STUDIES ON THE PATHOGENESIS AND DIAGNOSIS OF INFECTION WITH THE EQUINE LUNGWORM *Dictyocaulus arnfieldi*

by

ELIZABETH M. HALYBURTON, B.V.M.S., M.R.C.V.S.

A thesis submitted for the degree of Doctor of Philosophy
in the Faculty of Veterinary Medicine, University of Glasgow

Department of Veterinary Parasitology,
University of Glasgow.
March, 1991.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

TABLE OF CONTENTS

	Page no.
ACKNOWLEDGEMENTS	i
LIST OF TABLES	ii
LIST OF FIGURES	iv
SUMMARY	viii
GENERAL INTRODUCTION	1
MATERIALS AND METHODS	8
PARASITOLOGICAL TECHNIQUES	9
Faecal Sampling	9
Faecal Examination	9
Worm Recovery at Necropsy	10
Culture and Administration of *Dictyocaulus arnfieldi* larvae	11
ANIMALS	11
Housing	11
Worm-Free Foals	12
PATHOLOGICAL TECHNIQUES	12
Post Mortem Examination	12
Histological Techniques	12
Scanning Electron Microscopy Techniques	13
HAEMATOLOGICAL TECHNIQUES	15
Collection and Storage of Samples	15
Packed Cell Volume (PCV)	15
STATISTICAL ANALYSIS	16

TABLE OF CONTENTS (Cont'd) Page no.

IMMUNOLOGICAL TECHNIQUES 16

Preparation of Reagents 16

Immunisation of Rabbits 17

Purification and Concentration of anti-*D. arnfieldi* Antibodies 18

ELISA TECHNIQUES 22

Coating of Plates 22

ELISA Buffers and Substrates 23

Blocking Agents 24

Biotinylation of Rabbit anti-*D. arnfieldi* Antibody 24

EDTA Treatment 25

Autoclave Treatment 25

Periodate Treatment 25

PREPARATION OF *Dictyocaulus arnfieldi* SECTIONS 26

SECTION 1 - EXPERIMENTAL AND FIELD STUDIES WITH *Dictyocaulus arnfieldi* IN HORSES, PONIES AND DONKEYS 27

INTRODUCTION - EXPERIMENTAL STUDIES 28

LABORATORY CULTURE OF *Dictyocaulus arnfieldi* 28

EXPERIMENTAL INFECTION WITH *Dictyocaulus arnfieldi* 35

INTRODUCTION 35

EXPERIMENT 1 - INFECTION OF ADULT PONIES 41

Animals 41

TABLE OF CONTENTS (Cont'd) Page no.

Experimental Design 42

RESULTS 43

DISCUSSION 45

EXPERIMENT 2 - INFECTION OF PONY MARES AND FOALS 47

Animals 47

Experimental Design 48

RESULTS 49

Clinical Signs 49

Worm Recoveries 51

Pathology 51

DISCUSSION 53

INTRODUCTION - FIELD STUDIES 56

RESULTS 60

Donkey Sanctuary Survey 60

West of Scotland Survey 63

Investigation of an Outbreak of Coughing in Horses Associated with Donkeys Infected with *D. arnfieldi* 65

Donkey Grazing Study 67

DISCUSSION 69

SECTION 2 - STUDIES ON THE DEVELOPMENT OF AN ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA) FOR THE DETECTION OF *Dictyocaulus arnfieldi* INFECTION 75

INTRODUCTION 76

TABLE OF CONTENTS (Cont'd) Page no.

ELISA FORMATS 78

THE D. arnfieldi ELISA 82

IMMUNOHISTOCHEMICAL LOCALISATION OF D. arnfieldi ANTIGENS 82

Reactions with anti-D. arnfieldi Antibody Fractions Raised in Rabbits 85

Summary of Procedure 86

RESULTS 86

CONCLUSIONS 88

Reactions of anti-D. arnfieldi Antibody Fractions with Antigens of Fresh and Formalin Treated Worm Sections 89

Summary of Procedure 90

RESULTS 90

CONCLUSIONS 90

COMPOSITION OF THE D. arnfieldi ANTIGEN 93

EDTA Treatment 93

Autoclave Treatment 94

Periodate Treatment 94

RESULTS 95

CONCLUSIONS 95

ELISA PROCEDURE FOR D. arnfieldi ANTIGEN DETECTION 95

Antigen Titration 98

TABLE OF CONTENTS (Cont'd) Page no.

USE OF THE ELISA FOR DETECTION OF *D. arnfieldi* ANTIGEN IN EQUINE SERUM SAMPLES 100

RESULTS 102

USE OF AN ELISA FOR DETECTION OF *D. arnfieldi* ANTIBODIES 109

Summary of Procedure 109

RESULTS 110

DISCUSSION OF ELISA STUDIES 112

GENERAL DISCUSSION 116

REFERENCES 134

APPENDICES 143

ACKNOWLEDGEMENTS

I am most grateful to Professor J.L. Duncan under whose supervision this work was carried out and especially to Dr. H.V. Smith whose help was invaluable.

I would like to thank Professor H.M. Pirie and Mrs. M. Pirie with whom I collaborated in the pathological and endoscopic studies.

For their expert assistance I wish to thank Mr. K. Bairden and all the technical staff of the Parasitology Department, Mr. A. May for his photographic help and Mr. J. Murphy and Mr. R. Irvine for their help in the post mortem room.

Special thanks are due to Mr. A. Hinson of the Scottish Parasite Reference Laboratories, Stobhill Hospital for his unstinting help and encouragement and to Mrs. J. Nybo for her skilled typing of this manuscript.

Finally I am indebted to Mrs. E. Svendsen, MBE, and the staff of the Donkey Sanctuary for their support, both financial and technical, which was most generous.

LIST OF TABLES

		Page no.
SECTION 1		
Table 1.1	Experimental Design: A Study of D. arnfieldi Infection in Adult Ponies. Details of Infective Dose and Time of Necropsy	42
Table 1.2	Experimental Design: A Study of D. arnfieldi Infection in Pony Mares and Foals. Details of Infective Dose and Time of Necropsy	48
Table 1.3	Clinical Signs, Pathology and Worm Recoveries from Pony Foals and Mares after Infection with 2,500 - 10,000 Dictyocaulus arnfieldi L_3	50
Table 1.4	Donkey Sanctuary Data for Three Years Showing Total Numbers of Donkeys Tested, Numbers Positive, Percentage Positive and Mean Faecal Dictyocaulus arnfieldi Larval Counts	61
Table 1.5	Donkey Sanctuary Data over a Three-Year Period Showing Total Numbers of Animals of Different Age Groups Tested, the Numbers and Percentage Positive and the Mean Faecal Dictyocaulus arnfieldi Larval Counts	62
Table 1.6	Donkey Sanctuary Data Showing Numbers of Animals Tested and Numbers Positive in Years, Seasons and Age Groups	64
Table 1.7	Clinical and Parasitological Findings in Animals Involved in an Outbreak of Coughing Related to Recent Contact with D. arnfieldi	66
Table 1.8	Donkey Grazing Study - Results of Faecal Examination	68
SECTION 2		
Table 2.1	Comparison of Enzyme Immunoassay (EIA) and Radioimmunoassay (RIA)	77
Table 2.2	Incubation of D. arnfieldi Section with Primary Antibody (1:10 - 1:20) then 1:100 Conjugate	87

LIST OF TABLES (Cont'd) Page no.

Table 2.3	Incubation of *D. arnfieldi* Sections with Primary Antibody (1:10 - 1:80) then 1:50 Conjugate	87
Table 2.4	Incubation of Either Formalin Fixed or Unfixed *D. arnfieldi* Sections with FrRB	91
Table 2.5	Mean Optical Densities Obtained Using the ELISA for *D. arnfieldi* Antibody at Varying Serum Dilutions	111

LIST OF FIGURES

SECTION 1

Figure 1.1 Gross appearance of the lungs of a pony on Day 20 post-infection with 2,000 D. arnfieldi L_3. A few discrete areas of overinflation are evident

Figure 1.2 Gross appearance of the lungs of a pony on Day 34 post-infection with 2,000 D. arnfieldi L_3. Several discrete and obvious areas of overinflation are present

Figure 1.3 Areas of overinflation evident in the lung of a pony on Day 34 post-infection with 2,000 D. arnfieldi L_3

Figure 1.4 Well defined areas of overinflation in the lungs of a pony on Day 48 post-infection with 2,000 D. arnfieldi L_3

Figure 1.5 Marked areas of overinflation in the lung of a pony on Day 48 post-infection with 2,000 D. arnfieldi L_3

Figure 1.6 Marked areas of overinflation in the lungs of a pony on Day 62 post-infection with 2,000 D. arnfieldi L_3

Figure 1.7 Grossly normal lung of a pony foal 42 Days post-infection with 2,500 D. arnfieldi L_3

Figure 1.8 Grossly normal lung of a pony foal 56 Days post-infection with 2,500 D. arnfieldi L_3

Figure 1.9 Marked areas of overinflation in the lung of a pony mare 42 Days post-infection with 6,000 D. arnfieldi L_3

Figure 1.10 Gross appearance of the lung of a pony mare 56 Days post-infection with 10,000 D. arnfieldi L_3 showing marked areas of overinflation

Figure 1.11 Histological sections of normal bronchiole and surrounding alveolar pattern (left) compared with mild bronchiolitis in an area of moderate overinflation (right) in a pony mare necropsied on Day 42 post-infection with 6,000 D. arnfieldi L_3. H & E x 25.

LIST OF FIGURES (Cont'd)

Figure 1.12 Histological sections of small bronchi (left) compared with a small bronchus showing chronic bronchitis and a dense infiltration of lymphocytes, plasma cells and some eosinophils (right) in a pony mare necropsied on Day 56 post-infection with 1,000 D. arnfieldi L_3. H & E x 10.

Figure 1.13 Histological section of a bronchiole with mucus and eosinophils in the lumen and lymphocytes, plasma cells and eosinophils in the lamina propria from a foal necropsied on Day 70 post-infection with 2,500 D. arnfieldi L_3. H & E x 100.

Figure 1.14 Histological sections of a normal bronchiole (left) compared with a severe bronchiolitis with thickening of the bronchiolar wall due to infiltration by lymphocytes and plasma cells with some eosinophils in a foal necropsied on Day 70 post-infection with 2,500 D. arnfieldi L_3 (right). H & E x 25.

Figure 1.15 Scanning electron micrograph of the ciliated surface of airways in a pony experimentally infected with D. arnfieldi

Figure 1.16 Scanning electron micrograph of the trachea of a pony mare experimentally infected with 6,000 D. arnfieldi L_3 showing a patch of non-ciliated microvillous cells

Figure 1.17 Scanning electron micrograph showing patches of non-ciliated cells in a bronchus of a foal experimentally infected with 2,500 D. arnfieldi L_3

Figure 1.18 Scanning electron micrograph showing non-ciliated epithelial (Clara) cells in a terminal bronchiole of a foal experimentally infected with 2,500 D. arnfieldi L_3

SECTION 2

Figure 2.1 Direct ELISA using labelled antibody or labelled antigen

Figure 2.2 Indirect ELISA

Figure 2.3 Direct Sandwich ELISA

LIST OF FIGURES (Cont'd)

Figure 2.4 Indirect Sandwich ELISA

Figure 2.5 Direct Antibody Competition ELISA

Figure 2.6 Direct Antigen Competition ELISA

Figure 2.7 Indirect Antibody Competition ELISA

Figure 2.8 Indirect Antigen Competition ELISA

Figure 2.9 Diagram of the indirect assay used in the immunohistochemical localisation of Dictyocaulus arnfieldi reacting with anti-Dictyocaulus arnfieldi IgG antibody fraction raised in rabbits

Figure 2.10 Photomicrograph of a section of an adult D. arnfieldi showing a brown colourisation indicating the recognition by antibodies of both epicuticle and gut antigens

Figure 2.11 Diagram of Immune Complex

Figure 2.12 Optical densities obtained in the D. arnfieldi ELISA with untreated, periodate treated and autoclaved D. arnfieldi antigen

Figure 2.13 Diagrammic representation of the ELISA for Dictyocaulus arnfieldi antigen

Figure 2.14 Diagrams showing action of blocking agent

Figure 2.15 The optical densities using raw and EDTA-treated serum in the ELISA for D. arnfieldi together with larval counts in 50 grams of faeces (Da/50 g) from the donkeys admitted to the Donkey Sanctuary from 1986-1988

Figure 2.16 The optical densities using raw and EDTA-treated serum in the ELISA for D. arnfieldi in mature ponies experimentally infected with 2,000 (Ponies 1-8) or 5,000 (Ponies 9 and 10) D. arnfieldi L_3

Figure 2.17 The optical densities using raw and EDTA-treated serum in the ELISA for D. arnfieldi in mares and foals experimentally infected with 6-10,000 and 2,500 D. arnfieldi L_3 respectively

LIST OF FIGURES (Cont'd)

Figure 2.18 The optical densities using raw and EDTA-treated serum in the ELISA for D. arnfieldi together with faecal D. arnfieldi larval counts (l.p.g.) from donkeys at Glasgow Veterinary School

Figure 2.19 The optical densities using raw and EDTA-treated serum in the ELISA for D. arnfieldi together with faecal larval D. arnfieldi counts (l.p.g.) from animals referred to Glasgow Veterinary School

Figure 2.20 Mean optical densities obtained using the ELISA for D. arnfieldi at varying dilutions of serum from mares and foals experimentally infected with D. arnfieldi (Section 1 - Experiment 2) and a D. arnfieldi-naive foal

SUMMARY

Various studies involving D. arnfieldi were undertaken to further existing knowledge of lungworm infection in horses and donkeys. These fall into two main categories: first, a number of parasitological investigations were carried out including experimental and field studies of infection and second, immunological investigations were undertaken on the development of an ELISA to detect infection with D. arnfieldi.

In Section I, following a review of the literature concerning experimental D. arnfieldi infection, various techniques for the culture of D. arnfieldi infective larvae (L_3) were compared.

The technique which proved most successful involved the Baermannisation of faeces to recover first stage larvae (L_1) which, following washing and sedimentation to clean the larval suspension of faecal debris and concentrate the larvae, were then incubated in water in shallow flat bottomed trays for 10-14 days until they reached the infective L_3 stage. Larvae obtained by this method were used for experimental infections.

Two experimental studies were conducted. In the first, ten naturally reared adult ponies were infected with 2,000 - 5,000 D. arnfieldi L_3. These ponies were monitored for any clinical response to infection and pairs of animals were examined at necropsy at various time intervals post-infection. While gross lesions were present in the lungs of the first pair of ponies

killed three weeks post-infection, no D. arnfieldi were found until the second pair of ponies were necropsied five weeks post-infection. The lesions increased with the increase in time between infection and necropsy and most ponies exhibited varying bouts of coughing.

In the second experiment the response to D. arnfieldi infection was compared in three adult pony mares given 6,000 - 10,000 L_3 and three foals given 2,500 L_3. At necropsy of one mare and one foal six, eight and ten weeks post-infection gross lesions attributable to D. arnfieldi infection were found and histologically there was overinflation, bronchitis, cellular infiltration and excess mucus in the lumena of the airways. These lesions were generally less severe in the foals than in the mares and the clinical signs in both groups were confined to mild coughing.

In the second part of Section I which deals with lungworm field studies the literature concerning naturally occurring D. arnfieldi infection is reviewed. A survey of the infection rate in 698 donkeys arriving at the Donkey Sanctuary, Sidmouth, Devon over a three-year period showed that this has fallen to 34% compared with 73% in a previous United Kingdom survey in 1973. This decrease was deemed to be due mainly to increased use of improved modern anthelmintics. In a survey of the level of patent D. arnfieldi infection in 681 horses and 39 donkeys in the West of Scotland 28% of the donkeys proved positive while only

two horses (0.3%) had patent infections. Interestingly neither of these animals had any history of previous donkey contact. During the course of the latter survey an outbreak of coughing in a small group of horses/ponies following the introduction of two donkeys with patent D. arnfieldi infections was investigated. The major clinical signs were coughing and increased respiratory rates but none of the horses or ponies developed patent D. arnfieldi infections. Finally, in this section, a small study was undertaken to follow the pattern of faecal excretion of D. arnfieldi L_1 in three adult donkeys and two worm-free donkey foals grazing a paddock grazed the previous year by donkeys with patent D. arnfieldi infections. Contrary to expectations the foals failed to develop patent infections despite one of the adults in the group having a consistently positive faecal L_1 counts.

In Section II the basic principles and various formats of the Enzyme Linked Immunosorbent Assay (ELISA) are outlined followed by a description of the steps taken to develop the reagents necessary for a D. arnfieldi ELISA. The ELISA chosen was an antigen capture micro ELISA utilising the biotin/streptavadin development system: this allowed the same antibody to be used for antigen detection (non-biotinylated) and assay development (biotinylated).

To check the affinity of anti-D. arnfieldi IgG antibody prior to use in the test, antibody fractions raised in two

immunised rabbits were incubated with sections of D. arnfieldi worms on slides. This showed that the antibody fractions from both rabbits recognised worm antigens although the serum from one rabbit gave better results. A comparison of the antigenicity of fresh versus formalin-fixed D. arnfieldi adult worm sections showed that fresh worms were more antigenic and immunohistochemical studies indicated that antibody recognised both surface and somatic antigens on the epicuticle and gut lining.

To investigate the composition of the D. arnfieldi antigen the following procedures were carried out: firstly the antigen was autoclaved to destroy the tertiary structure if peptide in nature: secondly, periodate treatment was used to disrupt any terminal sugars if the D. arnfieldi antigen was a glycoconjugate. The results of these treatments suggested that 20% of the epitopes of the D. arnfieldi antigen were protein in nature while 30% were carbohydrate.

From checkerboard titrations the optimal concentrations of reagents for use in the ELISA were determined. Basically plates coated with non-biotinylated antibody were washed prior to incubation with test antigen. Following washing, biotinylated antibody was added, incubated, washed and streptavadin peroxidase conjugate added. After incubation and washing the chromogen/substrate was added and incubated in the dark before reading. Overall this enabled the detection of D. arnfieldi

antigen down to levels of 1.2 - 2.6 ng of protein. Serum samples from various animals including experimental and field infections were tested using the ELISA. Discrepancies between faecal counts and optical densities by ELISA were found. These did not follow any set pattern - animals with negative faecal counts could give high ELISA readings and vice versa.

An ELISA for D. arnfieldi antibody was also developed and although this correlated well with infection in samples from experimental ponies this would have limited diagnostic value in the field.

GENERAL INTRODUCTION

Dictyocaulus arnfieldi (Cobbold, 1884) is the only equine lungworm and is found in horses, donkeys, mules, hinnies and zebra. Although found in all five continents the only detailed reports of the prevalence of D. arnfieldi in donkeys have been from the United Kingdom.

The life cycle of D. arnfieldi is direct. The adults, living in the bronchi and bronchioles, lay eggs which are carried by the mucocilary blanket up the trachea to the larynx and then swallowed. The eggs, containing first stage larvae (L_1) are passed in the faeces and hatch within a few hours (Wetzel and Enigk, 1938; Round, 1972). Two moults occur to produce the infective third stage larvae (L_3) and this stage can be reached, under ideal conditions, in three days. The L_3 can survive up to 14 days in sunlight and 24 - 42 days in the shade (Borovkova, 1948). Following ingestion by a susceptible host the L_3 moult to the fourth stage larvae (L_4) in the intestine and migrate, via the mesenteric lymph nodes to the lungs where the final moult and maturation occur in the bronchi toward the periphery of the lobes (Wetzel and Enigk, 1938). Adult worms can be up to 10 cm in length and are thread-like. The minimum prepatent period is 9 - 10 weeks (Britt and Preston, 1985).

The donkey is considered to be the natural host for D. arnfieldi as many animals develop patent infections which appear to be non-pathogenic: These may persist for the life of the donkey if no effective anthelmintic treatment is given. In contrast, in adult horses most D. arnfieldi infections do not become patent but clinical signs of a chronic cough, increased

respiratory rate and rhonchi, heard on lung auscultation, usually accompany infection (Clayton and Murphy, 1980). Horse foals appear to behave more like donkeys and develop patent infections without clinical signs: Unlike donkeys however, the duration of patency is limited, the maximum length of patency recorded in the United Kingdom being 31 weeks in foals and eight months in adult horses (Round, 1972). In parts of the Soviet Union up to 70% of horses have been shown to have patent infections (Akramovskii, 1952; Borovkova, 1948; Koulikov, 1935) and the plane of nutrition is considered to exert an influence on the length of patency: this ranged from six to seven months for horses on a high plane of nutrition to 21 months for animals on a poor diet (Akramovskii, 1952).

Round (1972) showed that donkey foals usually acquire infection in their first grazing season. Regardless of age or the time at which these foals start grazing, patent infections do not appear until August with the majority reaching patency in the autumn. This tends to support the theory that D. arnfieldi larvae do not overwinter on pasture (Akramovskii, 1952), and that infection of donkey foals originates from larvae passed in the spring and summer by infected adult donkeys.

The level of infection in individual donkeys tends to increase during the first 2 - 3 years of life. After this faecal larval output usually remains fairly constant. It has been found that after anthelmintic treatment with fenbendazole and mebendazole, which reduces faecal larval counts to zero, adult

donkeys are resistant to reinfection: this is not the case in donkeys under 3 years of age (Round, 1972).

In the Soviet Union horse to horse transmission has been reported (Akramovskii, 1952) while in this country, although horse to horse transmission is possible the horse usually acquires infection from the donkey.

Despite the differences in clinical response between the donkey and the horse the pathological findings are similar (Nicholls, Clayton, Duncan and Buntain, 1979). At necropsy D. arnfieldi infection is characterised by pale, raised areas of overinflated pulmonary tissue, often wedge-shaped, around the lung periphery. These circumscribed lesions are most numerous in the caudal lobes, a distribution which is consistent with an agent arriving in the lungs via the bloodstream. In many cases the small bronchi supplying the overinflated areas are occluded by coiled worms and mucus exudate, often greenish in colour. An intense mucopurulent reaction with mucus, polymorphonuclear leukocytes and eosinophils, surrounds any hatched L_1 found in the bronchi while adults in the bronchi stimulate little exudation. There is epithelial hyperplasia with an increase in both size and numbers of goblet cells. The lesions tend to be localised with a diffuse eosinophilia being the only change in areas of lung exempt from gross parasitological changes.

