples were positive for virulent *R. equi*. Almost 75% (29/39) of the 250 l samples were positive for virulent *R. equi*, whilst 50% (8/16) of the 100 l samples were positive.

Table 6.4: Virulent *R. equi* in exhaled air from foals with or without clinical *R. equi* pneumonia

Disease status	No. tested	No. exhaling virulent *R. equi*	Virulent *R. equi* (cfu/1000 l)		
			Median	Range	IQR
R. equi pneumonia	48	31 (64.6%)	8.0 (12.0)	0.0-160.0 (4.0-160.0)	0.0-16.0 (8.0-27.0)
Healthy	7	6 (85.7%)	8.0 (8.0)	0.0-60.0 (8.0-60.0)	8.0-16.0 (8.0-24.0)
Total	55	37 (67.3%)	(10.0)	(4.0-160.0)	(8.0-22.0)

IQR: interquartile range
Figures in brackets were obtained when only positive samples were included

NANAT medium was used unsuccessfully in a trial on 3 foals. All samples collected onto NANAT medium yielded numerous colonies (>1000) with a colonial morphology atypical of *R. equi*. *R. equi* were not detected in blots of the samples collected onto NANAT medium but were detected in blots of samples collected from the same foals onto mCAZ-NB medium.

6.3.3. Assessment of eleven foals on a farm with a high prevalence of *R. equi* pneumonia

a. Ultrasonographic and haematological examinations

Of the 11 foals examined on farm 3, five had lung lesions detectable by ultrasonography and in four of these the lesions were suggestive of *R. equi* pneumonia. Two of these four foals had a significant leucocytosis and three were hyperfibrinogenaemic. One of these foals had been diagnosed with *R. equi* pneumonia previously and had been treated with erythromycin and rifampicin for 6 days prior to sampling (Table 6.5).

Table 6.5: Ultrasonographic, haematological, microbiological and serological findings in 11 foals and their treatment

Foal	Description of lung lesions		Tx*	WBC (x 10^9 cells/l)	Fibrinogen (g/l)	Concentration of virulent *R. equi*		Serum titre against VapA
	Right lobe	Left lobe				Breath (cfu/1000 l)	Faeces (cfu/mg)	
1	NDL	NDL	No	NT	NT	8	13	NT
2	Single (<1 x 1cm)†	NDL	No	12.0	5	ND	11	128
3	NDL	NDL	No	8.8	4	8	ND	512
4	NDL	NDL	No	10.2	4	8	183	128
5	NDL	Single (2 x 1 cm)	Yes	15.5	6	8	ND	1024
6	Single (2 x 2 cm)	Single (2 x 2 cm)	Yes	9.8	4	8	5	512
7	NDL	NDL	No	9.8	5	8	ND	1024
8	NDL	NDL	No	10.1	4	60	NT	1024
9	NDL	Multiple (1 x 1 cm)	Yes	11.8	5	16	NT	2048
10	NDL	NDL	No	10.8	6	16	39	128
11	Multiple (3-6 x 2 cm)	Multiple (≤8 x 8 cm)	Yes	22.9	8	8	8100	16384

NDL: No detectable lesion
Multiple: > 5 lesions
Tx : Treatment
*All foals that received treatment were administered erythromycin and rifampicin
†Small lesion not typical of *R. equi* pneumonia
WBC: white blood cells (normal range: 5-12 x 10^9 cells/l)
NT: sample not taken
ND: not detected in sample
Fibrinogen (normal range: 1-4 g/l)

b. Serology

VapA specific antibody was detected in all 10 serum samples. Titres of IgG against the specific 20 amino acid epitope of VapA ranged from 128 to 16,384. All four foals with detectable lung lesions suggestive of *R. equi* pneumonia had titres >512. Three foals in which lung lesions were not detected also had titres between 512 and 1024. The highest titre was seen in foal 11. This foal had been diagnosed previously with *R. equi* pneumonia and was being treated at the time of sampling (Table 6.5).

c. Concentrations of virulent *R. equi* in the exhaled air and faeces of foals

Virulent *R. equi* were detected in the exhaled air from 10 of the 11 foals on farm 3. Concentrations of virulent *R. equi* ranged from 8 to 60 cfu/1000 l (Table 6.5). The highest concentration was detected in a clinically normal foal with an antibody titre against VapA of 1024. All foals with lung lesions suggestive of *R. equi* pneumonia had detectable virulent *R. equi* in a 250 l sample.

Faecal samples from 9 of the foals were examined for virulent *R. equi* and detectable concentrations were found in 6, ranging from 5 to 8,100 cfu/mg. The highest concentration was seen in the foal being treated for *R. equi* pneumonia, while two of the foals with the lowest antibody titre against VapA had the second and third highest concentration of virulent *R. equi* in their faeces (Table 6.5).

6.3.4. Comparison of the concentrations of airborne virulent *R. equi* in environmental and exhaled air samples

The frequency of detection of virulent *R. equi* in environmental air samples was almost half that of the frequency of detection in exhaled air samples from foals, even though the volume of air sampled from the environment was more than twice that sampled from foals. The concentration of virulent *R. equi* in the exhaled air was approximately eight fold higher than that in environmental air samples. When positive samples were compared the median concentration of virulent *R. equi* in exhaled air was five fold higher than that in environmental air (Table 6.6). The median concentration of virulent *R. equi* in exhaled air was significantly higher than in environmental air samples (P<0.001) and was also significantly greater when only positive samples were considered (P<0.001).

Table 6.6: Comparison of the concentrations of airborne virulent *R. equi* in environmental and exhaled air samples

Type	Sample			Virulent *R. equi* (cfu/1000 l)			
	No.	Positive	Volume (l)	Median	Range	IQR	P
Environmental air	48	19	24000	0.0 (2.0)	0.0-24.0 (2.0-24.0)	0.0-2.0 (2.0-4.0)	
Exhaled air	55	37	11350	8.0 (10.0)	0.0-160.0 (4.0-160.0)	0.0-16.0 (8.0-22.0)	<0.001*** (<0.001***)

IQR: interquartile range
P= probability that the median concentration of virulent *R. equi* in environmental air differs from that in exhaled air from the foal.
Figures in brackets were obtained when only positive samples were included

6.4. Discussion

Virulent *R. equi* were detected in air samples collected near the muzzle of foals and thus it was concluded that the exhaled air of foals contained virulent *R. equi*. This indicated that the respiratory tract of foals can generate aerosols of virulent *R. equi*. Comparison of the concentration of airborne virulent *R. equi* in lanes and holding pens with that in

exhaled air from foals with and without pneumonia allowed an assessment of the potential risk of transmission directly from foal-to-foal.