The differential diagnosis of coughing in the horse poses a problem for the practising veterinary surgeon (Clayton and Murphy, 1980). Infection with D. arnfieldi is one of the possible causes and although it usually produces chronic coughing

i.e. duration greater than three months, usually the veterinary surgeon is called in at the start of the episode and thus has to differentiate D. arnfieldi infection from other causes of acute and chronic coughing in the horse. Viral infections (equine influenza and equine herpes virus type 1) can be confirmed by serology and virus isolation while bacterial infections can be ultimately confirmed by examination of suitable material. The differential diagnosis of causes of chronic coughing often depends largely on the history. Cases of Chronic Obstructive Pulmonary Disease (C.O.P.D.) usually present with increased expiratory effort and a recent history of the horse being fed hay or bedded on straw which is of poor quality i.e. mouldy and dusty while Pharyngeal Lymphoid Hyperplasia (P.L.H.) usually occurs in horses up to four years of age in training and can be confirmed by endoscopy. Suspected cases of D. arnfieldi infection present special problems. Although there may be a history of grazing with donkeys currently there is no way to confirm the diagnosis in the live horse. For example faecal examination by Baermann technique, which is suitable in donkeys where patent infections are the norm, is of limited value in the horse where most infections do not reach patency. Endoscopic examination of the airways is also of limited value as it is difficult to detect the adult worms in the lower bronchi even in heavily infected donkeys. When worms have been detected in the bronchi the infection is usually patent and larvae can be found in the faeces (Clayton, 1987; Fischer, Deegan and Lieske, 1982); an added

complication is that, in the horse, there is retardation of development and the worms often remain less than 1 cm in length.

Examination of tracheal washings has been considered an aid to diagnosis of infection in horses (MacKay and Urquhart, 1979). On rare occasions D. arnfieldi have been detected but often the only significant finding is the presence of eosinophils which, although indicative of parasitic infection, is not specific.

Obviously the finding of D. arnfieldi eggs, larvae or worms by Baermann, endoscopy or tracheal washings is proof of infection but a negative result is meaningless.

In many instances the diagnosis of D. arnfieldi infection in the horse is retrospective in that if, following treatment with an anthelmintic effective against D. arnfieldi, the animal stops coughing then it is assumed the cough was indeed due to the presence of D. arnfieldi. In other cases diagnosis is simply based on coughing in association with a history of recent or previous grazing contact with donkeys.

Clearly this is a rather unsatisfactory situation and the objectives of the work presented in this thesis were first to provide more information on various aspects of D. arnfieldi infection and second to examine the possibility of developing a diagnostic test for lungworm infection in horses. In view of the increasing use of the Enzyme Linked Immunosorbent Assay (ELISA) for the diagnosis of parasitic infections in man and animals the ELISA was considered the test of choice. Although most ELISAs are used to detect antibodies, a diagnostic test for equine lungworm would have to differentiate current from previous

infection and therefore the ELISA would be one which could detect *D. arnfieldi* antigen.

MATERIALS AND METHODS

PARASITOLOGICAL TECHNIQUES

Faecal Sampling

All faecal samples were collected per rectum using separate plastic gloves for each animal. The samples were then placed in plastic cartons with lids and stored in the refrigerator until tested.

Faecal Examination

McMaster Flotation

Faecal samples were examined by a modified McMaster technique (Gordon-Whitlock, 1939). In this method, 3 g of faeces are homogenised in 42 ml water, passed through a 100 um mesh sieve, and the filtrate collected. A 15 ml flat-bottomed tube is filled with the well-mixed filtrate then spun in a centrifuge at 2000 rpm for 3 min. The supernatant is poured off, the sediment agitated using a whirlmixer and the tube filled to the previous level with saturated salt (NaCl) solution. The tube is inverted 6 times and sufficient fluid removed in a pipette to fill both chambers of a McMaster slide. The eggs rise rapidly in the floatation fluid hence the pipetting must be accomplished rapidly. The number of eggs in both chambers of the McMaster slide is multiplied by 50 to give the count in eggs per gram (e.p.g.) of faeces.

Baermann Sedimentation

The detection of Dictyocaulus spp. larvae in faeces was achieved using the modified Baermann method of Henriksen (1965) in which 10 g of faeces was suspended in a gauze bag in warm water contained in a conical measure (urine flasks). After a

minimum of 6 hours, the supernatant was drawn off leaving a final volume of 10 ml and the gauze plus faeces discarded. The numbers of larvae in 1 ml were determined and the results expressed as larvae per gram of faeces.

Worm Recovery at Necropsy

Lung Examination for Recovery of *D. arnfieldi*

A perfusion method similar to that of Inderbitzen (1976) was employed to recover worms from the lungs at necropsy. The procedure was as follows: The pericardial sac was opened to expose the pulmonary artery in which a 2 cm incision was made. A rubber tube was introduced pointing towards the tracheal bifurcation and fixed *in situ* by double ligatures. The remaining great vessels were tied off and water from a mains supply allowed to enter via the pulmonary artery. The water entering the vascular system eventually ruptured the capillary network to return via the bronchial tract and out from the trachea. Twenty litres of washings collected from each set of lungs were filtered through a 37 micron aperture sieve and subsequently examined for worms.

This method required intact pleura. If the pleura had been damaged on removal from the carcase or if samples of lung tissue had been removed for histological examination, an alternative method of lungworm recovery was necessary. The method used was as follows: The airways were opened by cutting along the bronchi with scissors. Each lung was then floated with the cut surface down for 12 hours in a bucket of warm saline, after which the

tissue was removed and the supernatant syphoned off. The remainder was spun in a centrifuge at 1,500 rpm for 3 minutes and the sediment examined microscopically for the presence of worms.

Culture and Administration of Dictyocaulus arnfieldi Larvae

Larval Culture

Infective third stage Dictyocaulus arnfieldi larvae (L_3) were cultured as described (page 33). Larvae in water were transferred from flat glass trays into 500 ml urine flasks and left to sediment for a minimum of eight hours. The supernatant was then carefully syphoned off to leave a volume of 50 ml. The numbers of larvae present in each sample were determined by counting 5 ml (10%) of a well-mixed larval suspension using an Eel counting slide (Hawksley) under the dissecting microscope. Doses were then made up by volume to give the desired number of larvae.

Administration

The animals were restrained to allow the passage of a polythene stomach tube (Portex Ltd., Hythe, Kent, England). The infective dose was then poured through a funnel into this tube then flushed through with water.

ANIMALS

Housing

Animals were housed in a unit of ten cement-lined brick loose boxes with access to a concrete run-out area for part of the day. Where required, different groups/individuals were kept separately. Straw bedding was removed and the loose boxes

thoroughly cleaned out daily: The run-out area was also swept and hosed daily. The animals were fed hay ad lib and proprietery horse and pony cubes or coarse mix (Spillers).

Worm-Free Foals

Worm-free donkey foals for the grazing study were obtained in the following manner. The in-foal donkey mares were wormed approximately every eight weeks while at grass with pyrantel embonate (Strongid-P, Pfizer, Kent) . Several weeks before they were expected to foal they were housed individually in clean boxes and subsequently wormed repeatedly with pyrantel every four weeks. The donkey mares foaled indoors and the foals were housed in their boxes with access to the run-out area. At weaning the two donkey foals were housed together with access to the run out area.

PATHOLOGICAL TECHNIQUES

Post Mortem Examination

All animals were shot using a humane killer and faecal and blood samples were collected at this time. The larynx, trachea, heart and lungs were carefully removed intact.

The lungs were examined visually and by palpation. The number, size, appearance and distribution of any superficial lesions were noted.

Histological Techniques

Tissues for light microscopy were obtained at necropsy from the lungs and bronchi both from gross lesions and randomly from the caudal lobes. Blocks about 2 - 3 mm thick were collected in

buffered neutral formalin, fixed for at least 24 hours, post-fixed in corrosive formol, dehydrated and cleared in a double embedding series. Tissue blocks were finally embedded in paraffin wax and cut at 5 - 6 um in a rotary microtome.

Haematoxylin and eosin (H & E) was the routine stain used for sections however selected sections were also stained with Alcian Blue/Periodic Acid Schiff (PAS) pH 2.5 for mucus and carbol chromotroph for eosinophils.

Scanning Electron Microscopy Techniques

Samples for Scanning Electron Microscopy (SEM) were taken from the following sites:-

1. Dorsal trachea midway between the larynx and the tracheal bifurcation.
2. Ventral trachea midway between the larynx and the tracheal bifurcation.
3. Right cranial lobar bronchus.
4. Right caudal lobar bronchus.
5. Right cranial segmental bronchus.
6. Right caudal segmental bronchus.
7. Small bronchus from the right cranial lobe of the lung.
8. Small bronchus from the right caudal lobe of the lung.
9. Lung slices from three different locations in the right cranial lobe for examination of bronchioles, alveolar ducts and alveoli.
10. Lung slices from two different locations in the right caudal lobe for examination of bronchioles, alveolar ducts and alveoli.

When samples 1-4 had been taken, the right cranial lobar bronchus was cannulated and the cranial lobe of the lung perfused with Karnovsky's fixative (2.5% paraformaldehyde/2% glutaraldehyde). The dorsal basal segment of the caudal lobe of the right lung was similarly perfused via a cannula in the appropriate segmental bronchus.

Samples 5-10 were then taken. From all sites, pieces of tissue with a surface area of not more than 1 cm square and approximately 2 mm thick, were immersed in Karnovsky's fixative for 24 hours. Tissues were rinsed in 0.2M cacodylate buffer for four hours before dehydration through a series of acetones then critical point dried in liquid CO_2, mounted on aluminium stubbs and coated with gold paladium. Specimens were examined by means of a Philips 501B scanning electron microscope.

Processing for Scanning Electron Microscopy

Tissue samples were treated according to the following regimen.

Wash the tissue gently on 0.1M Cacodylate buffer (sodium) to remove surface blood and mucus.

Fix in Karnovsky's EM fixative or 3% buffered Gluteraldehyde - a minimum of 24 hours.

Trim or slice tissue to expose the surfaces to be scanned. As the appearance of the tissue changes considerably during drying to ensure the correct surface is scanned the top right hand corner is cut diagonally.

Wash in buffer (0.1M sodium cacodylate buffer) for a minimum of 4 hours.

Wash in 70% acetone - minimum of 4 hours.

Wash in 90% acetone - minimum of 2 hours.

Wash in 100% acetone - minimum of 2 hours.

Leave in 100% acetone overnight.

Attach tissues to an aluminium stub with silver paint.

Sputter coat with gold for 4 minutes.

Store samples at 37°C.

HAEMATOLOGICAL TECHNIQUES

Collection and storage of samples

Animals were bled by jugular venupuncture into sterile vacutainers (Becton-Dickinson, New Jersey, USA).

For estimations requiring serum, 10 ml of blood was drawn into a plain vacutainer and left to stand for several hours to allow the formation of a firm clot. Serum which separated was decanted into polythene Eppendorf tubes, frozen and stored at -5°C.

Samples for haematological estimations were collected in heparinised vacutainers and tested within a few hours of collection.

All parameters were either measured in, or the results converted into SI units.

Packed Cell Volume (PCV)

The packed cell volume (PCV) was determined by the microhaematocrit method. The heparinised blood sample was mixed

gently before filling 2 capillary tubes, which were sealed by Cristaseal (Hawksley & Sons Ltd., London) at one end. The tubes were spun for 6 mins in a microhaematocrit centrifuge, the percentage PCV was then determined from the scale on a Hawksley Microhaematocrit Reader and converted to a 1/1 ratio.

STATISTICAL ANALYSIS

Where applicable data was analysed using the Chi square test. With one degree of freedom, the significance at the 5% level ($p = 0.05$) is above 3.84.

IMMUNOLOGICAL TECHNIQUES

Preparation of Reagents

Dictyocaulus arnfieldi Antigen

All stages of D. arnfieldi present in the lungs of a naturally infected donkey which had been humanely destroyed for chronic lameness, were recovered using the modified Inderbitzen method described earlier (page 10). The D. arnfieldi worm material was washed by centrifugation with TRIS-HCl buffer (4 washes at 1500 rpm for 3 min), before being frozen in a small volume of buffer.

At this point, the various stages of D. arnfieldi present were still intact so, following defrosting, the suspension was macerated using a Polytron. The suspension was kept on ice and "Polytroned" for 45 mins before most, but not all, of the parasites were disrupted. This suspension was then stored overnight at $4^{o}C$.

Following overnight storage, the suspension was centrifuged at 4200 rpm for 20 min, the supernatant decanted, and respun at 4200 rpm for a further 20 min. The resultant supernatant was decanted, the pellets resuspended using a vortex mixer, then combined and frozen at -5°C.

The final supernatant was then spun at 16,000 for 45 min in an ultracentrifuge and decanted to give the solution which was used as the "antigen solution". The pellet was again resuspended using the vortex and frozen.

The protein content of the antigen solution was determined by the Lowry method with precipitation (vide infra). Read at 680 nm using the tungsten light source, the protein content was 30 ug/ml. The antigen solution was aliquoted and frozen for later use.

Immunisation of Rabbits

To produce antibodies to D. arnfieldi two rabbits were immunised as follows:-

All stages of D. arnfieldi, recovered from the lungs of an infected donkey (vide supra) were washed by centrifugation in PBS - four changes spun at 1500 rpm. The pellet was resuspended in a small volume of PBS before being transferred to a Griffiths tube, kept in ice, and the parasites disrupted. The final volume (4 ml) was mixed with an equal volume of Freund's complete adjuvant. This was homogenised by taping a syringe containing the mixture onto a whirlmixer and mixing for 30 minutes until a creamy coloured homogenous solution was obtained. This solution was split into two equal volumes, one of which was frozen (at

-5°C) for later use. The other was used immediately to immunise two rabbits. Each rabbit received 2 ml of the solution. This was administered subcutaneously at multiple sites over the shoulder and back region of the rabbit to stimulate the maximum immune response.

Six weeks later the rabbits received a similar booster immunisation using the D. viviparus/Freunds mixture which had been previously frozen. Two weeks after the second immunisation the rabbits were bled out and their sera frozen separately.

Purification and Concentration of anti-D. arnfieldi Antibodies

Preparation of DEAE column

The column (Pharmacia 250 x 25) was loaded with DEAE prepared as follows:-

60 g of DEAE was added to approximately 500 ml of a 0.01M phosphate buffer, pH 7.6 and stirred well. This was left to equilibrate for 20 minutes at room temperature before being sieved through a Buchner funnel using 11mm filter paper.

The column was assembled, ensuring it was truely vertical. The solid DEAE retained by the filter paper was re-suspended in approximately 600 ml of the phosphate buffer and this was used to load the column. The excess fluid was allowed to drain away from the bottom of the column with more DEAE suspension being added at the top. Care must be taken not to disturb the loading edge nor to let the column dry out. When the column was full and white in appearance, the plug was put in and the column left overnight to settle.

In the morning the column required packing before use. The top flow filter was inserted and the phosphate buffer flushed through, flow rate approximately 120 ml/hr, to pack the column. Prior to the chromatographic separation the ultra violet detection system was switched on for one hour. This allowed the mercury in the mercury vapour lamp to vapourise completely. After one hour the flow rate was reduced to 60 ml/hr. The plotter was allowed to warm up and the fraction collecter set to collect 3 ml in each tube.

After 20-30 minutes at the reduced flow rate the pump was switched off and the buffer allowed to drain out. When the bottom of the meniscus had entered the column the top flow filter was removed and the sample (Rabbit 1 or 2) added with a syringe to the top of the column. The sample was allowed to drip through until the meniscus had entered the column then phosphate buffer was added by syringe before the top flow filter was replaced. The pump was switched on to give a flow rate of 60 ml/hr to flush through the sample which was collected in the fraction collector.

Amicon - Ultrafiltration Method

The Amicon stirred cell had a volume of 60 ml. The filter/membrane was a YM10 which had an exclusion range of 10,000 mol weight (Diaflow Ultrafilter Membranes). Before use the membrane had to be treated as follows:-

The membrane must be handled by the edge only with care not to scratch the glossy surface. The membranes are pretreated with glycerin to prevent drying and sodium azide is added as a preservative. These must be rinsed off before use by floating

the membrane glossy side down in a beaker of distilled water for at least one hour, changing the water three times. For use the membrane is placed in the amicon chamber with the glossy side toward the solution. For re-use the membranes should be stored in 10% ethanol/water solution in the refrigerator.

The amicon cell was set up in the refrigerator. The carrying gas used was nitrogen at a pressure of 55 psi (37 atm).

IgG Preparation by Caprylic Acid Precipitation

To one volume of serum add two volumes of acetate buffer (0.06 M, pH 4) in a beaker and stir with a magnetic stirrer. Measure out 7.5 ml caprylic acid (also called octanoic acid) for every 100 ml of serum used. Slowly drip the caprylic acid into the serum/acetate buffer solution while stirring vigorously. Continue stirring for 30 minutes after the addition of all the caprylic acid. Centrifuge the solution for 30 minutes at 2000 rpm and 20^{o}C. Decant the supernatant and adjust the pH to 5.7 using 0.02M phosphate buffer pH 6.2. Dialyse the supernatant against 0.02M phosphate buffer, pH 6.2 at 4^{o}C for two nights. If any precipitate has formed spin it down at 2000 rpm and 20^{o}C for as long as needed. Concentrate supernatant in an amicon to give a small volume of high IgG concentration.

Acetate Buffer 0.06M pH 4

9.84 g sodium acetate (anhydrous)

6.2 ml glacial acetic acid

Make up to less than 2 litres and adjust pH to 4 if necessary. Make up to 2 litres.

Phosphate Buffer 0.02M pH 6.2

Made as a 1 : 100 dilution of stock 2M phosphate buffer pH 6.2

Phosphate Buffer 2M pH 6.2

119.38 g $Na_2HPO_4.12H_2O$

or 59.4 g $Na_2HPO_4.2H_2O$

Make up to less than 500 ml and adjust to 6.2 if necessary. Make up to 500 ml.

Concentration of purified anti-D. arnfieldi antibody solution

After the protein concentration of the fractions collected from the column had been calculated, all tubes with significant amounts of protein were selected for concentration. Concentration of this fluid was performed as before by ultrafiltration in an Amicon stirred cell ultrafilter using a filter of exclusion range 10,000 mol. wt. Ultrafiltration of the antibody solution was performed at 3.5 bar using nitrogen. Once the antibody solution fluid had been reduced to the desired level, the filtration was stopped and the concentrated antibody solution removed from the cell.

The cell was then washed with 40 ml distilled water and the filter stored in 10% ethanol. The resultant concentrated antibody solution was aliquoted and frozen at $-20^{o}C$ until use.

Protein determination using the modified Folin-Lowry technique

To determine the protein concentrations of the final antibody and antigen solutions, known dilutions of these

were made up in distilled water to give final volumes of 1 ml. These dilutions were then tested for protein concentration using the modified Folin-Lowry technique (Sigma Kit No. P5656). This protocol involved the preparation of a standard curve using solutions of known protein concentrations then processing the test solutions and comparing those with the standard curve. Absorbence values of all solutions were read at 680 nm on a Cecil Digital UV Spectrophotometer (model no. CE 2922).

ELISA TECHNIQUES

Coating of Plates

The antibody to D. arnfieldi raised in rabbits was diluted to 1:100 in 0.5M bicarbonate/carbonate coating buffer pH 9.6 (page 23). Fifty microlitre aliquots of the appropriately diluted antibody were pipetted into each well of a 96 well microtitre plate which was then sealed with a clear adhesive sheet and incubated for 2 hrs at 37°C. After incubation the plates were washed 3 times with PBS (pH 7.2) containing 0.05% Tween 20 (PBST) to remove any unadsorbed antibody. Tween 20 was incorporated into the wash buffer because of its mild detergent properties, which facilitate the removal of any unabsorbed reagents during the washing procedures, and its ability to block non-specific binding of assay reagents. The plates were then shaken dry and stored at -30°C until use. Before use the plates were thawed and washed a further 3 times with wash buffer and tapped dry.

The ELISA plates used throughout this study were Dynatech 129B (Dynatech, Virginia, USA) 96 well polystyrene microtitre plates. The microtitre plates were of the format shown below, and when referring to ELISA plate formats, the notation of columns 1-12 and rows A-H will be used.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

ELISA Buffers and Substrates

Coating Buffer (Carbonate-bicarbonate, pH 9.6)

1.50 g Na_2CO_3

2.93 g $NaHCO_3$

Make up to 1 litre with distilled water.

Wash Buffer (PBS-Tween 20, pH 7.2)

42.50 g NaCl

5.35 g NaH_2PO_4

1.95 g Na_2HPO_4

2.50 ml Tween 20 (polyoxyethylene (2) - sorbitan monolaurate)

Make up to 5 litres with distilled water.

This can be stored at room temperature but must be discarded if it becomes cloudy.

Diluting Buffer

Add 800 µl bovine serum albumin to 100 ml of wash buffer.

Store at +4°C.

Peroxidase Substrate - Stock Solution

100 mg orthophenylene diamine (O.P.D.)

10 ml absolute methanol

Mix thoroughly. Should be stable for 1-2 weeks if stored in the dark at +4°C.

Peroxidase Substrate - Working Solution

50 ml	distilled water
24.7 ml	0.1 M citric acid
25.3 ml	0.2 M Na_2HPO_4
1 ml	stock O.P.D.
0.05 ml	6% H_2O_2

Blocking Agents

A solution (5 or 2.5%) of dried skimmed milk (Marvel) in diluting buffer (page 24) was used as a blocking agent. Marvel acts as a blocking agent to prevent non-specific binding of test reagents thereby reducing non-specific adsorption of serum proteins and test reagents to the plate, which eliminates background "noise" in the system.

Biotinylation of Rabbit anti-*D. arnfieldi* Antibody

The antibody solutions were biotinylated to provide a suitable method for developing the proposed assay by using streptavidin conjugated enzymes.

The antibody solutions were dialysed against three changes of 0.1M Na_2HCO_3 at pH 8.2-8.6, then the protein concentration of the samples were adjusted to 1.0 mg/ml. N-hydroxysuccinimidobiotin (Sigma H1759) was first allowed to reach room temperature (stored at -20°C) to prevent hydrolysis and then weighed out and dissolved in freshly made up dimethyl sulphoxide to 1 mg/ml. This was then added at 120 µl per ml of antibody sample and rotated at room temperature for 4 hours on a rotary mixer (Eschmann; Sussex, model BSM). Following this the mixture was dialysed against PBS (pH 7.2) overnight before being used in the development of the capture sandwich assay.

EDTA Treatment

Equal volumes of EDTA solution (0.2M EDTA in 0.2M Tris 1M HCl, pH 8) and serum were boiled for 20 minutes in a stoppered glass test tube. The tubes were centrifuged (15,000g, 15 minutes) and the supernatant analysed for the presence of D.arnfieldi antigen in the ELISA.

Autoclave Treatment

The antigen solution/serum was diluted as required in PBS and autoclaved at 121°C, 15 psi for 15 minutes.

Periodate Treatment

Equal volumes of antigen solution/serum and sodium periodate solution (100m Molar) in acetate buffer pH 5.5 were mixed in a stoppered glass test tube and incubated at room temperature in the dark overnight.

Preparation of *Dictyocaulus arnfieldi* Sections

Whole *D. arnfieldi* worms recovered from the lungs of an infected animal were blocked in paraffin wax to enable five micron sections to be cut. These five micron sections were then mounted on glass microscope slides.

Dewaxing of *D. arnfieldi* Sections

The *D. arnfieldi* sections mounted on glass slides in paraffin wax were dewaxed using a series of graded alcohols. The slides were immersed in each alcohol solution for three minutes. The initial immersion was 100% alcohol and the final immersion was 100% distilled water.