There was a significantly higher concentration of virulent *R. equi* in the exhaled air from foals than in the environmental air. The breath of foals, whether they had clinical signs of *R. equi* pneumonia or not, was a concentrated source of virulent *R. equi*. In general, the concentrations of virulent *R. equi* in exhaled air were much higher than those seen in air from areas found to have relatively high concentrations (Chapter 4).

Of the 55 foals sampled, 37 were exhaling detectable concentrations of virulent *R. equi*. The age of these foals generally ranged from 4 to 16 weeks, when disease predominantly occurs (Section 4.3.1) (Zink *et al.* 1986; Prescott 1991; Horowitz *et al.* 2001). Virulent *R. equi* were detected in exhaled air from 65% of foals with *R. equi* pneumonia. Similarly, Hillidge (1987) recovered *R. equi* from lesions from 7/11 (67%) foals with *R. equi* pneumonia at necropsy and from 57/89 (64%) tracheal lavages. In a more recent study *R. equi* were recovered from 14/21 (67%) transtracheal and 59/96 (61%) nasotracheal lavages from foals with clinical signs of *R. equi* pneumonia (Hashikura *et al.* 2000). Thus, culture of exhaled air samples appears likely to be as sensitive for detection of clinically affected foals as culture of tracheal lavages, which is still seen to be the 'gold standard' for diagnosis of *R. equi* pneumonia.

Three apparently healthy foals in this study were found to have lesions suggestive of *R. equi* pneumonia by ultrasonographic examination. Ultrasonographic examination of lungs has been used widely on horse farms as a diagnostic aid for investigating suspected cases of *R. equi* pneumonia and as a screening tool on properties with a consistently high prevalence of disease. The technique is being used to detect small lung abscesses prior to the onset of clinical disease. The early treatment of these foals may account for the increased survival rates and decreased durations of treatment on farm 3 (Section 4.3.1). However, ultrasonographic examination of lungs has limitations in the diagnosis of *R. equi* pneumonia. Other bacterial causes of pneumonia, for example *Streptococcus equi* subspecies *equi*, may induce similar lesions. Furthermore, only superficial lesions are

detectable. Deep or mediastinal lesions and small lesions that do not involve the pleural surface of the lung may not be detected (Reef 1998).

Clinically normal foals with no obvious ultrasonographically detectable lung abscesses yielded virulent *R. equi* from samples of their exhaled air (6/7 foals). This suggests that these foals either had lung abscesses that were undetectable by ultrasonographic examination, or that these foals were subclinically infected and carrying virulent *R. equi* in their respiratory tract without any obvious lung pathology. As these foals did not develop obvious respiratory illness after sampling, it appears likely that they had subclinical infections with virulent *R. equi*. Subclinical rhodococcal disease has been noted previously. *R. equi* were recovered from transtracheal lavages from 77/216 (36%) clinically normal foals in one study (Ardens *et al.* 1986). This study was performed prior to the distinction between virulent and avirulent *R. equi* and hence did not assess the number of clinically normal foals carrying virulent *R. equi* in their respiratory tract. Subclinical infection may be more common on farms with a high disease prevalence and elevated environmental burdens of virulent *R. equi*. The reasons subclinically infected foals do not develop clinical disease and the possible effects these foals may have as sources of virulent *R. equi* in the herd needs to be explored.

Analysis of faecal samples has been suggested as a potential diagnostic tool (Takai *et al.* 1986a). Most foals between the ages of 4 and 12 weeks, whether affected by *R. equi* or not, have an increased concentration of *R. equi* in their faeces. A dramatic increase in the concentration of virulent *R. equi* has only been demonstrated in foals with overt clinical disease (Takai *et al.* 1994c), and the recovery of *R. equi* from the faeces from foals experimentally infected by the respiratory route has been inconsistent in other studies (Barton and Embury 1987). Among the 9 foals from which faecal samples were taken, the most severely affected foal had 8.1×10^6 virulent *R. equi* per gram of faeces. However, two other foals that had only just been diagnosed with disease had far lower concentrations in their faeces and one recently diagnosed case had no detectable virulent *R. equi* in its faeces. These findings illustrate the difficulty of relying on the recovery of virulent *R. equi* from the faeces of foals for diagnosis, especially in the early stages of clinical disease.

Serological assays currently available for diagnosis of *R. equi* pneumonia have also been found to be unreliable as a sole diagnostic tool (Martens *et al.* 2002b; Giguere *et al.* 2003b). With most assays distinguishing between residual maternal antibody and an active response is a problem, especially when testing foals <2 months of age (Hietala *et al.* 1985). The serological assay used in this study to assess 10 foals is based on a VapA epitope, rather than a whole protein as in the previous assays. Residual maternal antibody may still be a problem with this assay, but antibody to this epitope appears to be induced only during inflammatory processes associated with disease due to *R. equi* (Vanniasinkam *et al.* 2001). The assay detected extremely elevated concentrations of VapA epitope antibody in the one foal that was already suffering from *R. equi* pneumonia, but was unable to detect similar concentrations in the other three recently diagnosed cases. These three foals had antibody titres ≥512, two had hyperfibrinogenaemia (>4g/L) and only one had leucocytosis. One foal with no evidence of lung pathology had the highest concentration of virulent *R. equi* in its exhaled air (60 cfu/1000 l) and also had an antibody titre >512. Two other foals that yielded virulent *R. equi* from exhaled air samples had VapA epitope antibody titres >512, without high faecal burdens of virulent *R. equi*. These findings suggest that both exhaled air sampling and VapA epitope antibody titres may enhance the sensitivity of detection of infected foals above that achievable using haematological and ultrasonographic examination, and faecal culture. Previous studies have found that concentrations of the different classes of IgG directed against the Vap proteins differ between sick and healthy foals. Concentrations of IgGb and IgGT against Vap proteins were higher in the serum of sick foals than in that of healthy, immune foals or adults, both of which had higher concentrations of IgGa against Vap proteins (Hooper-McGrevy *et al.* 2003). The incorporation of assays to detect specific classes of IgG in the VapA epitope ELISA may improve the diagnostic potential of the assay.

The concentrations of airborne virulent *R. equi* detected in the pens and lanes in this study differed from those reported in the early ecological study (Chapter 4), which used NANAT medium rather than mCAZ-NB. The differences between the two selective media were also noted when comparing concentrations of *R. equi* and virulent *R. equi* in soil samples inoculated onto both media in work reported in Chapter 5, and has been noted

when comparing CAZ-NB and NANAT media in other studies (Martens *et al.* 2000). The mCAZ-NB medium was used to detect virulent *R. equi* in the exhaled air of foals after an unsuccessful trial of NANAT medium.

On the basis of the results of this study the significance of aerosol transmission of virulent *R. equi* from foal-to-foal must be considered. The inhalation of the pathogen in dust contaminated with virulent *R. equi* may still be an important source, as high environmental burdens of virulent *R. equi* are correlated with higher disease prevalence (Takai *et al.* 1991c; Takai 1997; Chapter 4 in this thesis). However, foals are often congregated, sometimes in crowded areas such as holding pens, for hours in a day while awaiting veterinary attention and farriery, and even within large paddocks the likelihood of aerosol transmission from foal-to-foal may be relatively high. In one study, foals kept in small groups (≤5 mare-foal pairs) were shown to have a reduced risk of developing *R. equi* pneumonia. However, once year and farm effects were accounted for, the association was not significant (P=0.071) (Chaffin *et al.* 2003c). Even though this finding was not statistically significant, the effect of group size on prevalence warrants further investigation. Management strategies to limit the likelihood of foal-to-foal aerosol transmission, such as decreasing the time foals spend congregating in crowded areas and reducing group sizes, may need to be considered as methods to reduce the prevalence of *R. equi* pneumonia on farms.

Chapter 7

General discussion, conclusions and further work

The studies reported in this thesis have shown that both the airborne concentration of virulent *R. equi* and the proportion of airborne *R. equi* that were virulent were associated with the prevalence of *R. equi* pneumonia on Thoroughbred horse breeding farms. These results confirm the anecdotal and experimental evidence that inhalation of virulent *R. equi* is the main route of pulmonary infection of the foal. Furthermore, these observations support the hypothesis that it is the size of the challenge with aerosolised virulent *R. equi* that influences the likelihood of a pulmonary infection manifesting as clinical pneumonia (Prescott and Yager 1991; Giguere and Prescott 1997). No association was observed between the concentration of virulent *R. equi* or the proportion of *R. equi* that were virulent in soil samples and the prevalence of *R. equi* pneumonia. This is in contrast to previous studies (Takai *et al.* 1991c; Takai 1997) that found an association between soil contamination and disease. These findings suggest that it is the capacity for aerosolisation of virulent *R. equi* that is the most critical aspect of the epidemiology of the disease.

This study identified multiple environmental and management factors that influenced the concentration of virulent *R. equi* on farms. Warm ambient temperature and low pasture height were associated with elevated airborne concentrations, as was dry soil. Dry soil was also associated with elevated proportions of airborne *R. equi* that were virulent. This supports the conclusions made by previous investigators that watering areas to increase soil moisture and maintaining good levels of pasture cover on paddocks would be useful management practices to reduce the level of exposure to aerosolised virulent *R. equi* (Prescott and Yager 1991; Giguere and Prescott 1997). There were very few associations between environmental factors and the concentration of virulent *R. equi* in soil. Acidic soil was the one environmental factor that was weakly associated with increased proportions of *R. equi* that were virulent in soil. While this association was not

found to be statistically significant in this study, it may be worthy of further, more focussed investigation, as laboratory studies have shown that a variety of *R. equi* virulence genes are upregulated under acidic conditions (Benoit *et al.* 2001; Ren and Prescott 2003).

The lanes and pens sampled in this study were areas of low soil moisture and poor grass cover and were associated with high concentrations of airborne virulent *R. equi*. Consequently, greater time spent in these areas would be expected to increase the exposure of foals to higher levels of airborne virulent *R. equi,* which in turn is likely to increase the relative risk of foals contracting *R. equi* pneumonia. The high airborne concentrations of virulent *R. equi* in the lanes and pens make these areas dangerous places to allow foals to congregate for any extended period of time. Such areas could be considered potential infection 'hot spots'.

A striking aspect of the study was the relatively low concentration and frequency of detection of virulent *R. equi* in environmental samples. More than half of the air and soil samples taken were negative. This may be a reflection of the relatively small size of the soil sample and the use of selective media, a technique that may have contributed to lower recovery of the organism. During the course of this study the use of a modified version of the previously described CAZ-NB medium (von Graevenitz and Punter-Streit 1995) was found to be a better alternative to NANAT medium for the recovery of virulent *R. equi* (Woolcock *et al.* 1979), but this was only discovered after NANAT medium had been used for the ecological study. The mCAZ-NB medium was used to detect virulent *R. equi* in the exhaled air of foals. However, some variation between different virulent strains in their ability to grow on the different selective media suggests that there is a need for further development of selective media for *R. equi*.

Detection of virulent *R. equi* in the exhaled air of foals suggests that there is the possibility, which has previously been dismissed, for *R. equi* pneumonia to have a contagious epidemiology (Martens *et al.* 1982; Hillidge 1987; Barton and Embury 1987; Hondalus 1997). The detection of virulent *R. equi* in the exhaled air of infected foals and apparently healthy foals, at concentrations significantly greater than those observed in environmental air samples, suggests that aerosol transmission between foals can occur.

Foal-to-foal transmission may allow the spread of disease to continue in a foal population despite the implementation of management strategies (such as irrigation) or changes in environmental conditions (such as reduction in temperature and increased rain) that result in a reduced concentration of airborne virulent *R. equi*. This may go some way to explaining the variability of success in some environmental management strategies.

The low concentrations of virulent *R. equi* detected in air cannot be compared with any other previous study on *R. equi*. The only previous study of airborne *R. equi* (Takai *et al.* 1994a) did not quantitate concentrations of virulent *R. equi*. Based on the concentrations of airborne virulent *R. equi* detected in the studies reported in this thesis a foal would need to inhale somewhere between 10,000 and 1,000,000 l of air to be exposed to the minimal infectious dose (~10^3 cfu/foal) required to cause disease in experimental studies (Wada *et al.* 1997). The likelihood of a foal being exposed to such a dose in a single event is low. Studies have shown that the mean inspiratory flow rate (l/min) at rest of a foal between the ages of 2 days and 3 months ranges from approximately 60 to 80 l/min (Koterba *et al.* 1995). Therefore, in a paddock with an airborne virulent *R. equi* concentration of 1 cfu/1000 l the time required for a foal to inhale an infectious dose would be approximately 8-12 days. If the foal is confined to areas where the air is heavily contaminated with virulent *R. equi* and also in close proximity to other foals, in crowded holding facilities, a much shorter period of exposure would be necessary. For example, in the most heavily contaminated holding pen or lane observed in this study (72 virulent *R. equi* cfu /1000 l), the time required for a foal to inhale an infectious dose would be 3-4 hours.