Dehydration of *D. arnfieldi* Sections

The *D. arnfieldi* sections were dehydrated using a series of graded alcohols. Immersion was for three minutes each time starting with 10% alcohol and finishing with 100% alcohol.

SECTION 1

EXPERIMENTAL AND FIELD STUDIES WITH *Dictyocaulus arnfieldi* IN HORSES, PONIES AND DONKEYS

INTRODUCTION - EXPERIMENTAL STUDIES

As one of the major objectives of this work was to try to develop a diagnostic ELISA for *D. arnfieldi* it was important to have animals known to have been infected with lungworms. It was necessary therefore to have a reliable method of culturing *D. arnfieldi* L_3. The first part of Section I deals with the examination and modification of existing larval culture methods. Subsequently two experimental studies were carried out in ponies not only to provide material considered important for the development of a diagnostic ELISA but also to provide more basic information on the clinical response and pathogenesis of the infection in adult and young animals.

The results of this work will be presented under the following headings:-

Laboratory Culture of *D. arnfieldi*.

Experimental Infection with *D. arnfieldi*.

Experiment 1 - Infection of adult ponies.

Experiment 2 - Infection of pony mares and foals.

LABORATORY CULTURE OF *Dictyocaulus arnfieldi*

There have been only a few attempts to culture *D. arnfieldi* in the laboratory (Round, 1972; Britt and Preston, 1985) and no particular method has proved entirely successful with very poor or negative yields being a common occurrence. Previous culture methods described by Round (1972) have included the following:-

(1) **Faecal Thin Layer Method** (Jarrett, Jennings, McIntyre, Mulligan and Urquhart, 1957 - D. viviparus)

A 1 cm layer of faeces in a shallow plastic tray is covered by damp butter muslin and left at room temperature for 5-6 days. The surface is then washed gently with water to recover the larvae. Yields of D. arnfieldi L_3 by this method proved variable but never good.

(2) **Incubation of Faeces in Jars**

From infected animals faeces are put into jars with screw lids. Tap water may be added to a depth of approximately 1 cm before incubation at 15°C.

Jars with added water have produced better yields of D. arnfieldi L_3 than dry jars but maximum recoveries were only approximately 12% of the original contents.

(3) **Water Culture** (Parfitt and Sinclair, 1967; Michel, 1968 - D. filaria).

First stage larvae are cultured in shallow trays in tap water at 20°C. This method gave poor recoveries for D. arnfieldi.

(4) **Filter Paper Method** (Method cited by Round, 1972)

A thin layer of faeces containing L_1 is spread on filter paper in a petri dish and the surface sprayed with water to keep the culture moist. This method proved reasonably successful for small amounts of faeces.

(5) **Sawdust Thin Layer Method**

First stage larvae recovered by the Baermann technique are transferred to a Petri dish and tap water added to cover the

bottom of the dish. Sawdust is then added to cover the surface and to soak up all the water before the dishes are incubated at 20°C for four days. The wet sawdust is subsequently Baermannised overnight to collect the infective larvae. Although this was the most successful method adopted by Round (1972) and Britt and Preston (1985) it was inconsistent and poor yields were occasionally obtained.

As none of these methods for the culture of D. arnfieldi L_3 proved overwhelmingly successful it was decided to try several other techniques used routinely to culture other species of nematode larvae. The source of larvae for all of these techniques was faeces from four naturally infected donkeys with counts of 40-80 D. arnfieldi L_1 per gram of faeces. When faeces from more than one donkey were used these were bulked together and well mixed. A 10 g sample from each batch of faeces was examined to ensure the presence of reasonable numbers of larvae before setting up the cultures as follows:

(1) Approximately 100 g of faeces, pressed lightly into balls, were put into plastic cartons with lids. These were stored in the culture room at 22°C for one week. A sample of the faeces was then mixed with a little water in a petri dish and examined with the dissecting microscope before the remainder of the faeces were put in 30 g amounts and Baermannised. No infective larvae were recovered using this technique.

(2) First stage larvae collected following Baermannisation of faeces were placed in lidded petri dishes with:-

(a) sawdust and water;

(b) vermiculite and water;

(c) damp filter paper.

These were incubated at 37°C and sprayed with water to keep the cultures moist. On examination with the dissecting microscope two days later all larvae present in the cultures were dead.

(3) The following faecal samples were cultured in lidded petri dishes and kept at room temperature for six days:

(a) fresh faeces, sawdust and water;

(b) homogenised fresh faeces, sawdust and water;

(c) homogenised fresh faeces, vermiculite and water;

(d) homogenised fresh faeces, charcoal and water;

(e) homogenised fresh faeces and water;

(f) homogenised fresh faeces put onto moist filter paper.

After six days samples from all of the above were examined microscopically by Baermannisation but no viable D. arnfieldi were found.

(4) Fresh faeces were crumbled into a bucket and mixed with water to enable the faeces to be spread evenly, to a depth of approximately 1/2", on plastic trays. The trays were put into plastic bags, sealed to keep the faeces moist and put into the culture room at 22°C. The trays were examined daily and water sprayed on the surface when required to keep the cultures moist. After six days water was added to the surface of the trays and

faeces to try to collect any larvae. Unfortunately the faeces formed a slurry with the water making direct examination impossible. The slurry was therefore sieved and then filtered through a milk filter which was then inverted on to another milk filter and Baermannised. A few third stage D. arnfieldi were found by this method but unfortunately there was gross contamination with small strongyle (cyathostome) larvae. It would have been possible to eliminate the cyathostomes by Baermannising the original faecal sample to recover D. arnfieldi L_1, sterilising the faeces, then adding the L_1 suspension to the sterile faeces and culturing as before but due to the poor yields the extra stages involved were not thought to be justified.

(5) First stage D. arnfieldi larvae recovered by the Baermann technique were concentrated by sedimentation and put into tissue culture bottles with either tap water, distilled water or(Minimal Essential Medium (M.E.M): the culture fluid was changed twice daily.

These cultures were left on the bench and examined daily to assess larval viability and stage of development. After two days all of the larvae in MEM were dead. In distilled water larvae survived for a maximum of four days while in tap water a few L_3 were found after one week but most did not reach this stage of development.

(6) Fresh faeces were crumbled into a bucket and enough water added to enable the mixture to be spread onto glass pie dishes to a depth of < 1/2 inch as follows:-

(a) faeces and moist vermiculite;

(b) faeces and moist sawdust;

(c) faeces on top of damp filter paper;

(d) faeces.

No larvae were found in samples removed from (a) and (b) after five days while similar samples from (c) and (d) had a few D. arnfieldi second stage larvae (L_2). Further samples from (c) and (d) examined after seven days were negative.

(7) Faeces were mixed with warm water then passed through a coarse sieve. The filtrate was then poured through filter paper on a Buchner funnel using suction. This filter paper was then inverted on to a milk filter and Baermannised. The first 30 ml from each Baermann funnel was collected in 15 ml conical tubes the following morning then centrifuged at 1000 rpm for ten minutes. The supernatant was removed and the loose pellet of larvae resuspended by inverting the tube several times before pouring the larval suspension into a petri dish on the bench. The larval suspension was 'washed' by repeating this spinning process twice daily. Although this method did yield some clean L_3 after 10-14 days not all of the larvae had developed to L_3 and many were dead.

(8) Faeces were crumbled and 'Baermannised' by placing 30 g samples in gauze in 250 ml urine glasses filled with tap water for six hours. The water was then sucked off leaving a volume

of 25 ml. These 25 ml samples were bulked in 150 ml lots and transferred to 500 ml urine glasses which were then filled with fresh water and left to sediment overnight or for at least six hours. Sedimented 50 ml samples from these were then bulked again to 150 ml lots, transferred to 500 ml glasses, and then filled with fresh water. This process was repeated until the larval suspension was clean and when clean the suspensions were transferred to glass trays which remained on the bench. Water was added twice daily using a wash bottle to keep the depth of the water at approximately 1/2 inch and help aerate the cultures. The trays were examined regularly under the dissecting microscope to check for larval viability and contamination. It took 10-14 days for the larvae to reach the L_3 stage and having reached this stage they remained viable for approximately two weeks. When the cultures had reached the L_3 stage they were bulked together in 500 ml urine glasses and sedimented overnight before reducing to a volume of 50 ml. These 50 ml samples were bulked in glass trays.

Maintenance of larvae in water trays following Baermannisation and repeated sedimentation was found to be the most successful technique for culturing D. arnfieldi larvae from the L_1 stage present in faeces to the L_3 stage required for infection and this was the method used to produce larvae for all the experimental infections. Although this method was found to be the most successful of those tried, it was time consuming and produced adequate, but not large, yields.

EXPERIMENTAL INFECTION WITH *Dictyocaulus arnfieldi*

INTRODUCTION

Since there are only a few reports of experimental infections with *D. arnfieldi* there is a paucity of detailed information relating to lungworm infection in both donkeys and horses.

Round (1972) described the results of a series of experimental infections mainly in ponies.

In the first using worm-free animals, two yearling ponies which received 6,000 L_3 were compared with two uninfected controls. The infected animals started to cough after 17 days, continued to cough for ten weeks and in both the respiratory rate was also raised. Despite frequent examination no larvae were ever found in the faeces and there were no differences between infected and control animals with regard to haematology, serum protein levels or body weight gains. Ten months later all four ponies were infected with a dose of 12,000 L_3 and compared with a further two uninfected controls of the same age. The ponies which were experiencing a reinfection started coughing 12 days post challenge and had occasional adventitious lung sounds which were first heard on auscultation on day 13 post infection. The ponies undergoing primary infection were much more severely affected with clinical signs of severe coughing, pronounced adventitious lung sounds and forced respiration, from 15 days post-infection. In both pairs of infected ponies there was an increase in the number of circulating eosinophils during weeks three and four post infection/reinfection when compared with the

uninfected controls. None of the four infected animals developed patent infections and all six ponies were returned to pasture.

Eleven months later the two ponies which had been infected twice previously were each given a further dose of 24,000 D. arnfieldi L_3. There was no coughing or other clinical signs associated with infection and larvae were not detected at any time in the faeces.

From these experiments Round concluded that all ponies showed clinical signs of coughing, increased respiratory rates and adventitious lung sounds after their first exposure to infection with D. arnfieldi. These signs occurred from Day 11 and tended to be most obvious at 3-5 weeks post infection: Reinfection produced a diminished clinical response which Round suggested could be due to some form of acquired immunity.

A further experiment by Round (1972) in which 12,000 L_3 were administered to four worm-free mature ponies produced an increase in circulating eosinophils by week five and coughing, although mild, was persistent and notable from Day 20. Minor adventitious sounds were also present from Day 18 in two of the ponies but only persisted for a few days; these infections never became patent. From this study Round suggested that, in adult ponies, clinical signs relating to D. arnfieldi infection were due to immature stages and that the lack of patency was a result of this retardation of development.

Round also attempted experimental infection of younger animals. Eight Welsh Mountain Pony foals, naturally reared but

considered unlikely to have had previous experience of D. arnfieldi were used. Seven received 7,000 D. arnfieldi L_3 orally with one animal remaining as an uninfected control. The intention was to carry out weekly serial kills. By seven days post infection there had been no clinical signs and neither macroscopic lesions attributable to D. arnfieldi nor lungworms were found. By 14 days post infection only slight coughing had occurred which could be related at necropsy to pneumonic lesions of virus pneumonia; again no lungworms were found. Due to these negative results Round considered that the larvae used were non-infective and abandoned the experiment.

On subsequent checking of the infectivity of his larval culture by infection of four 20 month old ponies, increases in respiratory rates and mild adventitious sounds were observed in all animals associated in some with a circulating eosinophilia. This suggested that the original larval inocula may indeed have been infective and the six survivors from the "foal infection" were each given a further 10,000 L_3 from the batch used to infect the four 20 month old ponies. This was therefore a reinfection after 94 days for five animals and an initial infection for one. One reinfected foal was killed at 11 days post reinfection and patches of overinflation were seen in the diaphragmatic lobes. In other lung lobes there were consolidated areas which proved to be lesions due to infection with Streptococcus zooepidemicus. On dissection of the bronchi and overnight flotation of the lungs 30 D. arnfieldi were recovered, all of which were mature. The single foal experiencing a primary infection was necropsied at 13

days. Although there was some emphysema no *D. arnfieldi* were found. This indicated that the worms found in the reinfected animal were from the first infection of 7,000 L_3 and the remaining four ponies were then killed on Days 18, 20, 25 and 32 after what was now in effect a reinfection. Lesions and worms were found in the lungs of all animals, only immature worms being recovered on Day 18 while subsequently both immature and mature worms were found. Round presumed that the mature worms found were from the initial infection while the immature worms were from the reinfection.

In a further study eight pony foals with unknown histories and five donkey foals - three reared worm free and two with an unknown history - were given 6,000 L_3 orally. Twenty-three days post infection four ponies and three donkeys were necropsied followed by four ponies and one donkey on Day 57; the remaining donkey was killed on Day 63. The maximum establishment achieved was 0.75% of the infective dose and there was no significant difference in the numbers of worms recovered between the ponies and the donkeys but there were statistically significant differences in the size of worms recovered. The worms recovered from the donkeys were larger than those from the ponies and parasites recovered on Day 57 were larger than those recovered on Day 23. On Day 23 all worms were immature < 0.5 cm. By Day 57 the ponies had both immature and young adult worms while the donkeys on Day 57 and Day 63 had immatures and adults in the lungs which were producing L_1. This suggested a minimum

prepatent period of 8-9 weeks in donkeys Neither the ponies nor the donkeys exhibited overt clinical signs following infection.

Round (1972) also compared the response of ponies and donkeys to single and trickle infections with D. arnfieldi L_3. In one experiment, four worm-free ponies aged 20 months were used. Two received 23 doses of 1,000 L_3 orally over five weeks while two received a single oral dose of 24,000 L_3 during week 2 of the experiment. All the four ponies exhibited coughing from week 3 and when necropsied at the end of week 6 immature worms were recovered from all although the lungs appeared normal. There were no significant differences in size or numbers of worms which established from the two infection regimens.

In a second experiment two yearling donkeys and two yearling ponies, all reared worm-free, were given five daily doses of 1,000 L_3 orally each week for six weeks. Larvae were detected in the faeces of both donkeys on week 13 and their faecal larval output increased over the next four weeks at which time all four animals were necropsied. From the two donkeys 204 and 430 adult D. arnfieldi were recovered while 131 and 6 immature worms were recovered from the lungs of the ponies.

In a recent study Britt and Preston (1985) infected 12 yearling Fell ponies which had grazed pasture considered to be free from D. arnfieldi. Six animals received a dose of 20,000 L_3 while the remaining six were infected with 12,000 L_3. There was no coughing or other clinical signs related to infection apart from a slight mucopurulent nasal discharge 51-89 days post infection. Eight of the 12 animals developed patent infections

with lungworm larvae being detected in their faeces on at least one occasion; the earliest appearance of larvae in faeces was 68 days post infection. A further two animals had worms present in the lungs at post mortem. Three from each infection group were treated with ivermectin (Eqvalan, MSD AGVET) and the remaining six were untreated. All of the animals were killed 130 days post infection. The six control ponies had a mean of 10.1 D. arnfieldi (range 6-35) in their lungs at necropsy whereas no worms were found in the ivermectin treated group. In the animals which received a dose of 20,000 L_3, 44.4% of the parasites recovered were immature while in those receiving 12,000 L_3 only 2.3% had not reached maturity.

Although a few horse foals have been observed to develop clinical disease with spasmodic cough, dyspnoea, raised temperature and obstructive bronchitis on auscultation (Poncet, 1983; Lyons, Drudge, Zygmont, Twehues, Downing and Sutton, 1982) most reports suggest that they behave more like donkeys than adult horses in that most develop patent infections without clinical disease (Round, 1972). For example Clayton and Duncan (1981) grazed pony mares and foals on contaminated pasture and within 11 weeks, although none of the foals had shown any clinical signs, all had D. arnfieldi larvae present in their faeces: In contrast no larvae were found in the faeces of the mares but two of the four developed a cough 4 - 6 weeks post turnout and in one of these, a few D. arnfieldi were found in the lungs at necropsy.

The lung pathology associated with D. arnfieldi infection has been described by Nicholls, Duncan and Greig (1978) and Clayton and Murphy (1980). The lesions, which are similar in the horse and donkey, consist mainly of raised circumscribed areas of pale, overinflated pulmonary tissue with hyperplasia of the bronchial epithelium and infiltration of lymphoid cells around the airways which were often occluded by mucus exudate and coiled worms. The finding of worms in the airways is more common in the donkey than in the horse although the situation in the horse foal has not been recorded.

To examine further the pathogenesis of D. arnfieldi two experiments were carried out to investigate the clinical and pathological response to infection in adult and young ponies. These experiments were also designed to provide serum samples at various stages after known infections in both adult and young animals for use in the development of a diagnostic ELISA for the detection of D. arnfieldi antigen.

EXPERIMENT 1 - INFECTION OF ADULT PONIES

Animals

Ten adult ponies of Shetland type were purchased for this experiment. Although their history was unknown it was assumed that all had previous grazing experience. The group was loose housed in a concrete yard with free access to straw-bedded shelters. Identification was by numbered cattle ear tags attached to individual nylon head collars. Faecal sampling and examination, by Baermann and McMaster techniques, showed that all

ponies were negative for first stage larvae (L_1) of D. arnfieldi but positive for strongyle eggs. Each pony then received several anthelmintic treatments with fenbendazole and ivermectin which eliminated strongyle eggs in the faeces. During this period of anthelmintic treatment and faecal sampling the ponies became acclimatised to their surroundings and to being handled.

Table 1.1

Experimental Design: A Study of D. arnfieldi Infection in Adult Ponies

Details of Infective Dose and Time of Necropsy

Animal Number	Dose of D.arnfieldi L_3	Date Infected	Day of Necropsy
1 2	2,000	3/9/87	20
3 4	2,000	3/9/87	34
5 6	2,000	25/9/87	48
7 8	2,000	25/9/87	62
9 10	5,000	15/10/87	68

All ponies received a single dose of D. arnfieldi L_3. Due to difficulties in culturing large numbers of infective D. arnfieldi larvae simultaneously, they were infected in groups on three separate occasions (Table 1.1). Although from different

batches all infective larvae were cultured in the same manner from the faeces of the same two donor donkeys.

The ponies were weighed weekly, respiratory rates were recorded daily and blood samples taken weekly for haematology. The weekly serum samples were frozen at $-20^{o}C$ for future use in an ELISA test.

Faecal samples were taken weekly for examination by both Baermann and McMaster techniques.

Necropsies were carried out in pairs between 20-68 days post infection. At necropsy the lungs were removed and photographed immediately. Any gross lesions, for example patches of overinflation, were noted before the lungs were subjected to perfusion to recover any lungworms present.

RESULTS

Detailed results of body weights, PCV's and respiratory rates are given in Appendices 1.1 - 1.3.

There was no evidence of changes in body weight of five of the six ponies necropsied up to Day 48. One of the Day 48 ponies and those killed later in the experiment showed weight losses ranging from 7-21 kg.

No *D. arnfieldi* L_1 were found in the faeces of any of the ponies.

The clinical responses, lung pathology and lungworm recoveries at various stage post infection were as follows:

Day 20 - Ponies 1 and 2

Although pony 1 had a higher than normal respiratory rate there was no increase in the respiratory rates of either animal nor was any coughing observed. In the lungs of both animals there were a few discrete areas of overinflation (Figure 1.1). No lungworms were recovered.

Day 34 - Ponies 3 and 4

No significant changes in respiratory rate were observed. Pony 3 showed mild coughing on on Days 28, 32 and 34 and pony 4 was heard to cough on Day 26 with harsh respirations on auscultation on Day 32.

The lungs of both animals had several discrete areas of overinflation (Figure 1.2 and 1.3). No worms were recovered from pony 4 but 16 small threadlike immature D. arnfieldi (< 5 mm long) were recovered from the lungs of pony 3.

Day 48 - Ponies 5 and 6

Although there was no increase in respiratory rate in either pony, pony 5 showed intermittent coughing from Day 7 until Day 32. Pony 6 was only heard to cough on Days 35 and 36.

The lungs of these animals had numerous overinflated areas which were much more marked than those observed earlier (Figures 1.4 and 1.5). On perfusion, 38 and 36 D. arnfieldi (< 1 cm long) were recovered from ponies 5 and 6 respectively.