The use of exhaled air sampling to detect virulent *R. equi* in clinically affected foals appeared likely to compare favourably with the more invasive transtracheal or nasotracheal lavage methods currently used to diagnose *R. equi* pneumonia. The detection of subclinically infected foals was an interesting finding and needs to be explored further in terms of its epidemiological significance and to investigate whether there is a threshold level of excretion between clinically affected and subclinically infected foals. The use of this non-invasive technique to detect virulent *R. equi* in the airways of foals, combined with improved serological assays and the use of ultrasonographic examination of lungs,

may improve early diagnosis of *R. equi* pneumonia. On one farm included in these studies the routine ultrasonographic examination of the lungs of foals was considered to be associated with reduced duration of treatment, due to early diagnosis, with the proportion of cases requiring more than 20 days treatment to effect a cure only 2/81 (2.5%) approximately 6.5 times lower (29/177 - 16.4%) than the proportion of cases requiring more than 20 days therapy on farms that did not routine examine the lungs of foals by ultrasonography. It may also be possible to use exhaled air sampling of foals to monitor the concentration of virulent *R. equi* in the respiratory tract of foals during antimicrobial therapy for *R. equi* pneumonia. This, combined with ultrasonographic monitoring during the course of therapy, may enable development of more efficacious therapeutic regimens and reduce the risk of recurrence.

Studies of the association between environmental conditions, the prevalence of disease due to *R. equi,* and the concentration of airborne virulent *R. equi* and the proportion of airborne *R. equi* that were virulent need to be expanded to address the issue of the age dependent susceptibility. The studies reported here confirm previous observations that the majority of cases of *R. equi* pneumonia are seen in foals between 1 and 3 months (Zink *et al.* 1986; Prescott 1991; Hashikura *et al.* 2000). Foals between one and two months were the age group in which *R. equi* pneumonia was most frequently diagnosed. High concentrations of airborne virulent *R. equi* occurred at particular times during the foaling season and this appeared to coincide with a higher number of new cases if the numbers of foals <4 months of age were also high. The concentration of airborne virulent *R. equi* was greatest later in the season, between November and February, when the majority of cases were seen. Few cases were diagnosed late in January or February, even though airborne concentrations of virulent *R. equi* were also high in these months. In the 2001 season in Victoria peaks in the concentration of airborne virulent *R. equi* and the proportion of airborne *R. equi* that were virulent were observed in January and February. Due to the restricted duration of the Thoroughbred breeding season the age of the majority of foals in a herd in January and February will be greater than 3 months of age and such foals seem to be less susceptible to development of *R. equi* pneumonia. The infrequency of *R. equi* pneumonia on Standardbred farms may be a reflection of the expanded duration of the breeding season compared to Thoroughbreds. The peak in the number of foals between 1

and 3 months of age will therefore be lower on a Standardbred farm than on a Thoroughbred farm of comparable size, possibly explaining the reduced disease risk in Standardbred foals. The lower prevalence of disease in the 2001 season appeared to be associated with a milder and wetter November and December compared to the previous season. These climatic conditions resulted in a reduction in the airborne virulent *R. equi* burden and the incidence of *R. equi* pneumonia did not increase as dramatically as in the corresponding period in the previous season, when conditions were drier and warmer and concentrations of airborne virulent *R. equi* were higher.

Management strategies that reduce the likelihood of susceptible foals encountering highly contaminated air appear to be important in controlling disease prevalence. On two of the Victorian farms in this study the disease prevalence altered between the two study seasons and this was associated with environmental and management changes that altered the airborne concentrations of *R. equi* on these farms. Variations in disease prevalence and mortality rates between seasons were also associated with environmental changes that appeared to influence the airborne *R. equi* population. This suggests that management strategies targeting environmental factors that influence the concentrations of airborne virulent *R. equi* may effectively reduce the prevalence and severity of *R. equi* pneumonia on farms. Despite these observations, a closer examination of management strategies that may affect the environmental burden of *R. equi*, and thus the likelihood of exposure to high concentrations of airborne virulent *R. equi* (i.e. irrigation, harrowing, liming and management of feeding areas), did not find any significant direct association with disease prevalence. The relatively small number of farms in this study and the limited quantitative analysis of the frequency and extent of implementation of such management strategies may explain the failure to detect an association. The association between smaller foal populations, lower disease prevalence and reduced risk of mortality, despite, in some cases, the presence of high airborne virulent *R. equi* environmental burdens on these farms, may implicate other factors, such as a reduced risk of contagious transmission, or a reduced prevalence of highly susceptible foals, associated with reduced prevalence and severity of disease on smaller farms. The prevalence of mortalities associated with *R. equi* pneumonia was not significantly associated with the concentrations of airborne virulent *R. equi* on farms, even though a high prevalence of disease and of mortalities were associated with

each other. This suggests that foal related factors may play an important role in the ultimate outcome of infection, including the ability of the foal to respond to exposure and to respond to treatment, as suggested by the observation that the majority of mortalities occurred within the first 10 days of antimicrobial therapy. On farms with a history of high prevalence of *R. equi* pneumonia the staff vigilance would be high, so diagnoses of cases may be expected to be made earlier and therapy initiated promptly. Such increased vigilance may have contributed to the lower overall case fatality rate seen on NSW farms (9/202 - 4.5%) compared to Victorian farms (6/94 - 6.4%), where fewer farms experienced a high disease prevalence. It appears that factors other than the concentration of the soil-derived virulent *R. equi* in the air might influence disease prevalence and severity.

The lack of significant associations between management and environmental factors and the concentrations of virulent *R. equi* or proportion of *R. equi* that were virulent in the soil prevents any conclusions being drawn about soil factors that selectively influence the proliferation or survival of virulent *R. equi*. It has been shown in *Legionella pneumophilia* that the genes responsible for virulence are also required for the bacteria to survive and replicate in their protozoan hosts (Segal and Shuman 1999; Solomon *et al.* 2000). It may be possible that genes on the virulence plasmid in virulent *R. equi* allow them to live within protozoa, at least in the soil, and therefore it might be expected that climatic conditions resulting in high soil moisture and high concentrations of protozoans in soil (Foissner 1987) might result in higher concentrations of virulent *R. equi*. This was not seen, but high concentrations of total *R. equi* in soil were associated with high soil moisture, in contrast with observations by Barton and Hughes (1987). This may imply a role for protozoa in the proliferation of *R. equi* in soil or it may be that soil moisture directly enhances the proliferation of the avirulent organism. Therefore, while the results of these studies give no indication that protozoa might be important in the survival or replication of virulent *R. equi* in soil, *in vitro* experiments comparing growth curves of the avirulent and virulent strains in different soils enriched with different protozoa, including those from the foal's intestine, might enable a better evaluation of the significance of protozoa in the life cycle of virulent *R. equi.*

The direct association between soil moisture and the concentration of *R. equi* in soil and the inverse relationship between soil moisture and the concentration of virulent *R. equi* and the proportion of *R. equi* that were virulent in the air suggest significant differences in the ecology of virulent *R. equi* in the soil. As the amount of available water in soil increases with soil depth (McLaren and Cameron 1996b), total *R. equi* concentrations may increase with soil depth, while concentrations of virulent *R. equi* may not. However, past studies indicate that at a depth of greater than 30 cm the concentration of soil *R. equi* is generally <10 cfu/g (Takai *et al.* 1986b). Therefore, there maybe a critical soil depth at which proliferation of soil *R. equi* is not feasible. A plausible hypothesis is that virulent *R. equi* grow better on the soil surface and so are more easily aerosolised. It may be that virulent *R. equi* compete better in the most superficial soil layers. The genes on the virulence plasmid associated with virulence are upregulated under conditions of micronutrient restriction and lower pH (Benoit *et al.* 2001; Ren and Prescott 2003). These harsh conditions are most likely to occur in superficial soil layers and in sandy soils, where less water is retained and nutrient concentrations are likely to be lower. The most superficial soil layers will also be where there are higher concentrations of equine faecal matter and as this is the most likely source of contaminating virulent *R. equi*, it may be that the survivability and replication in soil of virulent stains away from equine faecal material, in the deeper soil layers, is reduced in comparison to avirulent starins. Nevertheless, superficial soils of dry and sandy areas are more easily disturbed and hence generate a greater volume of dust. As airborne concentrations of virulent *R. equi*, rather than airborne concentrations of total *R. equi*, were associated with these factors, it is reasonable to hypothesise that virulent *R. equi* may have an ecological niche in the superficial soil layers. *In vitro* experiments comparing the growth rates of avirulent and virulent *R. equi* in soils with or without faecal contamination and at specific soil depths may be useful in identifying factors important to the survivability and multiplication of virulent *R. equi* in soil.

Genes responsible for conjugation have been identified on the virulence plasmids (Takai *et al.* 2000), but transformation of an avirulent strain to a virulent one by acquisition of the plasmid has not been documented. As many of the virulence genes are upregulated under acidic conditions, it is plausible to hypothesise that acidic conditions may enhance

the replication of, and transformation of *R. equi* by, the plasmid in the soil. Therefore, the association between increased proportions of soil *R. equi* that were virulent and acidic soils may be a result of selective pressures allowing the transformation of *R. equi* by the plasmid. *In vitro* experiments comparing plasmid concentrations in *R. equi* cultures at differing pH and with micronutrient restrictions would enable this to be further investigated.

Future work on the epidemiology of *R. equi* pneumonia needs to focus on reducing the prevalence and severity of *R. equi* pneumonia on farms with a high prevalence of disease. The potential of specific management changes to reduce the environmental burden of virulent *R. equi* and foal-to-foal transmission needs to be explored. The use of continual monitoring of soil moisture levels to guide irrigation, especially in environmental 'hot spots' such as holding pens and lanes, the control of group sizes in paddocks and of the time foals spend in crowded areas awaiting veterinary attention and farriery, the monitoring and alkalinisation of soil in 'hot spots', and the maintenance of good pasture cover are all strategies that promise to reduce the likelihood of foals inhaling higher concentrations of virulent *R. equi*. Methods for detection of susceptible foals on farms, by examining titres of antibody specific for virulent *R. equi* in the colostrum of mares and the serum of foals (Fontanals *et al.* 1997) and examining specific T cell subset ratios in foals (Chaffin *et al.* 2004), may also be useful in identifying 'at risk' foals. However, these strategies need to be implemented, assessed and compared carefully on farms with a high prevalence of disease before a global recommendations can be made to farm managers about the best management strategies to reduce the prevalence and severity of *R. equi* pneumonia.

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Appendices

Appendix 1

Horse farm questionnaire for the 2000 foaling season.

Q1. Please supply a property map, indicating the approximate size of paddocks and indicate paddocks that house mares and foals.

Q2. Are mares and foals rotated from paddock to paddock? If so, describe the timing and frequency of rotation?

Q3. Are specific paddocks designated for foals within certain age groups or foals undergoing medical treatment? Please indicate these paddocks on the property map and their purpose on the property map.

Q4. Are feeding bins rotated within a paddock? If so, indicate which paddocks and how frequently this is performed.

Q5. Describe the feed type given to mares and foals?

Q6. Are weaner / creep feeds used? If so, describe the feed used, duration of feeding and any tapering measures used to wean onto adult feed.

Q7. Describe the vaccination protocol used on foals? Please indicate timing of vaccination and vaccine(s) used.

Q8. For mares arriving on the property, are their vaccination histories known? If so, what are they vaccinated against? If not, are they vaccinated on arrival and against what are they vaccinated?

Q9. Describe the vaccination protocol used on mares? Please indicate timing of vaccination and vaccine(s) used. Indicated if any specific pre- or post-parturition vaccinations are given.

Q10. Describe the worming protocol used on foals? Please indicate timing of worming and wormer(s) used.

Q11. For mares arriving on the property, are their worming histories known? If so, what wormer(s) was used? If not, are they wormed on arrival and what wormer(s) are used?

Q12. Describe the worming protocol used on foals? Please indicate timing of worming and wormer(s) used. Indicate if any specific pre- or post-parturition worming is performed.

Q13. Are prophylactic antibiotics administered to foals as neonates? If so, describe the antibiotic used and the timing and duration of administration.

Q14. Are supplemental nutrients supplied to foals, apart from that which is already given in normal or weaner feed? If so, describe type, quantity and duration of nutritional supplementation.

Q15. Are paddocks which house mares and foals cleaned of horse dung? If so, how frequently? Specify frequency and protocol differences, if they exist, between different paddocks.

Q16. Are paddocks which house mares and foals irrigated or watered? If so, how frequently? Specify frequency and protocol differences, if they exist, between different paddocks.

Q17. Are chemical additives used on paddocks that house mares and foals? If so, when is this performed? Describe the chemicals and quantity used. Specify differences in chemicals used and timing of application, if they exist, between different paddocks.

Q18. Indicate the number of foals on the farm in the following time period:

Start of foaling to first sampling date (September 2000)

Start of foaling to second sampling date (October 2000)

Start of foaling to third sampling date (November 2000)

Start of foaling to fourth sampling date (December 2000)

Start of foaling to fifth sampling date (January 2001)

Start of foaling to sixth sampling date (February 2001)

Q19. How many cases of *Rhodococcus equi* pneumonia has the farm experienced this season? Please indicate the age of the foal when the disease was first noted, whether the foal survived or died as a consequence of the disease, and how the diagnosis of *Rhodococcus equi* was made in each case.