Day 62 - Ponies 7 and 8

Fairly persistent coughing was observed in pony 7 from Days 17-34 associated with an increased respiratory rate between

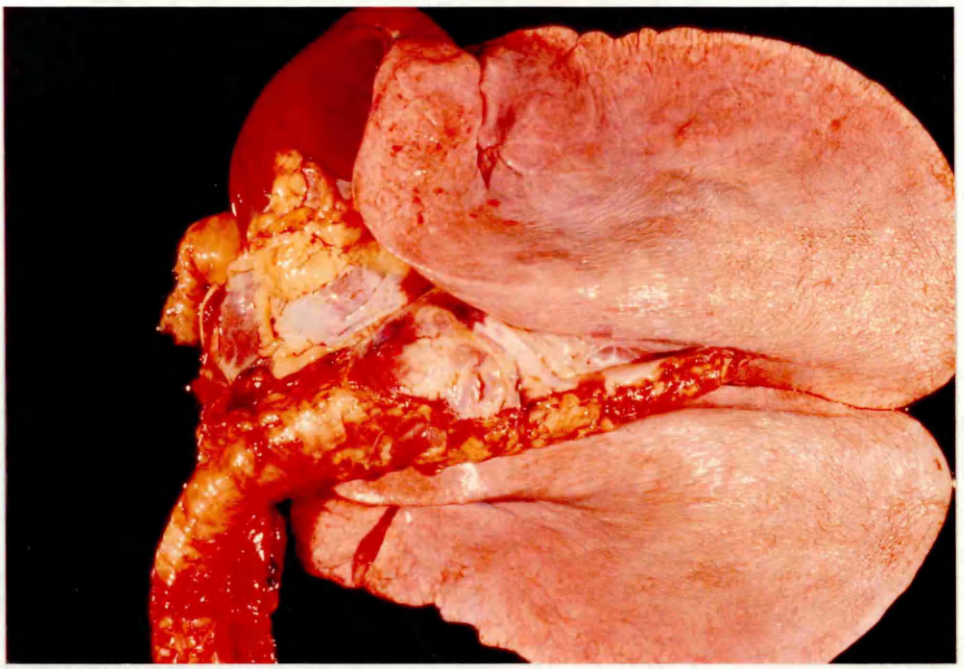


Figure 1.1

Gross appearance of the lungs of a pony on Day 20 post-infection with 2,000 D. arnfieldi L_3. A few discrete areas of overinflation are evident

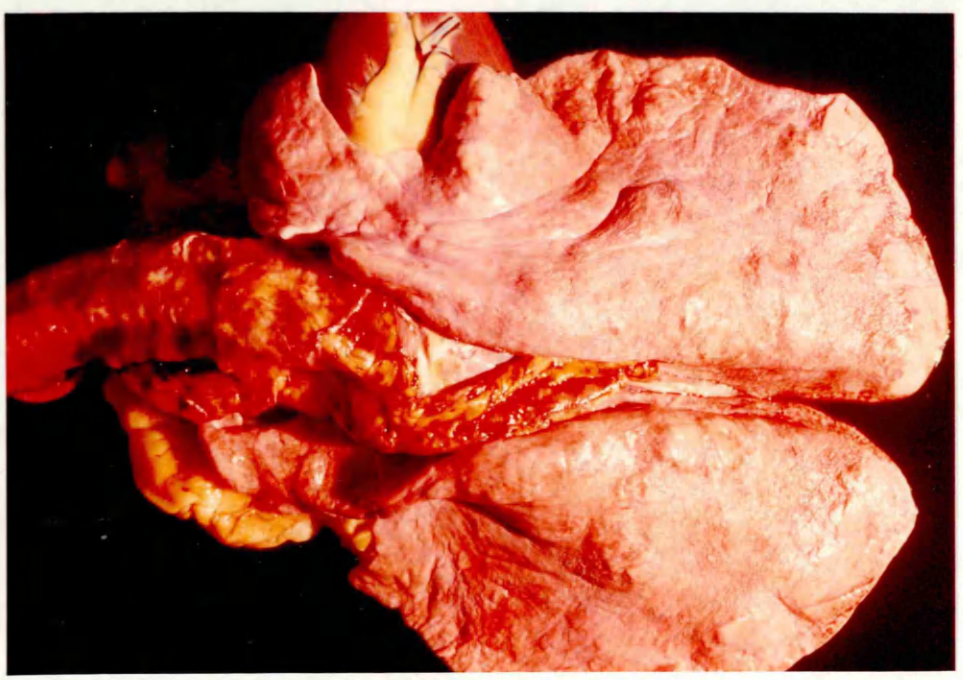


Figure 1.2

Gross appearance of the lungs of a pony on Day 34 post-infection with 2,000 D. arnfieldi L_3. Several discrete and obvious areas of overinflation are present

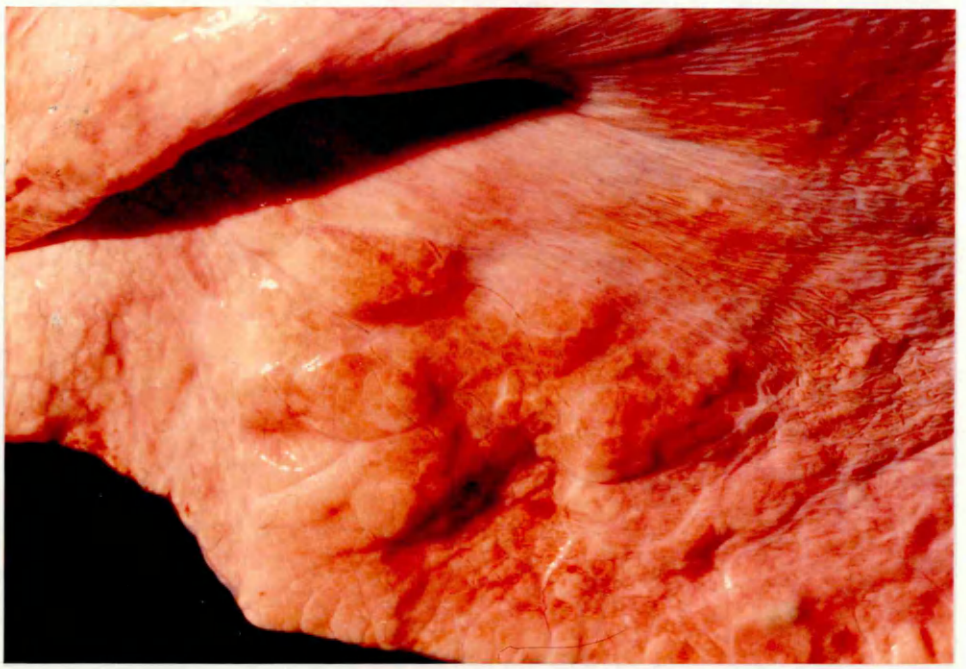


Figure 1.3

Areas of overinflation evident in the lung of a pony on Day 34 post-infection with 2,000 D. arnfieldi L_3

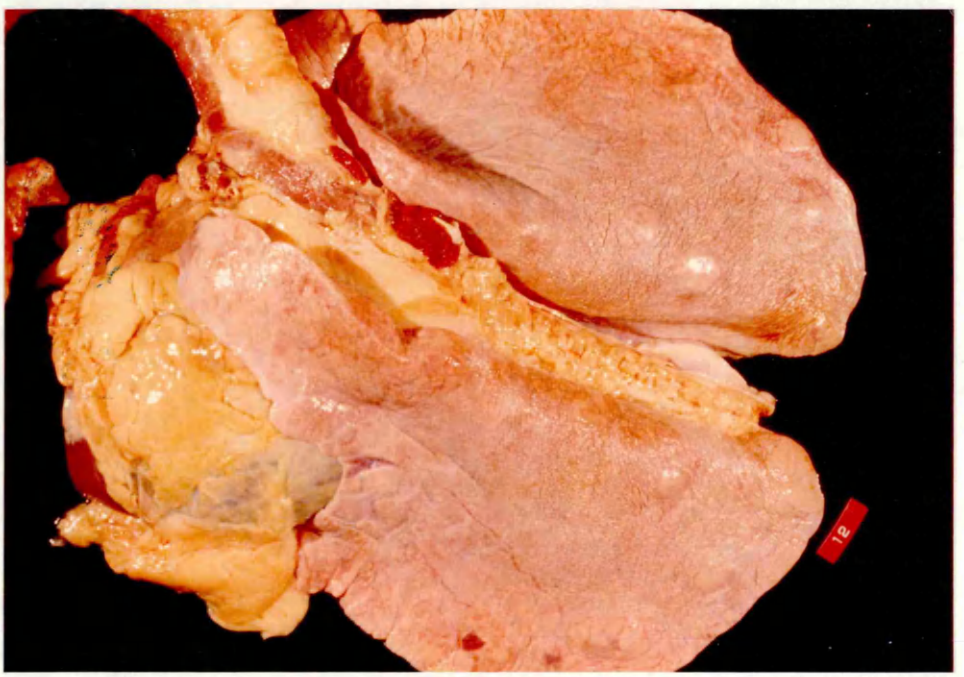


Figure 1.4

Well defined areas of overinflation in the lungs of a pony on Day 48 post-infection with 2,000 D. arnfieldi L_3

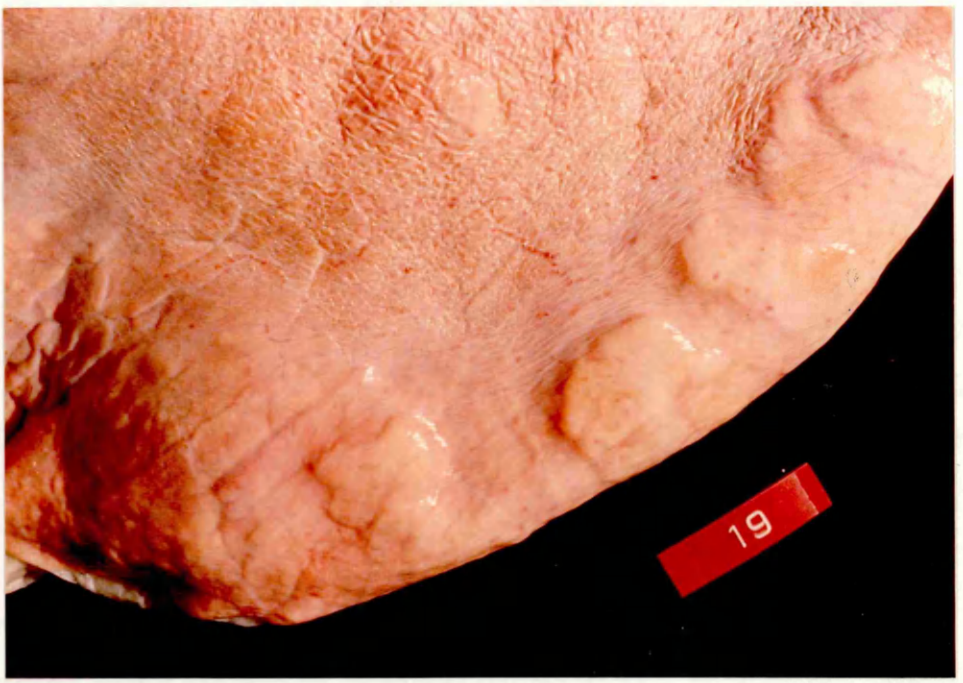


Figure 1.5

Marked areas of overinflation in the lung of a pony on Day 48 post-infection with 2,000 D. arnfieldi L_3

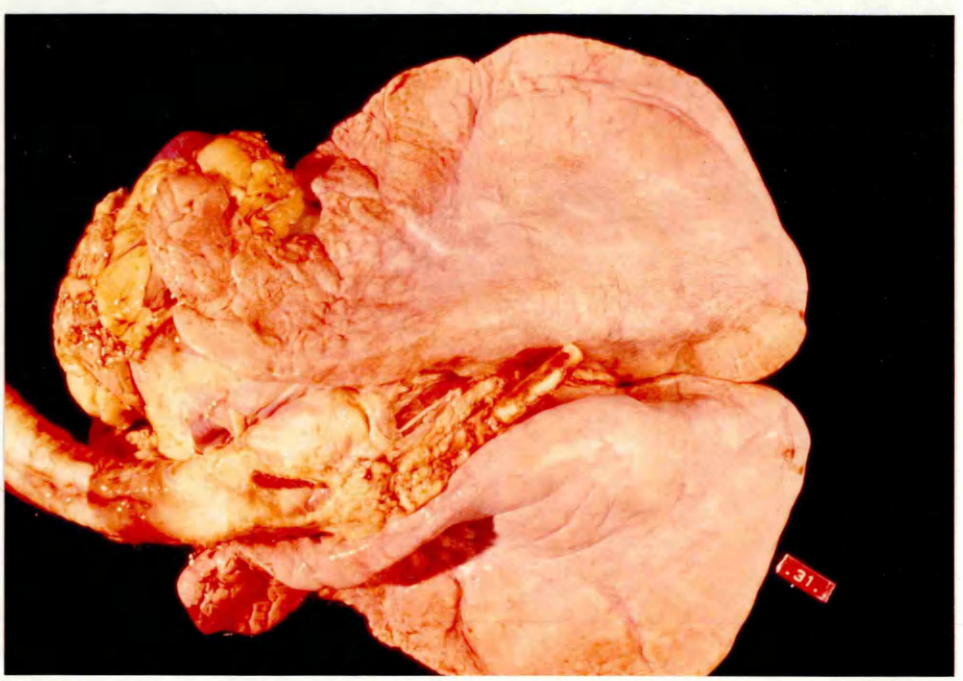


Figure 1.6

Marked areas of overinflation in the lungs of a pony on Day 62 post-infection with 2,000 D. arnfieldi L_3

Days 25-33. Sporadic coughing was heard until Day 50. Pony 8 showed no change in respiratory rate but was heard to cough on Day 24 and 26 and between Days 40 -43.

The lungs showed marked areas of overinflation (Figure 1.6) and 24 and 12 D. arnfieldi (< 2 cm long) were recovered from pony 7 and pony 8 respectively.

Day 68 - Ponies 9 and 10

Pony 9 showed an increased respiratory rate from Day 32 onwards and although heard to cough on Day 33, coughing was more frequent from Days 41-64. Pony 10 showed an increased respiratory rate from Day 18 onwards and coughing, first noted on Day 5, was relatively persistent from Day 20 until necropsy.

At necropsy the lung lesions were similar to those observed in the Day 62 ponies, although they were less severe in pony 9 than in pony 10: One and 102 young adult D. arnfieldi (< 4 cm long) were recovered from the lungs of ponies 9 and 10 respectively.

DISCUSSION

Although Round (1972) reported a consistent increase in respiratory rate preceded by coughing from around 2-3 weeks after a primary D. arnfieldi infection, which increased in severity between three and five weeks, the clinical response to infection in this experiment was variable. For example although coughing was noted in all eight ponies necropsied from 34 days onwards in only three of these animals (ponies 7, 9 and 10) was coughing marked and accompanied by increases in respiratory rate. These

signs were most evident from three weeks post infection and tended to increase towards the end of the experiment. The most severe clinical response was seen in one of the two ponies (pony 10) which received the dose of 5,000 L_3 and the highest number of worms (102 D. arnfieldi) was recovered from this animal at necropsy. The variation in clinical response between this study and Round's is most probably a reflection of variability in larval challenge, infectivity and worm establishment.

Despite the fact that no clinical signs were observed up to 20 days post infection and no worms were recovered from the two ponies necropsied at this time, grossly visible areas of overinflation were found in the lungs of both animals. The fact that worms were not recovered is probably due largely to the technical difficulty associated with recovery of a low number of very small worms from their situation in the lung parenchyma. In one of the remaining ponies (pony 4 - Day 34) no worms were recovered but gross lesions, typical of D. arnfieldi infection, were found at necropsy of all eight animals. The severity of the pulmonary lesions, which consisted of overinflated areas located on the periphery of the lung lobes, appeared to increase with the increasing period of infection becoming larger, more numerous and distributed over a wider area of the lung. No histopathology was possible since an intact pleura was required for worm recovery by perfusion and this technique completely disrupts the lung tissue.

None of the worms recovered in this study were sexually mature although this is not surprising since the minimum prepatent period reported for D. arnfieldi is 68 days (Britt and

Preston, 1985) and in other studies it has been found to be significantly longer at 91 days (Round, 1972). In general the size of the worms recovered during this experiment increased with time after infection : very small thread-like worms were recovered on Day 34 while immature adult worms were present in the animals necropsied on Day 68 post infection.

In essence this experiment confirmed previous reports of the response of adult animals to experimental D. arnfieldi infection. A second experiment was then designed to compare the response to infection in young and mature ponies and to examine the associated pathology in more detail.

EXPERIMENT 2 - INFECTION OF PONY MARES AND FOALS

Animals

Three adult Dartmoor mares and their foals were used. Although the history of the mares was unknown, they were semi-wild and it was assumed they had spent most if not all of their lives, outdoors. The mares were in foal when purchased and on arrival they were treated with ivermectin. When they were close to foaling they were moved into individual clean straw-bedded concrete loose-boxes with daily access to a concrete yard. Hay and water were available ad lib. The mares foaled indoors and the foals were reared under conditions designed to prevent helminth infection. The foals were weaned around six months of age at which time the mares were treated with fenbendazole. A minimum period of two weeks elapsed post weaning/treatment before any animal was infected.

Table 1.2

Experimental Design: A Study of D. arnfieldi Infection in Pony Mares and Foals

Details of Infective Dose and Time of Necropsy

Animal No.	Dose of D.arnfieldi L_3	Date Infected	Date of Necropsy
13 mare	6,000	13/11/88	42
14 foal	2,500	13/1/88	42
11 mare	10,000	24/12/87	56
16 foal	2,500	29/1/88	56
12 mare	10,000	24/12/87	70
15 foal	2,500	13/1/88	70

All ponies received a single dose of D. arnfieldi L_3 by stomach tube. Due to the difficulties in culturing large numbers of infective D. arnfieldi larvae simultaneously they were infected on three separate occasions as indicated in Table 1.2. Although from different batches all infective larvae were cultured in the same manner from the faeces of the same two donor donkeys.

Respiratory rates were recorded daily but due to difficulties in handling these semi-wild ponies regular auscultation of lung fields and collection of blood and faecal samples was not undertaken.

Necropsies of one mare and one foal were carried out at 42, 56 and 70 days post infection: In the previous experiment no histological examination of the lungs was possible as the perfusion technique used to recover D. arnfieldi completely

disrupts the lung tissue. In this experiment the lungs were carefully divided, leaving the pleura intact, and only one lung was used to recover worms, the other providing samples for histology and scanning electron microscopy. Where perfusion was not possible because the pleura had been damaged at necropsy, the lung was dissected following the respiratory tree and any visible worms picked out manually. The lung tissue was then floated in a bucket of saline overnight and the sediment examined for the presence of worms.

RESULTS

A summary of the major clinical findings and pathology together with worm recoveries at necropsy is presented for each animal in Table 1.3.

Clinical Signs

Respiratory Rate (RR)

All of the mares but only one foal (foal 15) showed an increased respiratory rate. Individual respiratory rates are detailed in Appendix 1.4.

The RR of the foal increased from Day 11 with the marked rise over Days 17-26.

Mare 11 showed a marked rise in RR from Day 34 while mare 12 showed an increase from Day 18 with a more marked rise from Day 34. The RR of mare 13 showed an increase from Day 10 with a more marked increase from Day 14. In all three mares the RRs remained elevated until necropsy.

Table 1.3

Clinical Signs, Pathology and Worm Recoveries from Pony Foals and Mares after Infection with 2,500 - 10,000 *Dictyocaulus arnfieldi* L_3

Animal	Clinical Signs	Date of Necropsy	Worm Recovery from Lungs	Gross Lesions	Histopathology	SEM Findings
14 foal	None	42	0	-	-	Normal ciliated surfaces
13 mare	Increase in respiratory rate	42	0	+	Moderate overinflation	Small non-ciliated patches in the dorsal trachea
16 foal	None	56	0	±	-	Normal ciliated surfaces
11 mare	Coughing Increase in respiratory rate	56	7*	++	Overinflation Chronic bronchitis in small bronchi with lymphocytes and eosinophils Eosinophils in interstitium	Normal ciliated surfaces
15 foal	Occasional cough	70	2	+	Mild overinflation Eosinophilia Bronchitis Bronchiolitis Mucus in lumen	Small non-ciliated patches in ventral trachea and small bronchus from caudal lobe
12 mare	Marked coughing: Increase in respiratory rate	70	0	-	-	Small non-ciliated patches dorsal and ventral trachea

* recovered by dissection and saline flotation/sedimentation

Coughing

Only foal 15 was noticed to cough and only on one occasion (Day 35) while two of the mares both showed fairly persistent marked coughing from Day 34. Although both of the mares exhibited bouts of harsh, dry coughing at no time did any animal appear in respiratory distress. No nasal discharge was detected in any animal.

Worm Recoveries

Worms were only recovered from the single lungs examined from two animals. Seven worms were found in mare 11 and two worms in foal 15.

Pathology

Gross lesions indicative of D. arnfieldi infection were found in the lungs of most animals. They were least severe in foals 14 and 16 (Figures 1.7 and 1.8) and most marked in mares 13 and 11 (Figures 1.9 and 1.10). In contrast, in the animals necropsied on Day 70 both had obvious gross lesions. The lesions consisted of circumscribed patches of overinflated lung tissue which were mainly found towards the caudal border of the lung.

Histopathology

Histopathological changes associated with gross lesions were found in only three animals. Mare 13 showed moderate overinflation (Figure 1.11). Mare 11 showed overinflation, chronic bronchitis in the small bronchi with lymphocytes and eosinophils in the interstitium (Figure 1.12) while foal 15 showed mild overinflation, eosinophilia (Figure 1.13), bronchitis and bronchiolitis (Figure 1.14) and mucus was present in the

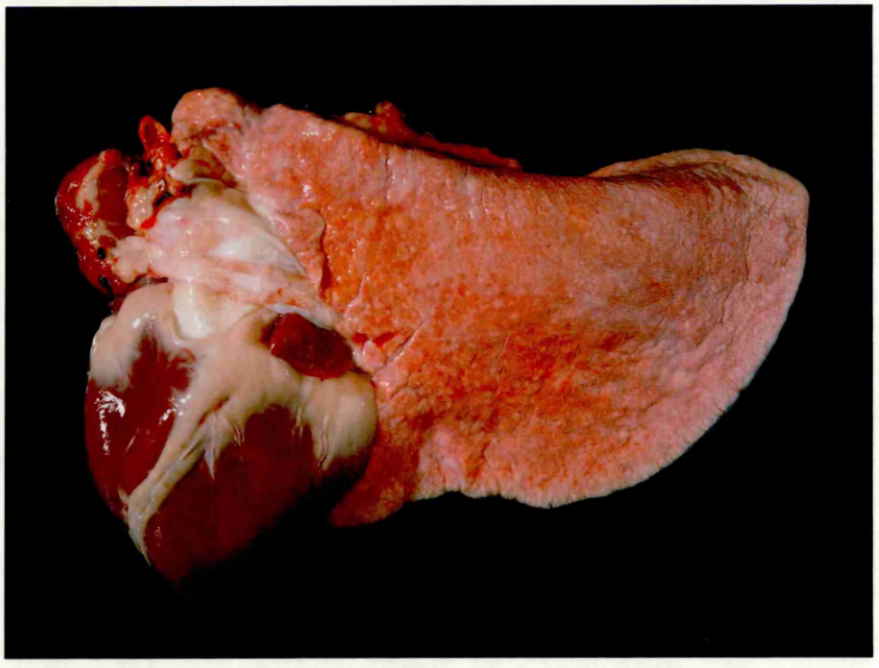


Figure 1.7

Grossly normal lung of a pony foal 42 Days post-infection with 2,500 D. arnfieldi L_3

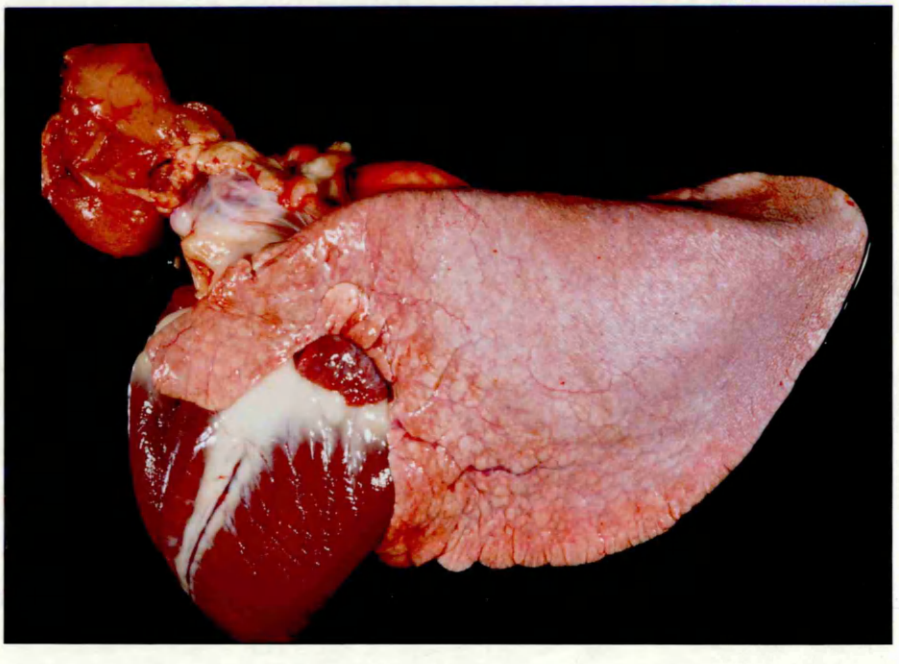


Figure 1.8

Grossly normal lung of a pony foal 56 Days post-infections with 2,500 D. arnfieldi L_3