Questionnaire modification for the 2001 foaling season

Q13. Are prophylactic antibiotics, probiotics or hyperimmune serum administered to foals as neonates? If so, describe the antibiotic, probiotic or hyperimmune serum used and the timing and duration of administration.

Q18. (Victoria) Indicate the number of foals on the farm in the following time periods:

Start of foaling to first of September 2001

Start of foaling to First of October 2001

Start of foaling to first sampling date (Middle of October 2001)

Start of foaling to second sampling date (End of October 2001)

Start of foaling to third sampling date (Middle of November 2001)

Start of foaling to fourth sampling date (End of November 2001)

Start of foaling to fifth sampling date (Middle of December 2001)

Start of foaling to sixth sampling date (Middle of January 2002)

Start of foaling to seventh sampling date (End of January 2002)

Q18. (New South Wales) Indicate the number of foals on the stud in the following time period:

Start of foaling to first of September 2001

Start of foaling to first of October 2001

Start of foaling to first of November 2001

Start of foaling to first sampling date (End of November 2001)

Start of foaling to second sampling date (Middle of December 2001)

Start of foaling to 15th of January 2002

Start of foaling to 29th of January 2002

Q19. How many cases of *Rhodococcus equi* pneumonia has the farm experienced this season? Please indicate the age of the foal when the disease was first noted, the date on which treatment (antibiotics) commenced, duration of treatment, whether the foal survived or died as a consequence of the disease and how the diagnosis of *Rhodococcus equi* was made in each case.

Q20. Indicate the date of first and last foaling for the season?

Q21. Please indicate the incidence or numbers of foals with following signs/diseases this season:

Respiratory Disease (other than *Rhodococcus equi* pneumonia)

Diarrhoea

Conformational problems

Traumatic injuries

Appendix 2: Soil conditions on Thoroughbred farms in the 2000 and 2001 seasons

State	Farm	Year	Location	Predominant soil texture	Soil moisture (%H_2O)		Soil pH	
					Median	Range	Median	Range
Vic	A	2000	Paddock	Clay	15.1	1.9-22.0	5.9	5.2-7.0
		2001	Paddock	Clay	12.2	1.8-20.0	5.8	4.9-6.5
			Pen/lane	Sand	4.1	0.8-9.0	6.7	6.1-7.8
			All		8.5	0.8-20.0	6.1	4.9-7.8
	B	2000	Paddock	Clay	15.0	1.4 -27.3	5.3	4.4-6.3
		2001	Paddock	Clay	9.3	2.1-24.0	5.0	4.3-6.0
			Pen/lane	Clay (Gravel)	11.2	2.1-24.6	6.4	6.0-7.3
			All		9.6	2.1-24.6	5.3	4.3-7.3
	C	2000	Paddock	Clay	8.4	2.1-24.3	6.6	5.8-7.5
		2001	Paddock	Clay	10.6	2.0-21.5	6.2	4.9-6.9
			Pen/lane	Sand	9.5	2.3-19.8	6.3	5.5-7.1
			All		9.6	2.0 -21. 5	6.2	4.9-7.1
	D	2000	Paddock	Clay	9.7	4.5-16.5	6.2	5.5-7.1
		2001	Paddock	Clay	15.4	3.5-30.5	5.7	4.8-7.0
			Pen/lane	Sand	9.8	1.8-15.8	6.5	5.2-7.1
			All		13.1	1.8- 30.5	5.9	4.8-7.1
	E	2000	Paddock	Clay/Sand	13.3	2.1-28.6	6.4	5.5-7.3
		2001	Paddock	Clay/Sand	12.5	2.4-24.8	5.8	4.5-7.0
			Pen/lane	Sand	4.5	0.8-9.5	6.4	5.8-7.1
			All		6.6	0.8-24.8	5.9	4.5-7.1
	F	2000	Paddock	Sand	8.7	1.7-18.1	6.5	5.4-7.3
		2001	Paddock	Sand	10.6	2.6-20.2	6.2	4.9-7.2
			Pen/lane	Sand	4.5	1.0-21.0	6.4	5.1-7.3
			All		9.7	1.0-21.0	6.2	4.9-7.3
	G	2001	Paddock	Sand	12.0	1.6-20.4	5.7	4.5-6.8
			Pen/lane	Sand	6.8	1.3-17.7	5.4	4.3-6.7
			All		8.5	1.3-24.0	5.5	4.3-6.8
	H	2001	Paddock	Clay	21.1	5.2-31.5	5.1	4.6-6.2
			Pen/lane	Sand	4.7	1.8-18.5	5.4	4.3-6.7
			All		15.4	1.8-31.5	5.3	4.3-6.7
	I	2001	Paddock	Clay	24.7	7.0-31.5	5.2	4.4-7.0
			Pen/lane	Sand/Clay	13.6	3.8-24.4	5.6	4.8-6.8
			All		18.6	3.8-31.5	5.2	4.4-7.0
	J	2001	Paddock	Clay	12.5	1.9-19.3	6.3	5.4-6.9
			Pen/lane	Sand (Gravel)	3.2	1.1-8.6	7.0	5.8-8.2
			All		8.1	1.1-19.3	6.4	5.4-8.2
NSW	1	2001	Paddock	Sand	10.3	5.6-22.3	6.3	5.5-6.5
			Pen/lane	Sand	8.3	6.6-9.8	6.1	5.2-6.3
			All		9.4	5.6-22.3	6.2	5.2-6.5
	2	2001	Paddock	Clay	10.9	5.3-17.8	5.7	5.4-6.2
			Pen/lane	Sand/Clay	5.4	2.0-9.3	6.3	5.9-6.9
			All		10.1	2.0-17.8	5.9	5.4-6.9
	3	2001	Paddock	Clay	21.9	17.0-28.0	6.6	6.1-7.4
			Pen/lane	Sand	9.5	6.2-11.2	7.7	7.0-8.5
			All		18.9	6.2-28.0	6.9	6.1-8.5
	4	2001	Paddock	Clay	23.6	15.9-40.1	6.0	5.5-6.8
			Pen/lane	Sand (Gravel)	3.1	1.2-6.6	6.7	5.7-8.0
			All		21.2	1.2-40.1	6.0	5.5-8.0
	5	2001	Paddock	Sand	9.6	5.3-15.9	6.1	5.6-7.3
			Pen/lane	Sand	5.1	2.6-6.8	6.6	5.8-7.7
			All		6.9	2.6-15.9	6.3	5.6-7.7
	6	2001	Paddock	Sand/Clay	10.4	3.6 -16.1	5.4	5.0-6.3
			Pen/lane	Sand	5.7	1.6-11.3	7.2	7.0-7.7
			All		7.9	1.6-16.1	5.5	5.0-7.7
	7	2001	Paddock	Sand/Clay	13.7	5.8-21.8	6.5	6.1-6.7
			Pen/lane	Sand	6.4	2.5-10.6	6.7	6.0-6.9
			All		10.7	2.5-21.8	6.6	6.0-6.9
	8	2001	Paddock	Clay	22.7	13.3-29.8	7.3	6.7-8.0
			Pen/lane	Sand	7.4	1.8-15.4	7.6	6.9-8.9
			All		20.0	1.8-29.8	7.5	6.7-8.9
	9	2001	Paddock	Clay	22.2	15.6-29.8	6.1	5.4-7.1
			Pen/lane	Sand/Clay	8.7	2.3-14.6	6.7	6.1-7.6
			All		17.2	2.3-29.8	6.3	5.4-7.6

10	2001	Paddock	Clay	19.3	11.2-24.2	6.6	5.8-7.1
		Pen/lane	Sand/Clay	6.9	3.0-13.1	7.6	7.1-8.2
		All		15.8	3.0-24.2	7.1	5.8-8.2
11	2001	Paddock	Sand/Clay	13.1	9.8-18.3	5.3	4.9-5.8
		Pen/lane	Sand	3.1	2.6-3.6	7.4	6.3-7.9
		All		10.8	2.6-18.3	5.6	4.9-7.9
12	2001	Paddock	Clay	17.4	14.7-20.8	5.8	5.3-6.3
		Pen/lane	Sand/Clay	11.8	3.3-20.6	6.2	5.7-6.7
		All		17.4	3.3-20.8	5.9	5.3-6.7

Appendix 3: Environmental conditions and pasture height on paddocks on Thoroughbred farms in the 2000 and 2001 seasons

State	Farm	Year	Pasture height (cm)		Humidity (%)		Wind speed (km/h)		Temperature (ºC)	
			Median	Range	Median	Range	Median	Range	Median	Range
Vic	A	2000	5.1	1.7-11.8	79.2	69.9-86.0	9.0	4.0 –19.0	18.0	10.2-26.9
		2001	11.7	1.6-26.1	71.9	66.5-79.4	15.0	12.0-25.5	18.2	13.9-27.0
	B	2000	3.4	1.6-12.8	66.7	55.7-81.4	19.0	14.0-28.0	27.0	16.7-35.7
		2001	7.9	2.3-21.5	75.8	65.6-85.7	17.5	4.3-21.0	17.4	10.9-30.9
	C	2000	2.6	0.0-9.2	68.3	60.4-77.5	24.0	12.0-39.0	20.2	14.2-34.8
		2001	4.6	2.1-9.2	77.7	67.2-84.3	17.3	2.7-35.7	15.4	11.1-20.3
	D	2000	9.3	2.5-18.1	70.4	60.4-73.1	26.0	13.0-43.0	17.5	13.0-29.1
		2001	6.2	3.0-15.0	76.5	70.7-84.9	10.0	5.0-26.5	16.2	12.3-18.1
	E	2000	9.6	2.5-18.1	79.2	64.5-96.1	13.0	4.0-17.0	18.5	6.8-28.2
		2001	10.5	3.1-22.1	74.9	50.2-97.5	16.3	9.5-23.3	18.1	11.6-28.3
	F	2000	11.8	6.0-18.2	63.3	61.1-76.3	15.0	5.0-30.0	22.8	16.2-28.6
		2001	10.9	4.9-22.3	69.4	59.5-83.7	20.0	8.0-32.3	20.7	13.0-22.4
	G	2001	17.7	4.9-29.9	82.4	67.7-94.9	18.5	9.0-26.7	15.9	11.4-21.9
	H	2001	16.1	6.5-26.7	87.0	77.8-94.9	25.0	8.3-29.8	11.9	6.8-18.0
	I	2001	17.7	5.5-26.2	87.3	65.1-98.2	23.5	11.0-28.8	10.3	7.6-20.8
	J	2001	10.6	3.6-18.7	75.2	66.5-97.9	16.7	9.8-28.8	18.5	12.6-25.8
NSW	1	2001	6.0	1.3-9.9	79.1	76.4-81.7	27.0	26.0-28.0	18.7	16.5-20.8
	2	2001	6.5	2.5-15.7	59.6	44.8-74.4	17.8	11.0-24.5	23.3	17.6-29.0
	3	2001	4.9	2.8-9.2	73.1	66.1-80.0	29.9	26.7-33.0	18.8	14.9-22.7
	4	2001	9.7	5.6-18.1	71.6	62.4-80.7	21.5	19.0-24.0	23.3	14.3-32.2
	5	2001	5.6	3.1-6.6	71.0	68.4-73.5	18.8	11.0-26.7	23.4	18.2-28.5
	6	2001	5.0	2.8-15.4	69.2	68.5-71.8	24.5	21.0-28.0	17.6	11.8-23.4
	7	2001	9.0	2.7-16.2	71.9	61.0 -82.8	19.8	17.7-22.0	20.1	11.0-29.1
	8	2001	12.3	6.2-19.6	68.9	56.1-81.6	20.5	11.0-30.0	20.3	13.7-26.9
	9	2001	10.8	7.9-18.8	67.8	59.2-75.9	10.3	10.3-10.3	18.7	14.6-22.8
	10	2001	8.9	4.1-14.2	79.4	70.4-88.4	10.0	7.0-13.0	18.5	18.1-18.9
	11	2001	5.2	2.4-8.0	67.8	67.8-67.8	13.0	13.0-13.0	21.8	21.8-21.8
	12	2001	5.0	2.3-14.0	71.4	55.6-87.1	6.8	6.8-6.8	21.2	14.5-27.9

Appendix 4: Maximal foal numbers in different age groups on 6 Thoroughbred farms in Victoria during the 2000 season

Farm	Age group (weeks)	September	October	November	December	January	February
A	<10	30	50	62	47	5	0
	<16	30	50	92	67	47	5
B	<10	9	32	46	28	5	0
	<16	9	32	55	51	28	5
C	<10	53	92	87	52	4	0
	<16	53	92	140	87	52	4
D	<10	23	54	76	50	5	0
	<16	23	54	99	76	50	5
E	<10	39	103	132	71	3	0
	<16	39	103	171	135	71	3
F	<10	79	130	129	80	2	0
	<16	79	130	208	131	80	2

Appendix 5: Maximal foal numbers in different age groups on 10 Thoroughbred farms in Victoria during the 2001 season