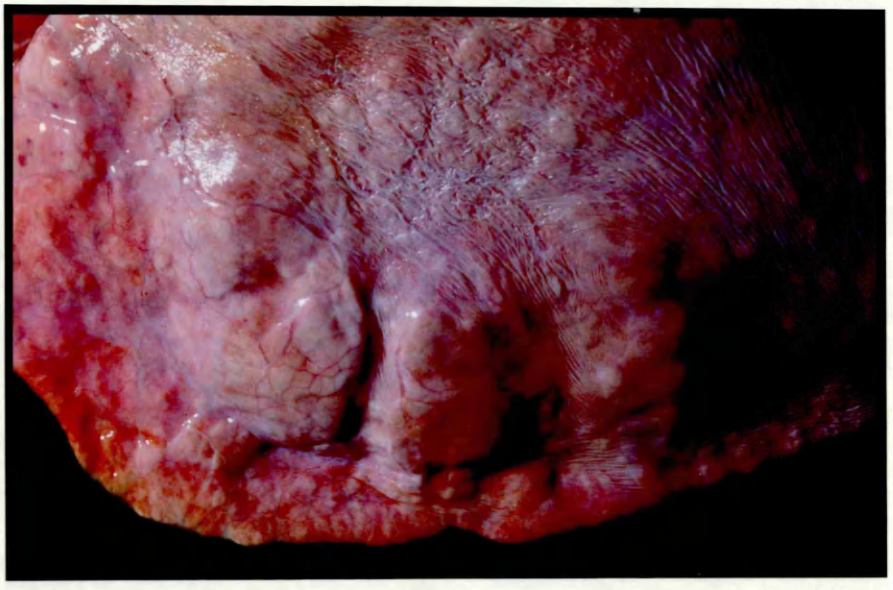


Figure 1.9

Marked areas of overinflation in the lung of a pony mare 42 Days post-infection with 6,000 D. arnfieldi L_3

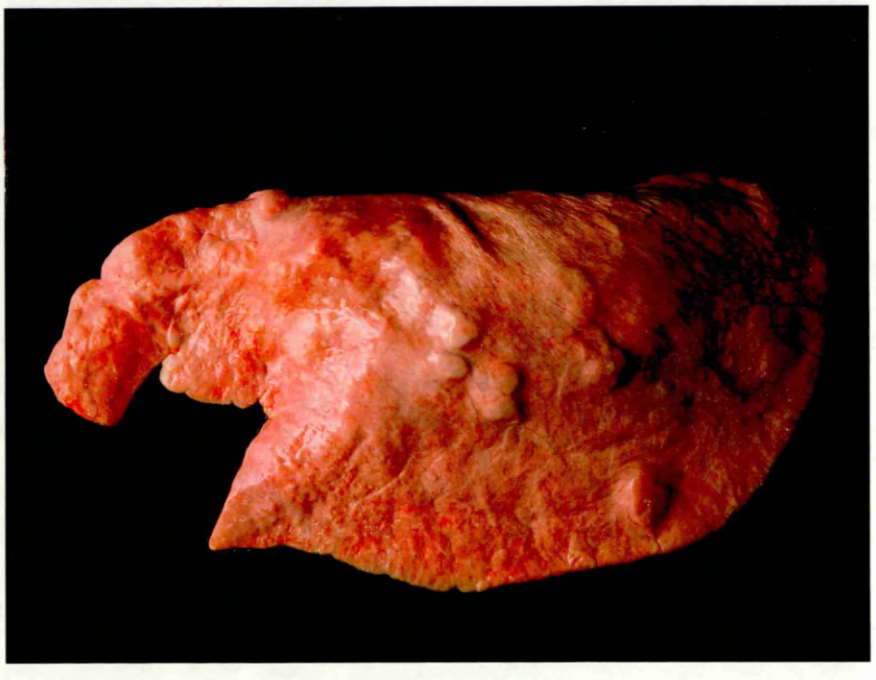


Figure 1.10

Gross appearance of the lung of a pony mare 56 Days post-infection with 10,000 D. arnfieldi L_3 showing marked areas of overinflation

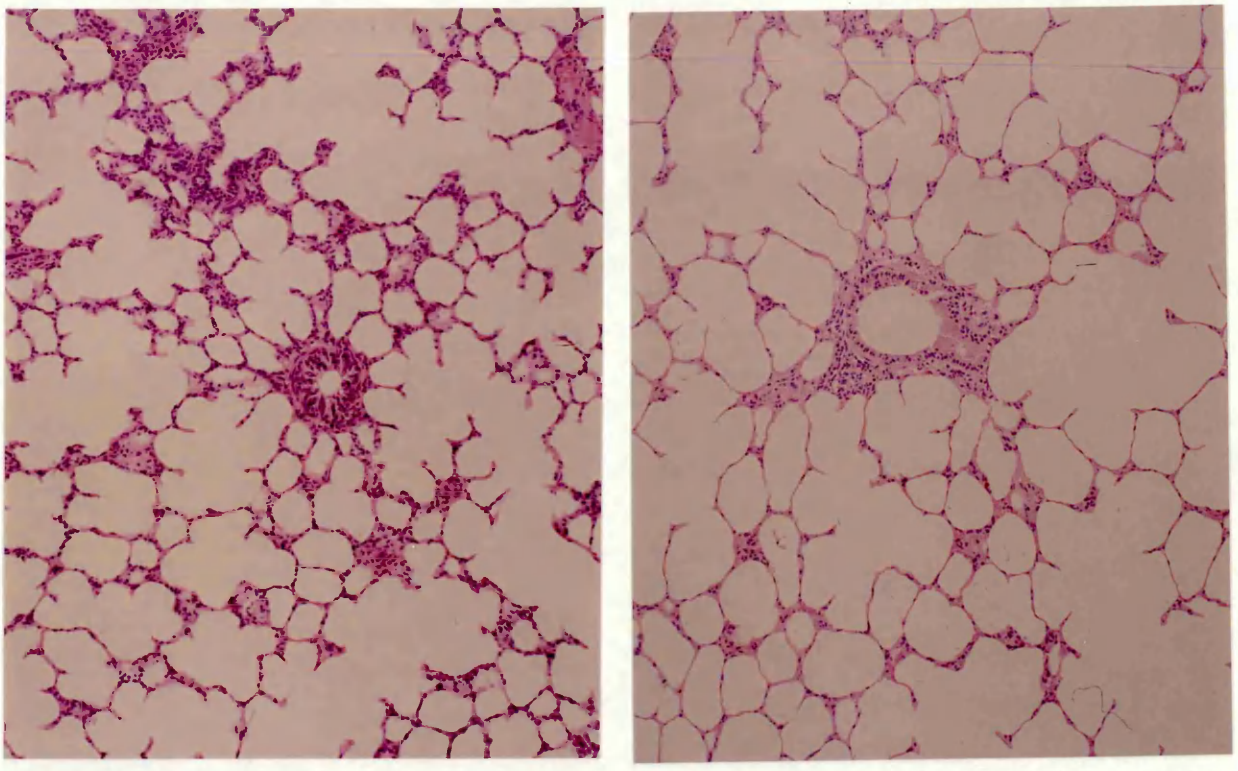


Figure 1.11

Histological sections of normal bronchiole and surrounding alveolar pattern (left) compared with mild bronchiolitis in an area of moderate overinflation (right) in a pony mare necropsied on Day 42 post-infection with 6,000 *D. arnfieldi* L_3. H & E x 25.

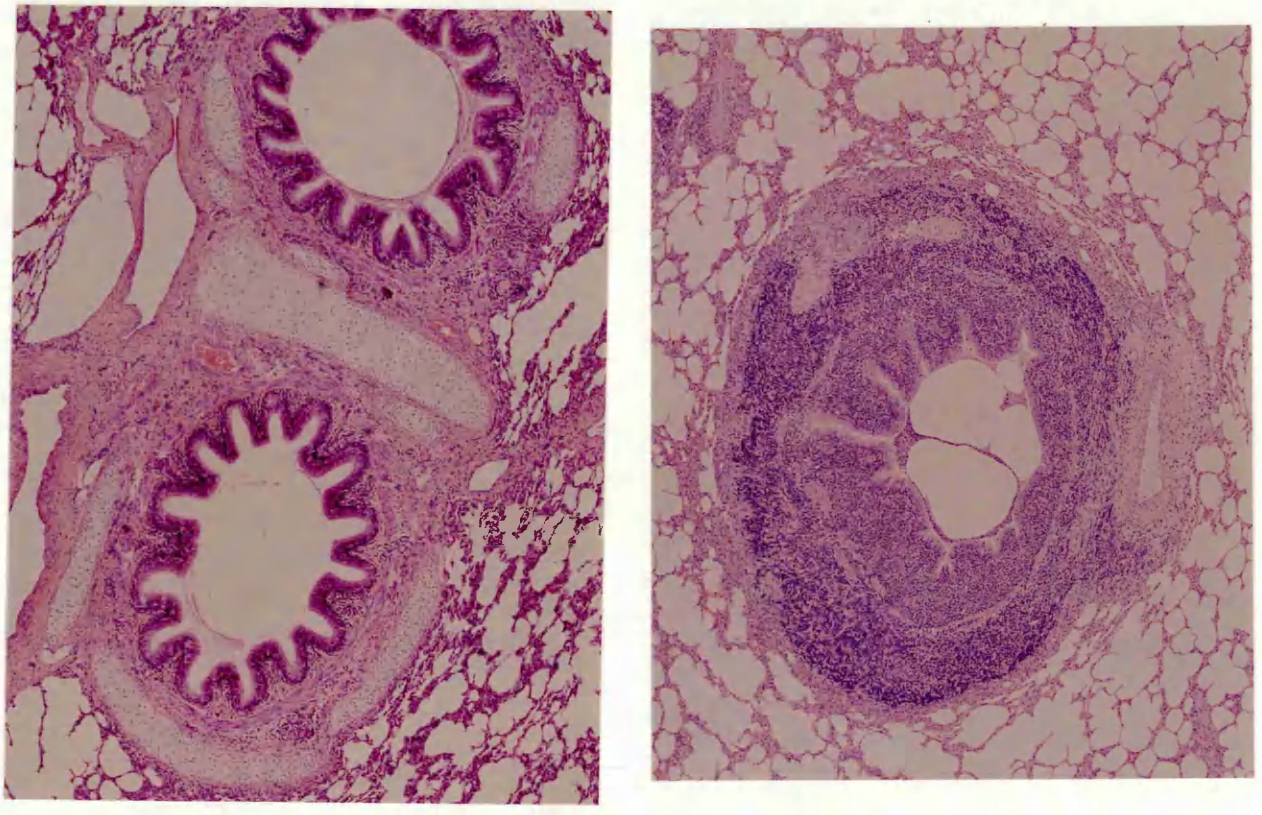


Figure 1.12

Histological sections of small bronchi (left) compared with a small bronchus showing chronic bronchitis and a dense infiltration of lymphocytes, plasma cells and some eosinophils (right) in a pony mare necropsied on Day 56 post-infection with 1,000 *D. arnfieldi* L_3. H & E x 10.

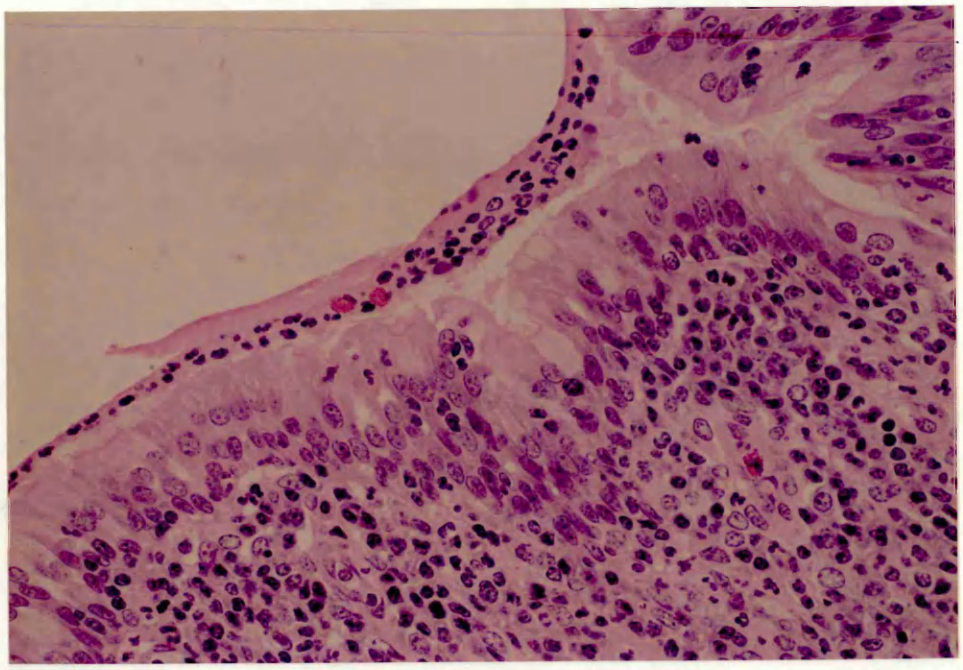


Figure 1.13

Histological section of a bronchiole with mucus and eosinophils in the lumen and lymphocytes, plasma cells and eosinophils in lamina propria from a foal necropsied on Day 70 post-infection with 2,500 *D. arnfieldi* L_3. H & E x 100.

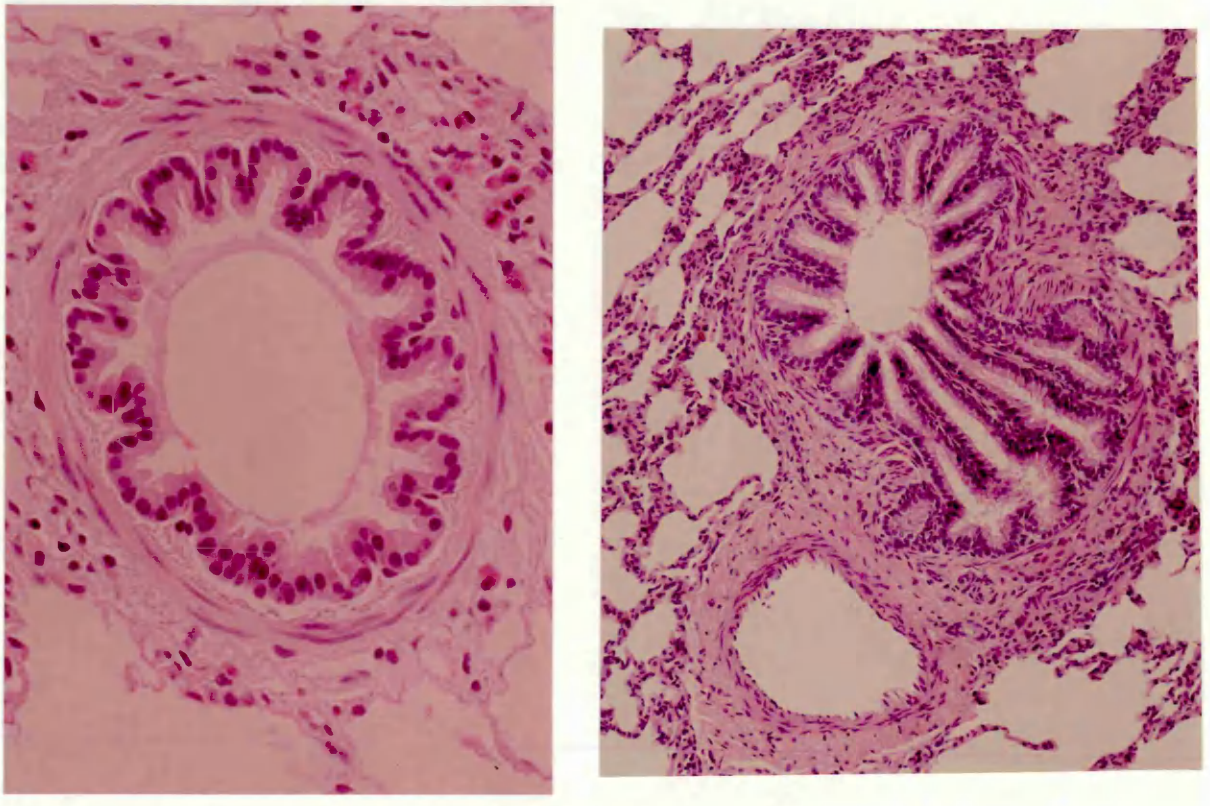


Figure 1.14

Histological sections of a normal bronchiole (left) compared with a severe bronchiolitis with thickening of the bronchiolar wall due to infiltration by lymphocytes and plasma cells with some eosinophils in a foal necropsied on Day 70 post-infection with 2,500 *D. arnfieldi* L_3 (right). H & E x 25.

lumen of the airways.

Scanning Electron Microscopy (SEM)

The SEM appearance of the airways in ponies infected with D. arnfieldi resembled those of normal horses (Pirie, Pirie, Cranston and Wright, 1990). The surface of the trachea, lobar and segmental bronchi were regularly folded and mucus, in varying amounts, was present. The surface cells were well ciliated and protruding from among the cilia were mucus-secreting cells, singly or in small groups (Figure 1.15). Small patches of non-ciliated microvillous cells (Figure 1.16), which have been seen in the larger respiratory passages of normal horses, were found in the trachea of mares 13 and 12. Similar patches occurred in foal 15 in the trachea and small bronchus from the caudal lobe of the lung. The latter site was usually well ciliated although the presence of non-ciliated cells gave a rather "moth-eaten" appearance (Figure 1.17). The surface of the small bronchi had shallow folds and some mucus was present. In the bronchioles, alveolar ducts and alveoli, the number of ciliated cells gradually decreased towards the terminal passages. On the other hand the non-ciliated bronchiolar epithelial (Clara) cells became more numerous until they were the dominant cell in the terminal bronchioles. The latter cells, which were dome-shaped and projected into the bronchiolar lumen, often had clefts on their surfaces (Figure 1.18). Under low power the alveolar surfaces appeared relatively smooth and interalveolar pores (of Kohn) were easily identified.

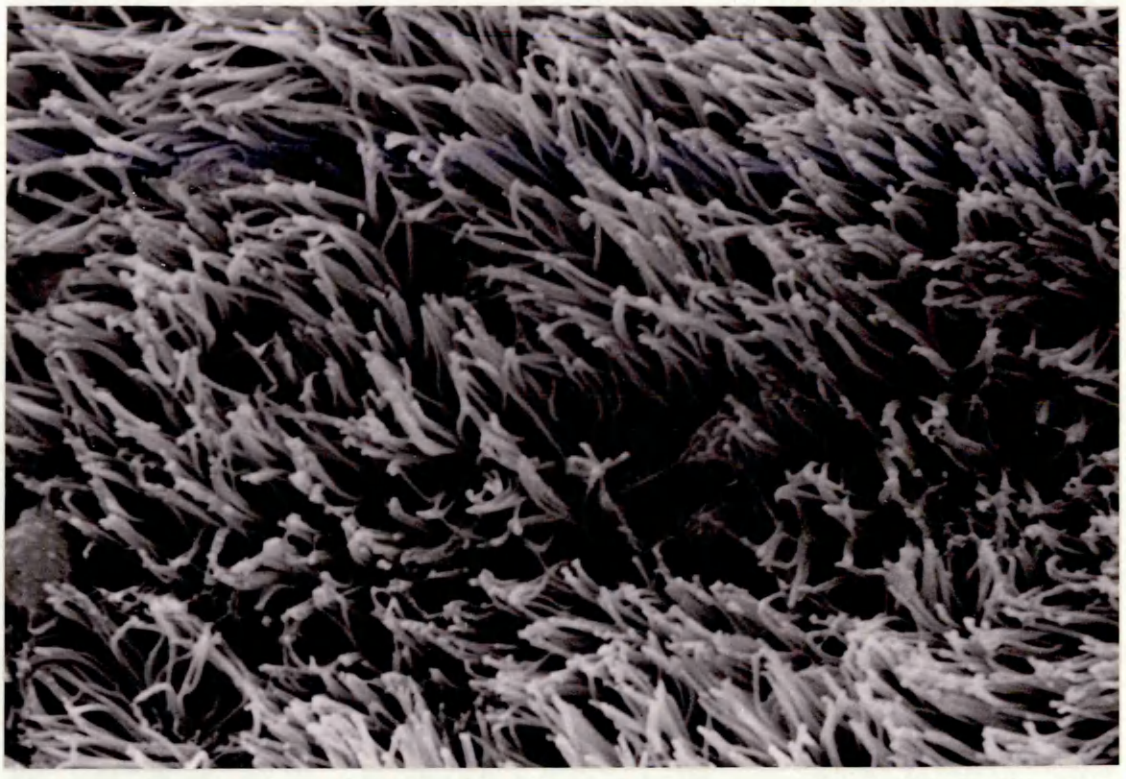


Figure 1.15

Scanning electron micrograph of the ciliated surface of airways in a pony experimentally infected with *D. arnfieldi*

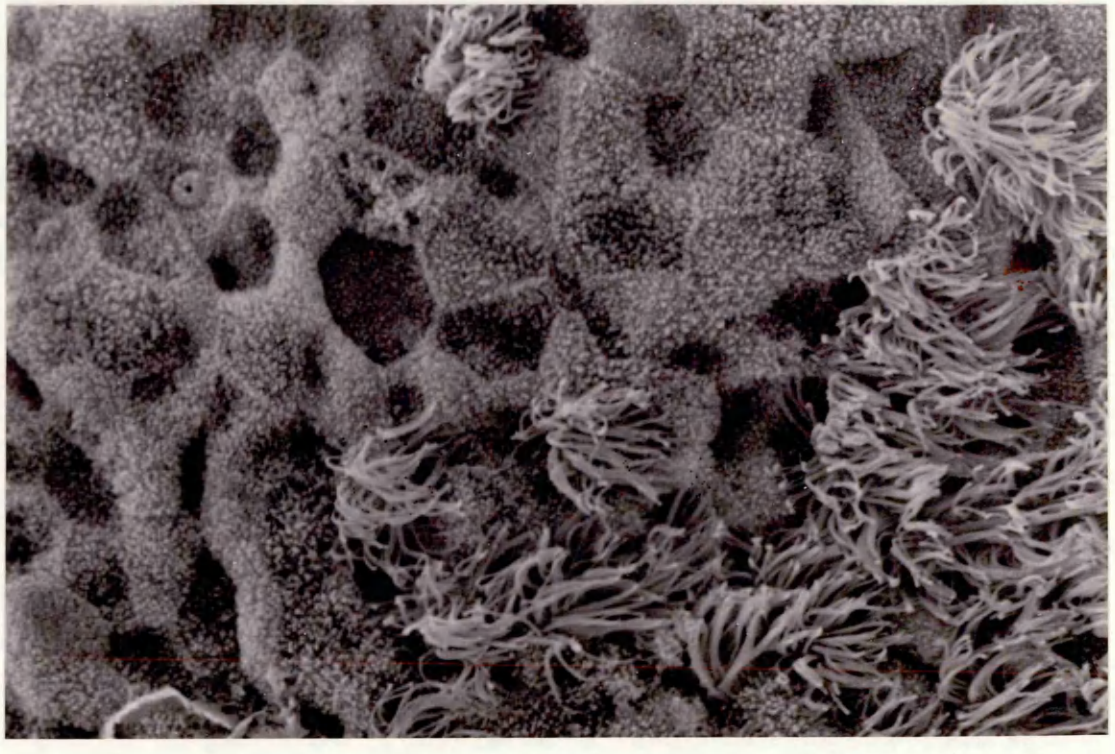


Figure 1.16

Scanning electron micrograph of the trachea of a pony mare experimentally infected with 6,000 *D. arnfieldi* L_3 showing a patch of non-ciliated microvillous cells