Farm	Age group (weeks)	October[1]	October[2]	November[1]	November[2]	December[1]	January[1]	January[2]
A	4-8	25	25	19	40	26	3	0
	4-12	37	37	34	65	46	29	9
	8-12	12	12	25	25	20	26	9
	<16	57	78	83	86	74	49	49
B	4-8	15	15	6	14	8	6	0
	4-12	24	24	21	29	14	14	6
	8-12	9	9	15	15	6	8	6
	<16	30	38	38	44	35	20	20
C	4-8	42	42	22	47	43	9	1
	4-12	63	63	85	89	65	52	27
	8-12	21	21	63	42	22	43	26
	<16	85	110	128	136	137	74	74
D	4-8	28	28	12	27	30	12	3
	4-12	40	40	40	55	42	42	27
	8-12	12	12	28	28	12	30	24
	<16	52	67	82	91	82	54	54
E	4-8	69	69	36	71	43	20	0
	4-12	110	110	105	140	89	63	20
	8-12	41	41	69	69	46	43	20
	<16	146	181	199	219	178	109	109
F	4-8	66	66	34	72	78	18	2
	4-12	108	108	100	138	112	96	56
	8-12	42	42	66	66	34	78	54
	<16	142	180	220	236	196	130	130
G	4-8	19	14	9	15	7	1	0
	4-12	28	25	23	29	16	8	2
	8-12	9	11	14	14	9	7	2
	<16	37	40	41	42	31	17	17
H	4-8	10	20	20	60	60	30	0
	4-12	40	50	30	70	80	90	50
	8-12	30	30	10	10	20	60	50
	<16	60	100	120	150	120	110	110
I	4-8	37	37	24	42	33	0	0
	4-12	45	45	61	79	57	33	15
	8-12	8	8	37	37	24	33	15
	<16	69	87	102	102	94	57	57
J	4-8	16	16	7	16	15	2	0
	4-12	19	19	23	32	22	17	8
	8-12	3	3	16	16	7	15	8
	<16	26	35	41	43	40	24	24

[1]middle of the month
[2]end of the month
Approximate time between dates within the same month was 2 weeks

Appendix 6: Maximal foal numbers in different age groups on 12 Thoroughbred farms in NSW during the 2001 season

Farm	Age group (weeks)	September[1]	October[1]	November[1]	December[1]	December[2]	January[2]	February[2]
1	4-8	0	30	32	36	20	2	0
	4-12	0	30	62	68	56	22	2
	8-12	0	0	30	32	36	20	2
	<16	30	62	98	118	90	58	22
2	4-8	0	32	88	66	47	0	0
	4-12	0	32	120	154	113	47	0
	8-12	0	0	32	88	66	47	0
	<16	32	120	186	233	201	113	47
3	4-8	0	118	163	140	55	12	0
	4-12	0	118	281	303	195	67	12
	8-12	0	0	118	163	140	55	12
	<16	118	281	421	476	370	207	67
4	4-8	0	88	112	123	34	19	1
	4-12	0	88	200	235	157	53	20
	8-12	0	0	88	112	123	34	19
	<16	88	200	323	357	288	177	54
5	4-8	0	40	45	57	29	5	1
	4-12	0	40	85	102	86	34	6
	8-12	0	0	40	45	57	29	5
	<16	40	85	142	171	136	92	35
6	4-8	0	47	56	76	27	0	0
	4-12	0	47	103	132	103	27	0
	8-12	0	42	47	56	76	27	0
	<16	47	103	179	206	159	103	27
7	4-8	0	29	11	13	5	3	0
	4-12	0	29	40	24	18	8	3
	8-12	0	0	29	11	13	5	3
	<16	29	40	53	58	32	21	8
8	4-8	0	40	101	71	20	0	0
	4-12	0	40	141	172	91	20	0
	8-12	0	0	40	101	71	20	0
	<16	40	141	212	232	192	91	20
9	4-8	0	11	19	13	4	0	0
	4-12	0	11	30	32	17	4	0
	8-12	0	0	11	19	13	4	0
	<16	11	30	43	47	36	17	4
10	4-8	0	43	62	49	19	1	0
	4-12	0	43	105	111	68	20	1
	8-12	0	0	43	62	49	19	1
	<16	43	105	154	173	131	69	20
11	4-8	-	-	-	-	-	-	-
	4-12	-	-	-	-	-	-	-
	8-12	-	-	-	-	-	-	-
	<16	-	-	-	-	-	-	-
12	4-8	0	7	20	16	10	1	0
	4-12	0	7	27	38	26	11	1
	8-12	0	0	7	20	16	10	1
	<16	7	27	43	53	47	27	11

[1]start of the month
[2]middle of the month
Approximate time between dates within the same month was 2 weeks. Time between January and February was approximately 2 weeks
Farm 11 did not provide sufficient information about the ages of foals

Appendix 7: Preparation of reagents

NANAT medium (Woolcock *et al.* 1979)

Tryptone soya broth (Oxoid, Basingstoke, UK)	30 g
Yeast extract (Oxoid)	1 g
Agar (Oxoid)	15 g

Dissolve in distilled H_2O to make 1 litre. Sterilise at 121^{o}C for 20 minutes and when cool add

Nalidixic acid	20 μg/ml
Novobiocin	25 μg/ml
Cycloheximide	40 μg/ml

Potassium tellurite to 0.005%

CAZ-NB medium (von Graevenitz and Punter-Streit 1995)

Mueller-Hinton agar (Oxoid)	38 g

Dissolve in distilled H_2O to make 1 litre. Sterilise at 121°C for 20 minutes and when cool add

Novobiocin	25 μg/ml
Ceftazidime	20 μg/ml

mCAZ-NB medium

As for CAZ-NB medium, but add cycloheximide (40 μg/ml).

Trypticase soy agar (TSA)

TSA base (Difco, Sparks, MD, US) 40 g

Dissolve in distilled H_2O to make 1 litre. Sterilise at 121^oC for 20 minutes

Phosphate buffered saline (PBS)

For 1 litre of 10 x solution:

80 g NaCl

2 g KCl

11.5 g Na_2HPO_4

2 g KH_2PO_4

Dissolve in distilled H_2O to make 1 litre. Sterilise at 121°C for 20 minutes

Phosphate buffered saline containing Tween 20 (PBST)

Dilute 10 x PBS to 1 x solution and add 0.5 µl Tween 20 per ml PBS.

Luria-Bertani broth (LB broth)

Tryptone (Oxoid)	10 g
Yeast extract (Oxoid)	5 g
NaCl	5 g

Dissolve in distilled H_2O to make 1 litre. Sterilise at 121°C for 20 minutes

Tryptic soy broth

Tryptic soy broth (Acumedia, Baltimore, MD, US) 30 g

Dissolve in distilled H_2O to make 1 litre. Sterilise at 121°C for 20 minutes

"Now this is not the end. It is not even the beginning of the end. But it is, perhaps, the end of the beginning".

Sir Winston Churchill, *Speech in November 1942*
British politician (1874 - 1965)