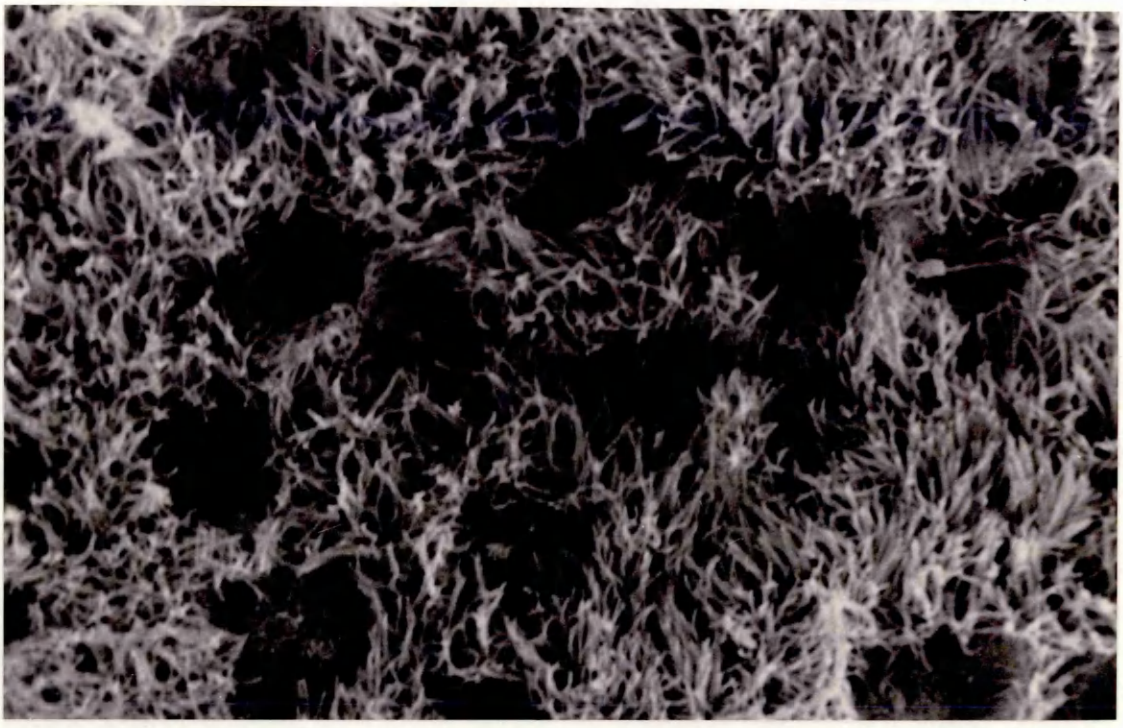


Figure 1.17

Scanning electron micrograph showing patches of non-ciliated cells in a bronchus of a foal experimentally infected with 2,500 *D. arnfieldi* L_3

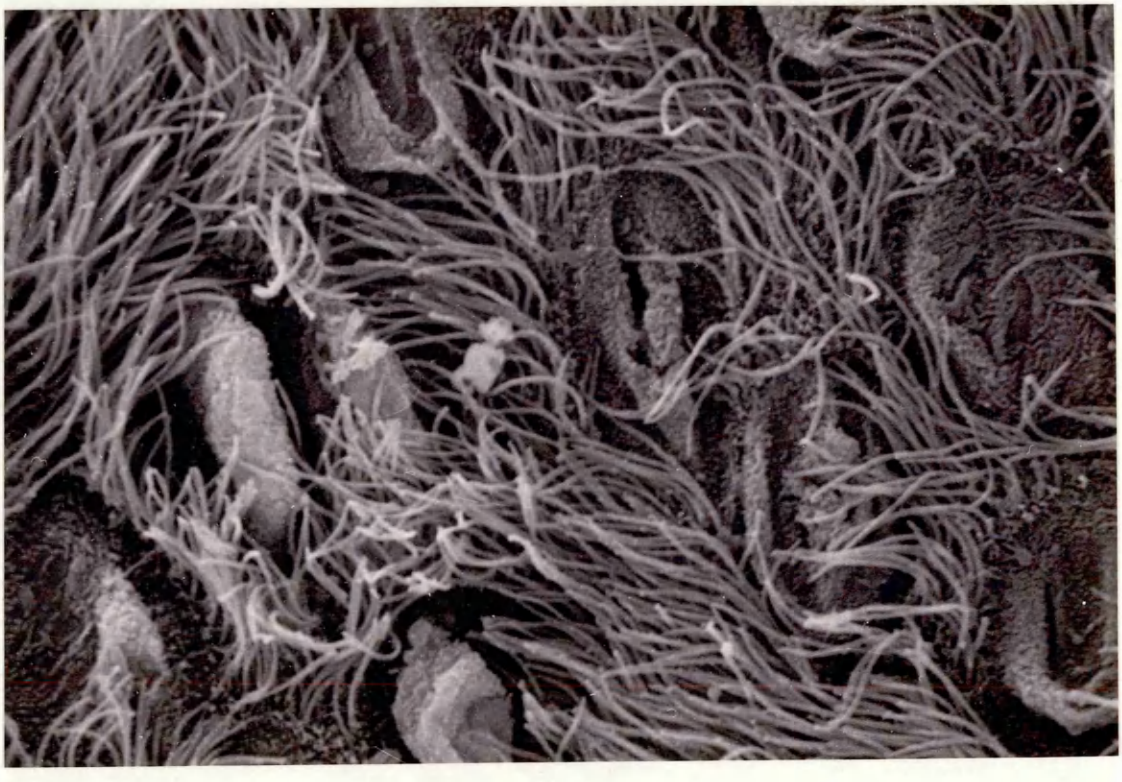


Figure 1.18

Scanning electron micrograph showing non-ciliated epithelial (Clara) cells in a terminal bronchiole of a foal experimentally infected with 2,500 *D. arnfieldi* L_3

DISCUSSION

Worm recoveries at necropsy in most previous studies of experimental D. arnfieldi infections have been generally variable and low (Round, 1972). In the previous study reported here (Experiment One) low numbers of worms were recovered from seven of the ten adult ponies infected, the three negative recoveries being from the lungs of animals killed three and five weeks post-infection, when any lungworms present would have been very small. The mares and foals in this experiment were necropsied in pairs at six, eight and ten weeks post-infection but very few worms were recovered from only one mare (mare 11 - seven worms) and one foal (foal 15 - two worms). The simplest explanation for this is poor infectivity of the D. arnfieldi larvae used in this study: This appears to have been a recurring problem in most experimental lungworm studies.

Despite the low worm establishment however, all three mares showed raised respiratory rates following infection and in two (mares 11 and 12) fairly persistent coughing was noticed from Day 34 until necropsy. The foal from which two worms were recovered showed a milder reaction with a slightly raised respiratory rate and occasional cough between three and five weeks post-infection.

Although these differences in response between the mares and foals in this experiment may have been related to the larger infective dose administered to the mares (6,000 - 10,000 L_3) compared with the foals (2,500 L_3) the findings tend to agree with a previous report of Clayton and Duncan (1981) where, after

exposure to natural infection, adult ponies developed marked clinical signs while foals grazing the same pasture showed no signs although they, unlike the adults, developed patent D. arnfieldi infections.

In spite of the apparent poor worm establishment in this experiment gross lesions of overinflation, commonly associated with D. arnfieldi infection in both horses and donkeys (Nicholls et al, 1979; Clayton and Murphy, 1980), were found in the lungs of the animals necropsied six and eight weeks post-infection although they were minimal in the foals. In contrast both the mare and foal necropsied at ten weeks (mare 12, foal 15) had obvious gross lesions of overinflation. Where present histopathological changes including overinflation, bronchitis, cellular infiltration and excess mucus production generally reflected the severity of both the gross lesions and clinical response and confirmed previous reports of lesions found in association with D. arnfieldi infection (Clayton and Murphy, 1980). An exception was the mare necropsied at ten weeks (mare 12) in which no significant microscopical changes were detected despite the fact that this animal showed the most marked clinical response and gross lung pathology. This latter finding together with the lack of significant changes attributable to D. arnfieldi in the lung tissues of all animals examined by scanning electron microscopy in this study may simply reflect the limited pathology associated with these infections or may be due to technical difficulties in location and selection of suitable

material from the distal airways and lung parenchyma.

The limited conclusions of this experiment are that adult ponies react more severely both clinically and pathologically to experimental D. arnfieldi infection although the results emphasise the unpredictability of infection and inconsistency in relationship between clinical response and underlying pathology.

INTRODUCTION - FIELD STUDIES

In 1952 Akramovski reported a high prevalence of patent D. arnfieldi infections in horses in the Soviet Union which was unrelated to any contact with donkeys. From the information on the epidemiology of D. arnfieldi infection in the Soviet Union it is suggested that larvae do not overwinter on pasture (Akramovski, 1952) hence infective stages of D. arnfieldi are not found until May each year : these larvae are derived from infected horses which are housed during the winter and contaminate the pasture when allowed out to graze the following spring. New patent infections are then seen in young horses from August with the majority appearing in September and October. This is the same timing to that found by Round (1972) in his study of when D. arnfieldi L_1 first appeared in the faeces of donkey foals in England. Round (1972) also reported the results of a limited survey of the prevalence of infection in equines in the U.K. Around this time the overall prevalence of infection in donkeys was found to be 73% although on one of the establishments where the horses and donkeys all grazed together, 80% of the donkeys had patent infections and 6% of the horses were patent. In contrast eight mature hinnies kept with the infected donkeys were found to be negative for D. arnfieldi infection on faecal examination. However one hinny colt introduced to infected donkeys when it was only a few weeks old subsequently developed a patent D. arnfieldi infection with a higher larval output compared with donkeys of a similar age (Round, 1972). Round (1972) also examined 57 Thoroughbred

horses, which had no known contact with donkeys and none of which exhibited any clinical signs of D. arnfieldi infection and found three (5%) were passing D. arnfieldi larvae in their faeces.

Of 55 horses examined at post mortem in Brazil (Ribeiro, Larangeira and Paiva, 1979) five animals (9%) were positive for D. arnfieldi with an average of 2-4 worms being recovered from their lungs. More recently in a post mortem survey of 488 horses in Kentucky (Lyons, Tolliver, Drudge, Swerczek and Crowe, 1985) 11% had D. arnfieldi present in the lungs. The average number of worms recovered was six and these were mainly fifth stage larvae (immature adults). Thirty per cent of those with D. arnfieldi in the lungs also had D. arnfieldi on examination of the faeces however the low numbers of D. arnfieldi found tends to suggest that although the potential for horse to horse transmission exists, it is low. In contrast six mature infected donkeys examined at post mortem (Tolliver, Lyons and Drudge, 1985) were found to have between 3 - 315 worms in the lungs.

In the Netherlands, Boersema and Kalis (1978) found 51% of 249 donkeys with an existing D. arnfieldi infection. No donkey under five months of age had a patent infection and although the number of larvae excreted increased with increasing age the percentage of infected animals did not show any such increase.

Of 423 donkey lungs examined at necropsy over a period of one year in Morocco 48% were found positive for D. arnfieldi (Pandey, 1980). Although D. arnfieldi infection was observed throughout the year there was evidence of increased incidence and

level of infection in donkeys in autumn and winter and to a lesser degree in June. These peaks appeared to be closely related to periods of rainfall and relatively low temperatures.

In a study of 176 donkeys kept on 59 premises in Denmark 87.5% had patent D. arnfieldi infections (Anderson and Fogh, 1981). Thirty-four of a total of 106 horses from 30 of these premises were hospitalised with dyspnoea and coughing although no patent D. arnfieldi infections were detected. Also in Denmark a group of horses was reported to have exhibited chronic coughing of five years duration which appeared three weeks after the arrival of a donkey. Three horses and the donkey had patent D. arnfieldi infection and lungworms were found in the bronchi at post mortem examination of all animals (Nielson and Anderson, 1981).

In Germany, faecal samples from 106 donkeys on 52 farms were taken every 6-8 weeks during 1982 (Heil, 1983). Examination of these showed that patent D. arnfieldi infections were lowest in yearlings (less than 40%) while 57% of 6-10 year olds were infected; only 28% of animals over ten years of age were positive for D. arnfieldi.

These studies indicate that D. arnfieldi is worldwide in distribution and, with the exception of the Soviet Union where there are large horse farms, the donkey appears to be the most significant host in the epidemiology of infection. Also from the information available it appears that retardation of development of D. arnfieldi occurs frequently in the horse regardless of breed or geographical location. As D. arnfieldi larvae

apparently cannot overwinter on pasture in temperate areas the donkey is undoubtedly important as a reservoir of infection (Heil, 1983).

In donkeys, the number infected with D. arnfieldi increases during the first two years of life after which time the number infected tends to remain static while the magnitude of the infection increases over the next few years. Round (1972) reported that 62% of donkey foals developed patent infections while donkeys over three years of age were resistant to reinfection following effective anthelmintic treatment, although an age immunity per se is not thought to occur. The earliest age at which Round found a foal to have a patent infection was 101 days and no patent infections were observed in foals before August of the year of their birth. Development of patency in foals tended to be synchronous and independent of the precise age of the foal. In a later study, Clayton and Duncan (1981) grazed four pony mares and foals and two infected donkey mares and foals together on D. arnfieldi contaminated pasture. In early August, within 11 weeks of turnout, all of the pony and donkey foals were positive for D. arnfieldi upon faecal examination. The general impression from these studies was that adult donkeys with patent infections continued to pass larvae for very long periods while those which did not have patent infections remained negative despite exposure to pastures grazed by heavily infected donkeys.

From the above it is obvious that, as with experimental infections, there is limited information on naturally occurring

D. arnfieldi infection. In order to assess the current situation in donkeys and horses in the UK two major surveys were undertaken. In the first, the Donkey Sanctuary in Devon was the main source of material and information. In the second survey, carried out in the West of Scotland, the majority of animals were horses or ponies but a few donkeys resident with these animals were included. During the Scottish survey there was an opportunity to investigate an outbreak of coughing, in a group of horses and ponies, which followed the recent introduction to the pasture of two donkeys with patent D. arnfieldi infections. Finally a small field study was carried out using donkeys resident at the Glasgow University Veterinary School in an attempt to provide more information on the acquisition of D. arnfieldi by young donkeys at pasture.

RESULTS

Donkey Sanctuary Survey

Detailed results of the faecal examinations for D. arnfieldi infection of all new arrivals at The Donkey Sanctuary from 1986 - 1988 are shown in Appendix 1.5. Of the total of 698 donkeys sampled 34% were positive and over the 3-year sampling period there was a decrease in the numbers positive from 40% in 1986 to 35% in 1987 and 23% in 1988 (Table 1.4). This reduction in positive cases in 1988 compared with 1986 and 1987 was significant ($p < 0.05$). When the numbers of positive animals within different age groups were compared (Table 1.5) these ranged from 30% - 42% but there were no significant trends.

Table 1.4

Donkey Sanctuary Data for Three Years Showing Total Numbers of Donkeys Tested, Numbers Positive, Percentage Positive and Mean Faecal *Dictyocaulus arnfieldi* Larval Counts

	Number Tested	Number Positive	% Positive	Mean *D. arnfieldi* Larval Count (L_1/50 g Faeces)
1986	264	105	39.8	80
1987	258	90	35.3	705
1988	176	41	23.3	783
Total	698	239	34.2	-

Table 1.5

Donkey Sanctuary Data over a Three-Year Period showing Total Numbers of Animals of Different Age Groups Tested, the Numbers and Percentage Positive and the Mean Faecal *Dictyocaulus arnfieldi* Larval Counts

Age	Number Tested	Number Positive	% Positive	Mean *D.arnfieldi* Larval Count (L_1/50 g Faeces)
0 - 2	41	15	36.6	931
3 - 5	78	26	33.3	323
6 - 10	117	49	41.9	213
11 - 15	125	50	40.0	160
16 - 20	117	36	30.8	284
21 - 25	75	24	32.0	1221
26 +	121	36	29.7	481
Unknown	25	2	8.0	314
Total	698	239	34.2	-

Although some statistically significant differences were found when age groups within sample years, age groups between sample years or season of sampling were compared (data presented in Table 1.6), no relevant trends emerged.

These results suggested that the Chi Square test was not an ideal method of statistical analysis especially as the number of variables examined required numerous repetitions hence increasing the risk of errors. A more accurate method of analysis to cope with the variables - age, year and season of testing - is log linear analysis, but this is a complex test and was not considered appropriate for the basic analysis of the results obtained in this survey.

West of Scotland Survey

To try to establish the prevalence of patent D. arnfieldi infections in a random equine population a survey was conducted of animals in various types of horse establishments in the West of Scotland : these included riding schools, livery yards, stud farms and dealers' yards as well as private stables each with a minimum of three resident animals. Fresh faecal samples were collected and examined by both Baermann and McMaster techniques. Although detailed histories were not available, all animals were known to have been at pasture at some time.

A total of 720 animals were sampled. Of these 681 were horses or ponies and 39 were donkeys. Eleven of the donkeys (28%) had patent D. arnfieldi infections, while samples from two horses (0.3%) proved positive for D. arnfieldi larvae. Fifty-three (8%) of the horses in the survey were known to have had

Table 1.6

Donkey Sanctuary Data Showing Numbers of Animals Tested and Numbers Positive in Years, Seasons and Age Groups

		Nos. Tested	Nos. +ve	Nos. Tested	Nos. +ve	Nos. Tested	Nos. +ve	Nos. Tested	Nos. +ve	Nos. Tested	Nos. +ve	Nos. Tested	Nos. +ve	Nos. Tested	Nos. +ve	Nos. Tested	Nos. +ve
	Year	Age of Donkey															
		0 - 2		3 - 5		6 - 10		11 - 15		16 - 20		21 - 25		26 +		Unknown	
Spring																	
March)	1986	5	1	10	5	20	10	16	8	20	7	5	1	14	10	2	0
April)	1987	4	3	6	2	11	7	10	4	7	3	4	4	10	1	2	0
May)	1988	2	0	4	0	3	0	2	1	5	1	4	1	9	1	3	0
Summer																	
June)	1986	8	4	9	3	14	7	12	5	12	2	5	2	9	3	1	0
July)	1987	8	2	12	6	8	3	11	5	14	6	8	3	11	3	3	0
August)	1988	3	2	3	1	2	0	13	3	13	1	11	4	10	2	7	1
Autumn																	
Sept.)	1986	3	0	7	3	22	8	20	9	10	6	7	2	5	1	1	0
Oct.)	1987	2	0	11	1	12	5	15	5	7	3	11	3	11	3	3	0
Nov.)	1988	0	0	1	0	8	1	11	5	5	0	7	2	10	2	3	1
Winter																	
Dec.)	1986	3	1	10	4	7	4	9	4	13	4	8	2	13	3	0	0
Jan.)	1987	3	2	5	1	9	4	5	1	11	3	5	0	19	5	0	0
Feb.)																	

contact with donkeys but none of these proved positive for D. arnfieldi while the two horses with patent infections, as far as was known, had no previous contact with donkeys and neither had shown any clinical signs of infection.

Investigation of an Outbreak of Coughing in Horses Associated with Donkeys Infected with D. arnfieldi

During the period of the West of Scotland survey reported above an outbreak of coughing occurred in a small group of five horses grazing with two donkeys. The donkeys had been purchased from a market in September four weeks prior to the first appearance of clinical signs in the horses. All seven animals were clinically examined and blood and faecal samples collected before they were treated by the owner with fenbendazole at 7.5 mg/kg daily for five days: the donkeys were then moved to separate grazing. Two weeks after they were first seen all seven animals were re-examined. A chestnut hunter gelding had been introduced into the field with the donkeys between visits but on examination no clinical abnormalities associated with D. arnfieldi infection were detected in this animal.

The results of the clinical and faecal examinations are presented in Table 1.7.

These limited observations suggest that the donkeys introduced D. arnfieldi infection to this group of horses which resuled in clinical signs which responded to the owners treatment with five daily doses of fenbendazole. Treatment reduced strongyle egg counts in all the animals and D. arnfieldi larvae in the faeces of the one donkey from which a post-treatment

Table 1.7

Clinical and Parasitological Findings in Animals Involved in an Outbreak of Coughing

Related to Recent Contact with *D. arnfieldi*

Animal Description	Clinical Findings Day 0			Clinical Findings Day 14			Faecal Worm Egg/Larval Counts			
							D 0		D 14	
	Respiratory Rate/Min	Coughing	Lung Auscultation	Respiratory Rate/Min	Coughing	Lung Auscultation	Strongyles e.p.g.	*D.arnfieldi* l.p.g.	Strongyles e.p.g.	*D.arnfieldi* l.p.g.
Shetland Mare	28	Frequent	Harsh/ Squeaks	36	Moderate	Squeaks	2,300	-ve	100	-ve
Chestnut 2 yr old Pony	30	Frequent	Harsh/ Squeaks	30	Occasional	Squeaks	2,550	-ve	50	-ve
Grey Pony Mare	36	Occasional	Normal	15	None	Normal	1,650	-ve	-ve	-ve
Bay Hunter Mare	20	None	Normal	18	None	Normal	2,600	-ve	50	-ve
Grey Hunter Gelding	40	None	Harsh	24	None	-	500	-ve	50	-ve
Donkey Mare	15	None	Normal	36	None	Normal	450	103	NS	NS
Donkey Gelding	32	None	Slightly Harsh	39	None	Normal	550	34	-ve	2

sample was obtained. The possibility remains, however, that transmission of some other respiratory pathogen from the donkeys to the horses may have been involved and that the apparent clinical response to anthelmintic treatment was fortuitous.

Donkey Grazing Study

The animals used in this experiment were three adult donkeys, all of which had a known grazing history and two donkey foals, nine and ten months old, which had been reared worm free. Although only one of the adults (No. 20A) had a current patent D. arnfieldi infection the other two were known to have had patent infections within the last three years. All animals had been housed over the winter and were turned out together, on 6th April, 1988, into a two acre paddock which had been grazed by infected donkeys the previous year.

The donkeys had blood and faecal samples taken weekly. The blood samples were stored for later use in the D. arnfieldi ELISA while the faecal samples were examined for D. arnfieldi L_1 and strongyle eggs. If the faecal strongyle egg counts became high the donkeys were treated with pyrantel embonate (Strongid-P, Pfizer, Sandwich, Kent), which is not effective against D. arnfieldi, at a dose rate of 19 mg/kg bodyweight. The donkeys were observed daily for any signs of respiratory disease.

The faecal strongyle egg counts and D. arnfieldi larval counts are shown in Table 1.8.

Table 1.8

Donkey Grazing Study - Results of Faecal Examination

Date	20A		21A		22A		23F		24F	
	Strongyles e.p.g	D.a. l.p.g.	Strongyles e.p.g.	D.a. l.p.g.	Strongyles e.p.g.	D.a. l.p.g.	Strongyles e.p.g.	D.a. l.p.g.	Strongyles e.p.g.	D.a. l.p.g.
6/4/88	200	64	0	0	50	0	0	0	0	0
13/4/88	250	109	350	0	0	0	0	0	0	0
20/4/88	250	100	300	0	ns	ns	0	0	0	0
23/4/88	200	115	250	1	200	1	0	0	0	0
30/4/88	50	36	50	0	0	8	ns	ns	0	0
12/5/88	200	79	240	0	450	0	0	0	0	0
23/5/88	150	101	600	0	50	0	0	0	ns	ns
25/5/88	150	119	1000	0	200	0	ns	ns	50	0
6/6/88	300	150	1750	0	100	0	ns	ns	100	0
8/6/88	0	101	0	0	0	0	0	0	0	0
15/6/88	0	138	0	0	0	0	0	0	0	0
21/6/88	0	138	0	0	0	0	0	0	0	0
6/7/88	ns	ns	150	0	0	0	ns	0	50	ns
12/7/88	50	36	300	0	800	0	150	0	900	0
19/7/88	300	161	1650	0	1550	0	750	0	1750	0
26/7/88	100	210	300	0	150	0	100	0	50	0
3/8/88	50	176	200	0	100	0	0	0	50	0
10/8/88	4	108	100	ns	ns	ns	0	0	0	0
17/8/88	0	70	0	0	350	0	50	0	0	0
23/8/88	0	97	0	0	56	0	50	0	0	0
1/9/88	0	121	0	0	0	0	0	0	0	0
14/9/88	0	48	100	0	550	0	650	0	ns	0
21/9/88	0	71	0	0	550	0	0	0	50	0
29/9/88	50	54	300	0	650	0	300	0	1100	0

ns - no sample

No clinical signs were observed in any of the five donkeys during the 4 - 5 month observation period. The adult animal with the patent D. arnfieldi infection remained positive throughout while the remaining two adult donkeys and the two donkey foals which were initially negative for D. arnfieldi, remained negative until the end of the study.

DISCUSSION

Recent surveys of the prevalence of D. arnfieldi infection in donkeys from different parts of the world show that, with the exception of one report from Denmark where Anderson and Fogh (1981) reported an 87.5% infection rate, this appears to be decreasing from the 73% level reported by Round in 1972. For example Boresema and Kalis (1978) found a prevalence of 51% in The Netherlands, Pandey (1980) found 48% of donkeys to be infected in Morocco while Heil (1983) in Germany found patent infections in less than 40% of yearlings, 57% of 6-10 year olds and only 28% of donkeys over ten years old. The overall prevalence found in the Donkey Sanctuary survey reported here was 34% and although, as reported by Heil (1983), the 6-10 year old group had the greatest proportion of animals positive (42%) and the younger donkeys the least (1-2 year olds - 37% and 3-5 year olds - 33%), the decrease to 28% found by Heil in animals over ten years of age was not seen in the U.K. survey where the percentage infection rates were - 11-15 years - 40%; 16-20 years - 31%; 21-25 years - 32% and over 26 years - 30%.

As both the U.K. surveys were based on the detection of larvae in faeces by the Baermann technique the considerable decrease in the level of infection between 1972 and 1986/88 cannot be attributed to differences in technique. The most likely explanation for the lower level of infection is the increased awareness of the importance of anthelmintic treatment of animals combined with the availability of new and effective anthelmintics which were not available in 1972.

In the early 70's the most modern anthelmintics commercially available were diethylcarbamazine, tetramisole/levamisole and some of the early benzimidazole compounds. Round (1972) found diethylcarbamazine by intramuscular injection, which was effective against D. viviparus in cattle, to be ineffective while tetramisole at 15 mg/kg gave variable reductions in faecal larval excretion. Thiabendazole, used at 440 mg/kg proved the most effective. A single dose gave promising but inconsistent results but two doses given 24 hours apart gave a reduction in larval output of 90-100% in samples taken 14 and 28 days post treatment.

At the same time trials using levamisole and cambendazole were carried out in donkeys in Germany (Enigk and Weingartner, 1973): a single oral dose of 15 mg/kg of levamisole produced a 78% reduction in faecal larval counts 41 days post treatment while intramuscular doses at 5 and 10 mg/kg gave reductions of 94% and 98%, although the higher doses produced slight reactions at the injection sites. Two intramuscular doses of levamisole at 5 mg/kg, 3-4 weeks apart, proved the most effective while cambendazole, given orally at 30 and 40 mg/kg, was unsuccessful

in eliminating infection.

In subsequent trials, oxibendazole was found to have no activity against D. arnfieldi (Kates, Colglazier and Enzie, 1975) while mebendazole, used at 15-20 mg/kg, was 99% effective in eliminating D. arnfieldi larvae from the faeces (Clayton and Neave, 1979).

In studies to define an effective therapeutic regimen against D. arnfieldi infection in donkeys to reduce the risk of transmitting infection to horses and, by removing worms from the lungs, to reduce the donkey's apparent susceptibility to other respiratory pathogens, Clayton and Trawford (1981) proposed minimum doses of 15 mg/kg mebendazole for five days. Mebendazole also proved effective in the treatment of yearling horses when two doses of 20 mg/kg given one month apart eliminated larvae from the faeces of these animals (Rickard and James, 1976).

Febendazole (FBZ) has been used in a number of anthelmintic trials in horses and donkeys (Enigk and Dey Hazra, 1976). A single dose of 7.5 mg/kg FBZ is effective against intestinal parasites in both donkeys and horses but neither single doses of 30 mg/kg nor two consecutive daily doses of 15 mg/kg eliminated adult lungworm infection in donkeys; both, however, produced a temporary 50-70% reduction in larval output but this had increased to pre-treatment levels within three weeks (Urch and Allan, 1980).

Duwel (1983) reported further studies on FBZ in donkeys at dose rates of 7.5 - 10 mg/kg for five days. This regime was superior to single doses but, in some cases, reduction in larval

output was less than 95%. Duwel suggested that the presence of mucus in the bronchi protected the worms from the action of the drug.

Perhaps the most significant development in anthelmintic therapy of D. arnfieldi was the recent introduction of ivermectin. When used in a trial involving 12 ponies experimentally infected with D. arnfieldi, mature D. arnfieldi were found in all six of the untreated controls at necropsy while no D. arnfieldi were found in the six ponies which had received ivermectin at 200 mcg/kg 15 days prior to necropsy (Britt and Preston, 1985). It is likely that over the last five to 15 years, the use of anthelmintics, especially ivermectin, has resulted in the observed decline in the prevalence of D. arnfieldi infection.

In our survey of horses and donkeys in the West of Scotland 28% of the donkeys but less than 1% of horses had patent infections. A surprising finding was that the horses with patent infections had no current or recent known contact with donkeys and that some of the horses with patent infections had not shown any coughing. Although horses can harbour patent infections without signs of disease, the history of the field outbreak was more typical: in this, the horses and ponies started coughing within a month of the introduction of the infected donkeys. This was not surprising since climatic conditions at the time (September) with moderate temperatures and adequate moisture, were ideal for the rapid development of larvae to the infective stage and, as shown by the experimental studies, lesions are

present in the lungs by three weeks post infection. This study reflected the variability in response to D. arnfieldi infection with some of the animals showing severe clinical signs while others in the group had either very mild or no clinical signs. As these animals were not necropsied it can only be assumed from the history, clinical signs and response to treatment that D. arnfieldi was responsible for the disease syndrome shown.

In the donkey grazing study the adult donkeys behaved as expected from previous studies (Clayton and Duncan, 1981; Round, 1972) in that the donkey with the patent infection (20A) maintained this with only minor fluctuations in output while the other two adult donkeys, known to have had previous exposure to D. arnfieldi infection, remained non patent throughout. In contrast the two naive young donkeys (23F and 24F) did not behave as expected in that they did not acquire detectable patent infections in late summer or by the end of September when the experiment was ended. Although the number of animals used in this experiment was small, the pasture was deliberately 'overgrazed' to try to ensure exposure to D. arnfieldi infective larvae by all the animals. It is possible that due to weather conditions, no infective larvae were available on the pasture during the grazing period which, although unlikely, makes the results difficult to interpret. Alternatively, since it was not possible to necropsy these donkeys to check for worms in the lungs, there is the possibility that the young animals, in which L_1 were not detected in the faeces, could have behaved like

horses and developed non-patent infections with retardation of development of D. arnfieldi.

The failure of these young donkeys to develop patent infections while grazing alongside an infected animal on pasture grazed by infected donkeys the previous year, was unexpected. One possible explanation is that the donkey 'foals' in this study were born the previous year and were around nine months old at turnout. Their initial exposure to infection, if it occurred, was therefore a lot later than most foals which graze alongside their dams from birth: also these 'foals' started grazing immediately upon turnout in April rather than gradually acquiring the grazing habit. Another possibility is that, due to the colder weather at this time, only few infective D. arnfieldi larvae were available which allowed these older donkey foals to acquire some immunity by trickle exposure without developing patent infections. Whatever the reasons, the fact that these young donkeys failed to show evidence of D. arnfieldi infection makes it impossible to draw any firm conclusions from this study.

SECTION 2

STUDIES ON THE DEVELOPMENT OF AN ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA) FOR THE DETECTION OF *Dictyocaulus arnfieldi* INFECTION

INTRODUCTION

Due to difficulties in the clinical diagnosis of lungworm infection in horses, an immunoassay was sought to provide a diagnostic laboratory test for D. arnfieldi infection. Two types of immunoassay, enzyme immunoassay (EIA) and radioimmunoassay (RIA), are currently used in the study and diagnosis of infectious diseases although EIA is rapidly superseding RIA. The first EIA was reported in 1971 and since that time EIAs have provided an extremely sensitive and precise method of analysing large numbers of samples rapidly for a variety of biological parameters including infectious disease agents such as viruses, bacteria, fungi and parasites. In general, EIA and RIA have similar sensitivities but the greater versatility and simplicity afforded by the EIA (Table 2.1) has led to its rise in popularity and it is now the assay of choice for detection and identification of disease agents; discrimination of disease agents; quantification of a disease agent; identification of specific antibodies and quantification of specific antibody isotypes. The difference between RIAs and EIAs is in their detection system; whereas RIAs use radionuclides which decay, EIAs use enzymes which are biological catalysts. EIAs operate via an enzyme attached to one of the reagents used in the test which, on addition of the appropriate enzyme substrate solution, then produces a colour change which can be measured by eye or by spectrophotometer. In most EIAs the solid phase is a plastic, onto which proteins and carbohydrates can be passively adsorbed; these are known as Enzyme Linked Immuno-Sorbent Assays (ELISAs)

Table 2.1

Comparison of Enzyme Immunoassay (EIA) and Radioimmunoassay (RIA)

	EIA	RIA
Handling of Reagents	Some mutagenic substances	Caution
Disposal of Reagents	No control	Strict Legislation
Storage of Reagents	Stable long term	Half-life dependent
Reading	By eye/Sophisticated machines	Sophisticated machines
Reading Time	Rapid	Slow
Availability of Reagents for Infectious Diseases	Good	Poor
Flexibility	Flexible	Flexible
Kits	Good	Poor
Expense	Moderate/Low	High
Training for Use	Simple	Simple
Field Use	Yes	No
Labelling in Laboratory	Simple	Difficult

and the most common format is a 96 well microplate for use in the 96 well micro ELISA. The advantage of the solid phase is that one of the components can be attached to it, further reagents added and, following suitable incubation, unreacted reagents washed away giving a simple separation of bound and free reagents. Assays involving such a separation step by washing are heterogeneous assays.

The basic principles which apply to all ELISAs are listed.

(a) Adsorption of antigen/antibody to the plastic solid phase (coating).

(b) Addition of sample and subsequent reagents.

(c) Incubation of reagents.

(d) Separation of bound and free reactants by washing.

(e) Addition of enzyme labelled reagents (conjugates).

(f) Addition of colour development system/enzyme detection (substrates).

(g) Reading of assay - visual or spectrophotometric.

Utilising these basic principles there are four types of ELISA.

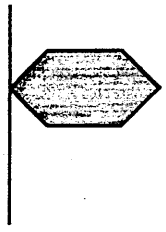
ELISA FORMATS

(1) Direct ELISA (Figure 2.1)

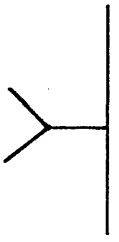
Antigen or antibody is adsorbed onto the solid phase and reacted directly with enzyme labelled antiserum or enzyme labelled antigens. The disadvantages of this assay are, with labelled antibody, low levels of antigen adsorption from crude samples due to competition for plastic sites by contaminants also

Figure 2.1

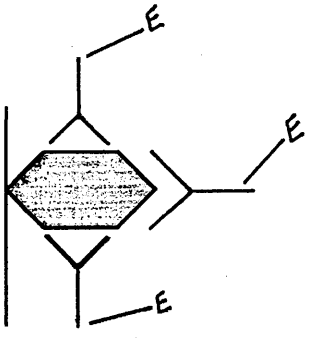
Direct ELISA using labelled antibody or labelled antigen.

1. 

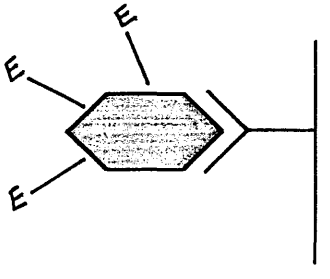
Passive adsorption of antigen/antibody to the plate



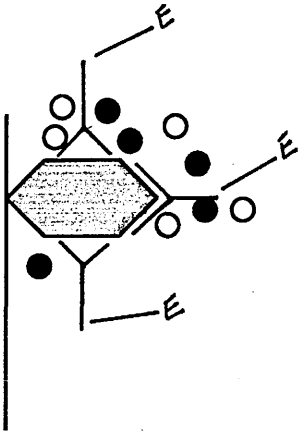
WASH

2. 

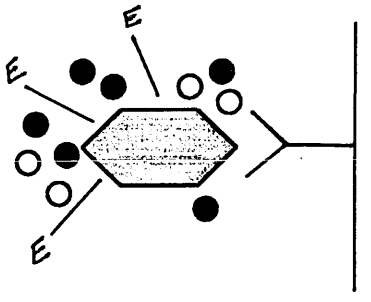
Addition and incubation of enzyme labelled antibody/antigen.



WASH

3. 

Addition and incubation of colour development system.



READ

all sera raised against different antigens have to be labelled giving poor applicability to diagnostic problems: In general antigens are rarely labelled.

(2) Indirect ELISA - (Figure 2.2)

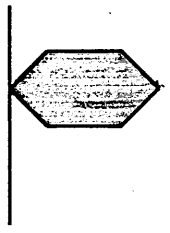
The antigen is adsorbed onto the solid phase and any non-adsorbed antigen is washed away before the addition of serum containing antibodies against the antigen in a blocking buffer which prevents the non-specific adsorption of protein onto any unoccupied sites on the plastic. After incubation any unbound antibodies are washed away enabling bound antibody to be detected, following incubation, with a single optimal dilution of anti-species antibody conjugated to an enzyme. The amount of specific antibody binding to the antigen is quantified after addition of the colour development system. This type of assay is used for the detection of specific antibodies present in serum samples. The specificity is dependant upon the purity of the antigen attached to the solid phase. Indirect assays have the advantage over direct assays in that only a single anti-species enzyme conjugate is required.

(3) Direct Sandwich ELISA - (Figure 2.3)

Antibody (IgG Fraction of whole serum) is attached to the solid phase at a constant optimal dilution and, following incubation, unadsorbed antibody is washed away before antigen is added in a blocking buffer. After incubation unbound antigen is washed away to enable the detection of bound antigen by the addition of enzyme labelled antibody specific for the 'captured' or 'trapped' antigen. This antibody can be the same as that used

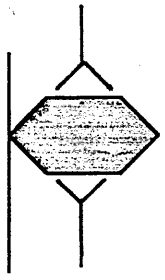
Figure 2.2.

Indirect ELISA

1. 

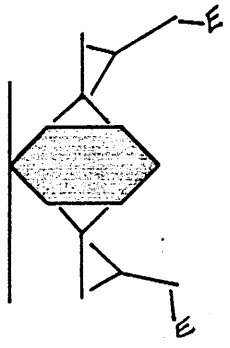
Passive adsorption of antigen.

WASH

2. 

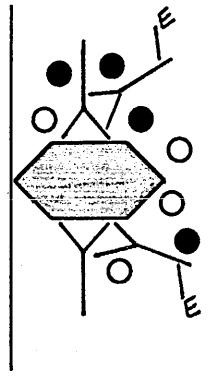
Addition of antibody directed against antigen.

WASH

3. 

Addition of enzyme labelled anti-species antibody.

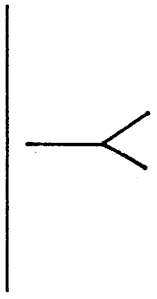
WASH

4. 

Addition of colour development system.

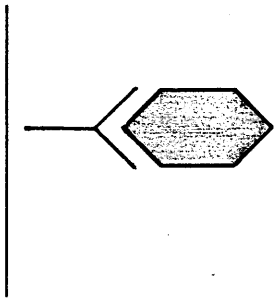
READ

Figure 2.3. **Direct Sandwich ELISA**

1. 

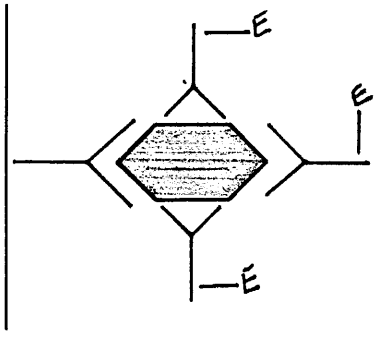
Passive adsorption of antibody.

WASH

2. 

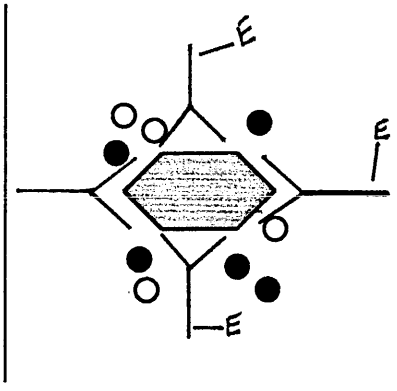
Addition of antigen.

WASH

3. 

Addition of enzyme labelled antibody against antigen.

WASH

4. 

Addition of colour development system.

READ

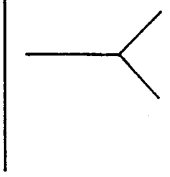
on the solid phase, produced in the same or a different species as the trapping antibody. After incubation and washing away of unbound conjugate, the colour detection system is added and the test read. This type of assay enables the detection of some antigens which, due to scarcity or contamination, cannot be attached directly to the solid phase. The 'trapping' antibody must bind specifically to the antigen to be detected which itself must contain a minimum of two epitopes to permit the formation of the 'sandwich'. The disadvantage is that all detecting antisera have to be enzyme conjugated, however this can be overcome by using an **indirect sandwich ELISA (Figure 2.4)** in which the second antibody is produced in a different species from the trapping antibody. This second antibody can be detected using a species specific antiserum conjugate which does not react with the antibody on the plastic. Any disadvantage of this extra step is outweighed by the advantage that many second antibodies may be titrated using one conjugate.

(4) Competition ELISA

(a) Direct Antibody Competition - (Figure 2.5)

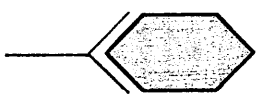
Adsorption of antigen onto the solid phase is followed by washing and the addition of specific antibody labelled with enzyme at a pre-titrated dilution to ensure the saturation of antigenic sites so none remain available for further antibody competition. This acts as a positive control. In this ELISA the interaction is disrupted by prior mixing of the test sample (non-labelled) with the labelled antibody. The non-labelled

Figure 2.4. **Indirect Sandwich ELISA**

1. 

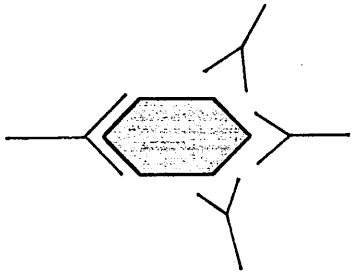
Passive adsorption of antibody.

WASH

2. 

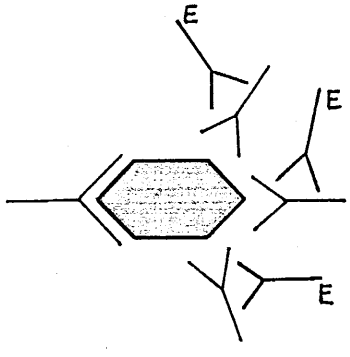
Addition of antigen.

WASH

3. 

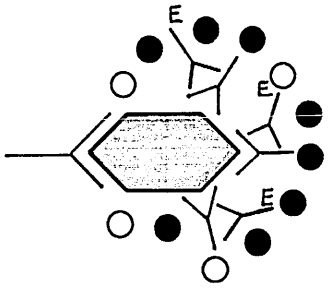
Addition of antibody from different species against antigen.

WASH

4. 

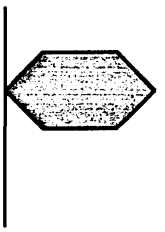
Addition of enzyme labelled anti-species (direct against antibody) antibody.

WASH

5. 

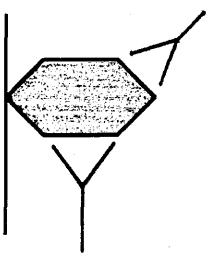
Addition of colour development system.

Figure 2.5. **Direct Antibody Competition ELISA**

1. 

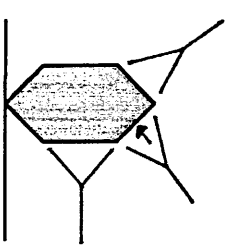
Passive adsorption of antigen.

WASH

2. 

Addition of antibody at various dilutions.

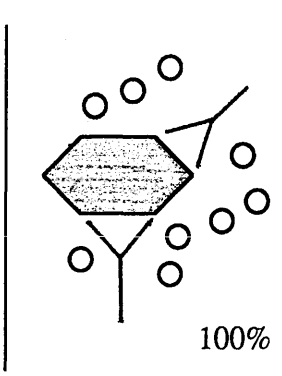
WASH

3. 

Blocked

Addition of enzyme labelled antibody (pre-titrated to give optimal colour development).

WASH

4. 

100%

Addition of colour development system.

READ

(competing) antibody, at a range of dilutions, replaces some or all of the conjugated antibody. After washing and development of the assay, the amount of 'competing' antibody is read as a reduction in the colour expected compared to the colour obtained without the addition of the non-labelled competing antibody. This type of assay is gaining in importance with the increase in the use of monoclonal antibodies (mAbs). Any species can provide the serum to be used as the competitor.

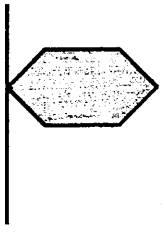
(b) Direct Antigen Competition - (Figure 2.6)

In this assay, antigen is the competing substance for pre-titrated conjugated antibody. If the labelled antibody reacts with the competitor antigen in the liquid phase it will not bind to the solid phase antigen and will be washed away after incubation, hence labelled antibody is no longer present to react with the antigen in the solid phase. This will reduce the expected colour.

(c) Indirect Antibody Competition - (Figure 2.7)

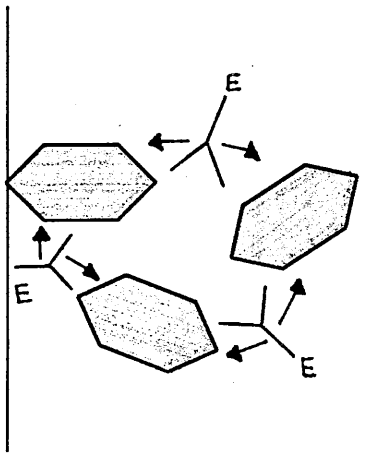
This is the same as the indirect ELISA except for the addition of competing (test) antibody to the solid phase antigen before or together with the pre-titrated specific antibody. The competing antibody must be produced in a different species from the pre-titrated antibody to prevent the anti-species conjugate reacting with both. The binding of the competing antibody to the antigen prevents the pre-titrated (labelled) antibody binding and thus reduces the colour produced. The greater the amount of antibody in the test sample the less colour will be produced.

Figure 2.6. **Direct Antigen Competition ELISA**

1. 

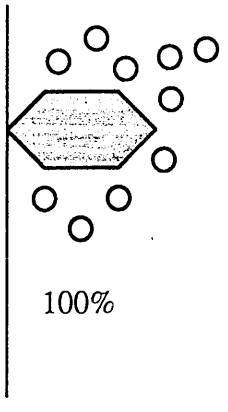
Passive adsorption of antigen.

WASH

2. 

Simultaneous incubation of free antigen with enzyme labelled antibody (pre-titrated) directed against antigen on plastic.

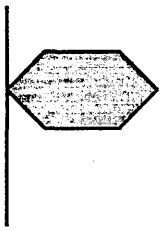
WASH

3. 

Addition of colour development system.

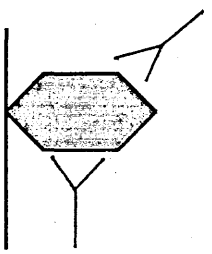
READ

Figure 2.7. **Indirect Antibody Competition ELISA**

1. 

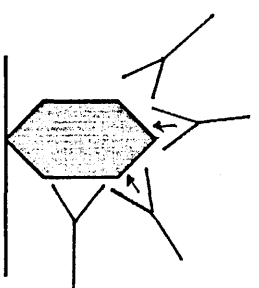
Passive adsorption of antigen.

WASH

2. 

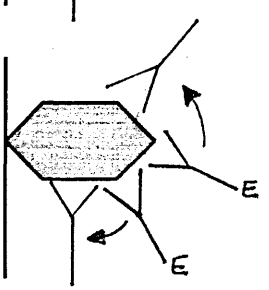
Addition of test antibody
at various dilutions.

WASH

3. 

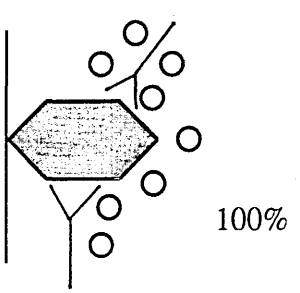
Addition of antibody
(pre-titrated) standard serum.

WASH

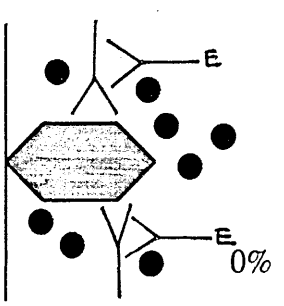
4. 

Addition of anti-species
conjugate against standard
antiserum.

WASH

5. 

Addition of colour
development system.



READ

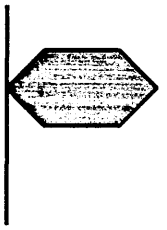
(d) Indirect Antigen Competition - (Figure 2.8)

In this type of assay, antibody is pre-titrated against the solid phase antigen by the use of anti-species conjugate. The addition of antigen in the liquid phase provides the competition which appears as a reduction in the colour produced.

THE *D. arnfieldi* ELISA

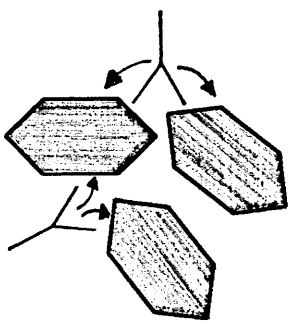
As helminth parasites are among the most complex infectious disease agents infecting mammals, the host's antibody response to helminth infection is the most diverse with the production of antibodies of all isotypes - IgA, IgE, IgG and IgM. Antibodies are produced against various antigens; those present within the parasite-somatic antigens; those on the outer surfaces - surface antigens; those contained in helminth excretions and/or secretions - excretory secretory (ES) antigens. The first components of the parasite to be recognised as foreign by the host i.e. to form the first host-parasite interface, are the surface antigens and ES antigens. Unlike the surface antigens which are of a more finite quantity, the ES antigens are produced for the life of the worm and so a more abundant antibody response might be expected. The somatic antigens do not form part of the host parasite interface until after the death of the worm when they become exposed and elicit an antibody response. Antigenicity is defined as the ability of proteins, carbohydrates or lipids to elicit the induction and production of antibodies which bind in a predetermined manner to the antigen. The region of the antigen to which the antibody binds is the antigenic

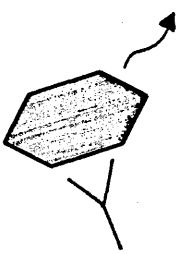
Figure 2.8. **Indirect Antigen Competition ELISA**

1. 

Passive adsorption of antigen.

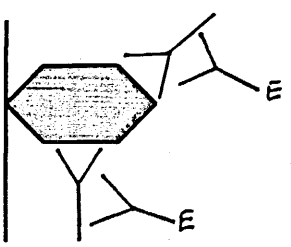
WASH

2. 



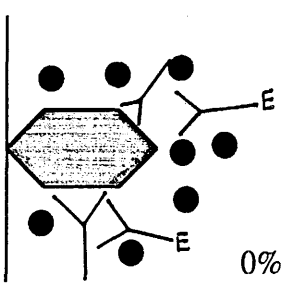
Simultaneous incubation of free antigen (test sample) with antibody directed against antigen on plastic at pre-titrated dilution.

WASH

3. 

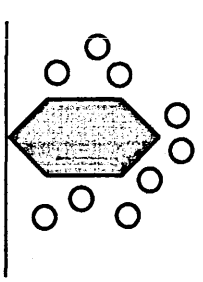
Addition of enzyme labelled antibody against antibody.

WASH

4. 

0%

Addition of substrate



100%

READ

determinant or epitope. Antibody specifically binds to the epitope of the antigen by multiple non-covalent interactions - electrostatic, hydrogen bonds, Van der Waals forces and hydrostatic forces. The multispecificity of antibody molecules is dependent not only on the heterogeneity of the epitope in question but also on the molecular construction of the antigen reactive sites (paratopes) of the antibody molecules. The strongest binding occurs when the paratope matches the epitope exactly - "best fit".

In the early stages of an infection there is little antibody available so an antigen excess prevails. This stage is normally superseded by antigen-antibody equivalence - a state which favours the formation of immune complexes. Stable antigen-antibody complexes can result when there are a sufficient number of short range interactions between both regardless of total fit. In the later stages of infection (chronic infections) the long term antigen challenge or exposure produces elevated levels of circulating antibody. As D. arnfieldi infection, like many helminth infections will be chronic at the time of diagnosis, it is most likely that a state of antibody excess occurs.

Due to commonness of some internal antigens e.g. enzymes, the more individually specific antigens are surface expressed. These antigens often show less cross-reactivity hence are more specific and therefore more useful for diagnosis. Antigens collected or extracted from stages of the parasite which do not occur in the final host are unlikely to be of use for detecting

antibodies to the parasite in that host.

Most ELISAs in current use in the veterinary field have been developed to detect antibody. As the serum of the host reflects the heterogeneous collection of immunoglobulin molecules produced in reponse to previous antigenic stimuli, the detection of antibodies to D. arnfieldi in a single sample would, due to the chronicity of the antibody response, only prove previous exposure which, since the horse is a grazing animal, may not be that uncommon. Such a result would be of little value in diagnosis of current infection. For this reason an antigen detection test for use on individual samples, would be superior.

While the most accessable body fluid to test is blood, the ELISA test can be used with a variety of fluids. Although it was considered that respiratory associated secretions would be the best source of antigens attempts to collect nasal secretions (Rouse and Angulo, 1970) from D. arnfieldi infected horses and donkeys proved to be impracticable. Serum was therefore chosen as the most readily available sample for use in the D. arnfieldi ELISA.

The ELISA selected was the antigen capture micro ELISA with a 96 well flat bottomed plate format. Flat bottomed wells were chosen as they give less steric interference when spectrophotometric reading is employed to assess colour development (Crowther and Smith, 1986). As only rabbit anti-D. arnfieldi antibody was available for use in the ELISA it was a prerequisite that the same antibody could be used for detection and, after biotinylation, development of the assay.

IMMUNOHISTOCHEMICAL LOCALISATION OF *D. arnfieldi* ANTIGENS

Reactions with anti-*D. arnfieldi* Antibody Fraction Raised in Rabbits

This procedure was undertaken to ensure the anti-*D. arnfieldi* IgG antibody fraction (FrR) raised in rabbits recognised *D. arnfieldi* antigen, prior to using this antibody in the ELISA test. The FrR was reacted with sections of *D. arnfieldi* worms as follows:-

Five micron sections of 10% formalin fixed adult *D. arnfieldi* worms blocked in wax were mounted on glass microscope slides and ringed with a diamond marker. The sections were then de-waxed and the slides air dried and outlined with yellow paint (Texpen, Marktex, Englewood, New Jersey) to contain the reactants. The sections were then covered with FrR at a range of dilutions (1:10 - 1.80) in phosphate buffered saline (PBS -pH 7.2, 100 mM) and incubated at room temperature for 30 minutes in a humid chamber. The slides were washed in three changes of PBS, each change lasting five minutes. The conjugate, sheep anti-rabbit IgG antibody conjugated to horseradish peroxidase (Sigma), at dilutions of 1:100 and 1:50 in PBS was added and incubated for 30 minutes as above. The slides were washed in PBS three times as above. The colour development system (50 ml PBS, 16 µl 30% H_2O_2 {substrate}, 15 mg DAB {diaminobenzidine}) was incubated on the slide for a maximum of 30 minutes, an insoluble brown colour product indicating a positive reaction. The reaction was stopped by rinsing the

slides in distilled water. Slides were then dehydrated by immersion through a series of alcohol concentrations counterstained with haemalum for less than a minute, blued in Scotts tap water substitute (pH 8), cleared in xylene and mounted with DPX (Distrene Plasticizer Xylene).

Summary of Procedure

(1) Apply antibody solution (FrR) to sections for 30 minutes.

(2) Wash three times with PBS.

(3) Apply conjugate for 30 minutes.

(4) Wash three times with PBS.

(5) Apply colour development system - colour - in 30 minutes.

(6) Stop with distilled water.

(7) Dehydrate through alcohols.

(8) Counterstain with haemalum.

(9) Mount.

RESULTS

The results of incubating D. arnfieldi sections with the primary antibody at either 1:10 or 1:20 dilution with 1:100 conjugate dilution are presented in Table 2.2 and the results with 1:50 conjugate dilution are presented in Table 2.3.

Table 2.2

Incubation of D. arnfieldi Section with Primary Antibody (1:10 - 1:20) then 1:100 Conjugate

Rabbit	Dilution of Antibody Solution	Degree of Brown Reaction
1	1:10	++
1	1:20	+
2	1:10	++
2	1:20	++
-	No primary antibody - 100 PBS	nil

Table 2.3

Incubation of D. arnfieldi Sections with Primary Antibody (1:10 - 1:80) then 1:50 Conjugate

Rabbit	Dilution of Antibody Solution	Degree of Brown Reaction
1	1:10	++
1	1:20	++
1	1:40	++
1	1:80	+
2	1:10	+++
2	1:20	+++
2	1:40	++
2	1:80	++
-	no primary antibody	nil

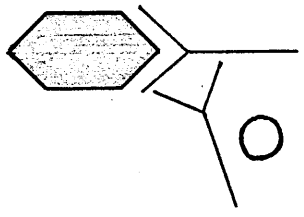
When the conjugate was used at a dilution of 1:50 a dark brown reaction was produced with FrR_1 at dilutions from 1:10 - 1:40. At a dilution of 1:80 the brown reaction product was less intense although still clearly visible. At the same dilution of conjugate (1:50) FrR_2 produced a very dark brown reaction product (see Table 2.3) at dilutions of 1:10 and 1:20. At the higher dilutions of 1:40 and 1:80 the intensity of the coloured product was reduced but still clearly visible.

CONCLUSIONS

The appearance of the brown product indicated a positive reaction. The colour was due to the reaction between DAB and hydrogen peroxide which was catalysed by horseradish peroxidase in the conjugate. To achieve this positive reaction the anti-rabbit antibody component of the conjugate must have reacted with the rabbit component of the anti-*D. arnfieldi* antibody which had bound to antigens present in the *D. arnfieldi* worm sections. This is in effect a modified type of indirect assay (Figure 2.9). In the control slides where no antibody solution was added, no brown reaction product was produced. The positive reaction in all the slides which had the antibody solution added indicated that the IgG fraction isolated from the serum of both rabbit 1 and rabbit 2 (FrR_1 and FrR_2 respectively) contained antibodies which recognised and reacted with antigenic sites on the *D. arnfieldi* sections. The more intense colour produced by FrR_2 compared with FrR_1 suggested the antibodies raised by rabbit 2 and present in FrR_2 had either a greater affinity for the

Figure 2.9.

Diagram of the indirect assay used in the immunohistochemical localisation of Dictyocaulus arnfieldi reacting with anti-Dictyocaulus arnfieldi IgG antibody fraction raised in rabbits.



Dictyocaulus arnfieldi antigen

anti-Dictyocaulus arnfieldi antibody raised in rabbits

Sheep anti-rabbit IgG

Horseradish peroxidase

antigenic sites or greater avidity for binding or were present at a higher titre than the antibodies present in FrR_1.

Reactions of anti-D. arnfieldi Antibody Fractions with Antigens of Fresh and Formalin Treated Worm Sections

The aims of this experiment were to establish the location of the antigens recognised by the FrR to enable an assessment as to whether the host animal (horse or donkey) was likely to have been exposed to these antigens in a natural D. arnfieldi infection and to examine the effect formalin treatment of the worms had upon recognition of its antigenic sites by the antibodies present in FrR_1 and FrR_2.

To determine the location of antigenic sites recognised by the antibodies in FrR_1 and FrR_2 within D. arnfieldi adults further experiments were performed using either sections of formalin fixed (Slides 1-5) or unfixed worms (Slides 6-10) mounted on glass microscope slides. The worms were ringed as before and the slides dewaxed through a graded series of alcohols. The biotinylated FrR_1 and FrR_2 (FrR_1B and FrR_2B) were applied onto sections of worms at dilutions of 1:10 and 1:20 in PBS and incubated in a humid chamber for 30 minutes at room temperature. Slides were then washed three times in PBS (as before) following which conjugate (streptavidin peroxidase - Sigma) at 1:1000 was added and the slides incubated as before. Slides were washed three times in PBS and the colour development system (50 ml PBS, pH 7.2, 0.1M; DAB {diaminobenzidene} 17 ug; H_2O_2 16 ul 30%) was added and incubated for 30 minutes to allow the brown reaction product to develop. The reaction was stopped

by rinsing the slide in distilled water and the sections dehydrated by immersion through a series of graded alcohols (10 - 100%), counterstained and mounted as before.

Summary of Procedure

(1) Add FrRB to section of D. arnfieldi worm on slide.

(2) Rinse with PBS three times.

(3) Add streptavidin peroxidase.

(4) Rinse with PBS three times.

(5) Add colour development system.

(6) Stop reaction with distilled water.

(7) Dehydrate slides.

(8) Counterstain and mount.

RESULTS

The results of incubating either formalin fixed or unfixed D. arnfieldi sections with either 1:10 or 1:20 dilution of FrRB and conjugate dilution 1:1000 are presented in Table 2.4.

CONCLUSIONS

All positive slides produced a brown reaction product. A more intense colour was produced in the slides with sections of the unfixed worm than with those with sections of formalin fixed worms indicating that more antigenic sites were available and accessible for binding with the antibodies present in FrR in the unfixed worm sections. The colour produced with the formalin fixed sections (Slides 1-4) showed no variation regardless of antibody source or dilution. In contrast the colour produced

Table 2.4

Incubation of Either Formalin Fixed or Unfixed *D. arnfieldi* Sections with FrRB

Slide	Source of Antibody	Dilution of Antibody	Intensity of Brown Reaction Product
1	FrR_1B	1:10	++
2	FrR_1B	1:20	++
3	FrR_2B	1:10	++
4	FrR_2B	1:20	++
5	none	100% PBS	nil
6	FrR_1B	1:10	+++
7	FrR_1B	1:20	+++
8	FrR_2B	1:10	++++
9	FrR_2B	1:20	++++
10	none	100% PBS	nil

Footnote:

1 - 4 Worm sections fixed in 10% Formol Saline

5 Negative control

6 - 9 Fresh worm sections

10 Negative control

with the unfixed sections was much more intense with FrR_2B than with FrR_1B although there was no variation in colour product at the different dilutions. From this it appeared that the formalin exerted a detrimental effect upon some D. arnfieldi antigens reducing their interactions with antibodies present in FrRB. This may have been due to formalin cross-linking protein epitopes thus rendering them unavailable for the antibody paratopes. As diluting the FrRB from 1:10 to 1:20 had no effect upon the intensity of coloured product which developed, the antibodies must have been in excess hence the availability of epitopes on the worm sections was the limiting factor.

Microscopic examination revealed the brown reaction product located on the epicuticle and gut lining of D. arnfieldi adults (see Figure 2.10) indicating that antibodies raised in the rabbits recognised both surface and somatic antigens. The finding that the antibodies in FrR recognised D. arnfieldi surface antigens increased the expectation that these antigens would be detectable in and recognised by the host as these surface antigens provide an extensive host-parasite interface. The antigens located on the gut lining of the worm could be part of the fabric of the worm or could be excretory/secretory antigens, in which case a greater immunological response would be expected as ES antigens are the first antigenic compartment recognised by the host's immune system and, unlike somatic antigens, ES antigens can be produced in large amounts within the host hence increasing the amount of antigen to which the host might be exposed.

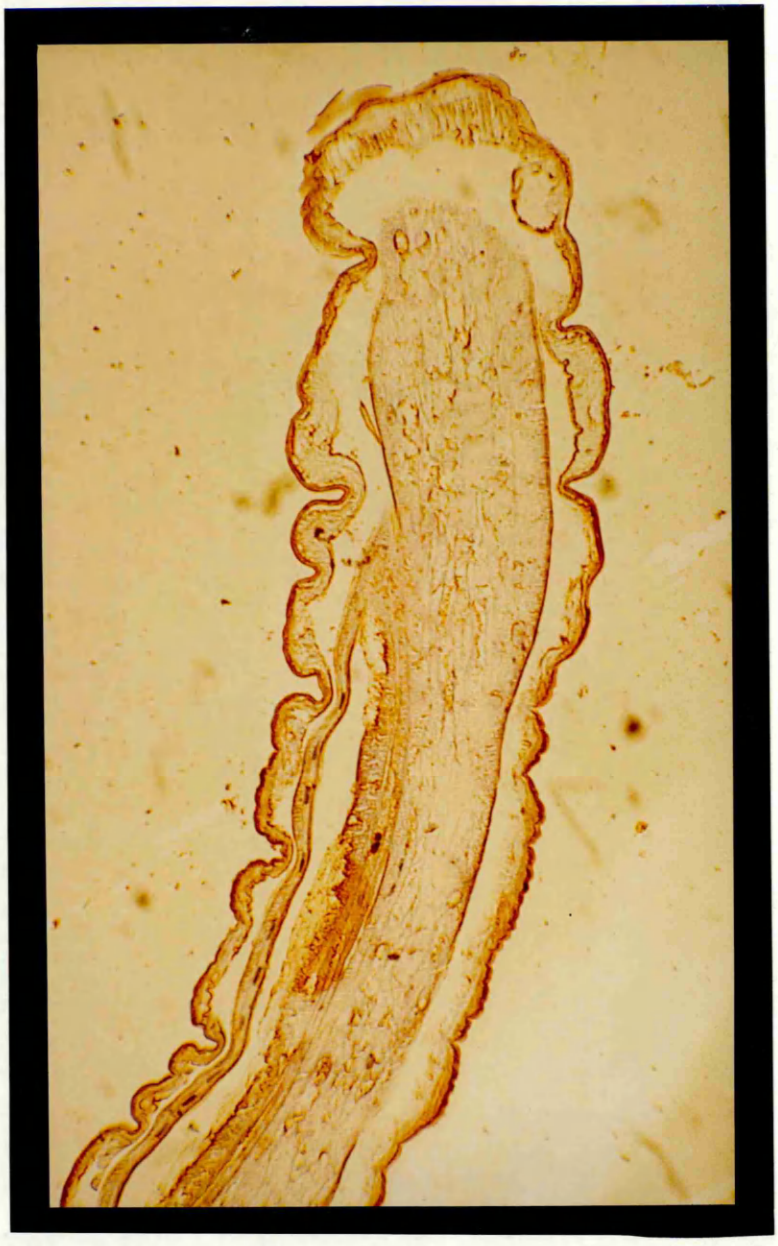


Figure 2.10

Photomicrograph of a section of an adult D. arnfieldi showing a brown colourisation indicating the recognition by antibodies of both epicuticle and gut antigens

COMPOSITION OF THE D. arnfieldi ANTIGEN

Having ascertained by immunohistochemical localisation that a circulating antibody response was produced in the immunised rabbits, further biochemical characterisation of the D. arnfieldi antigen was undertaken.

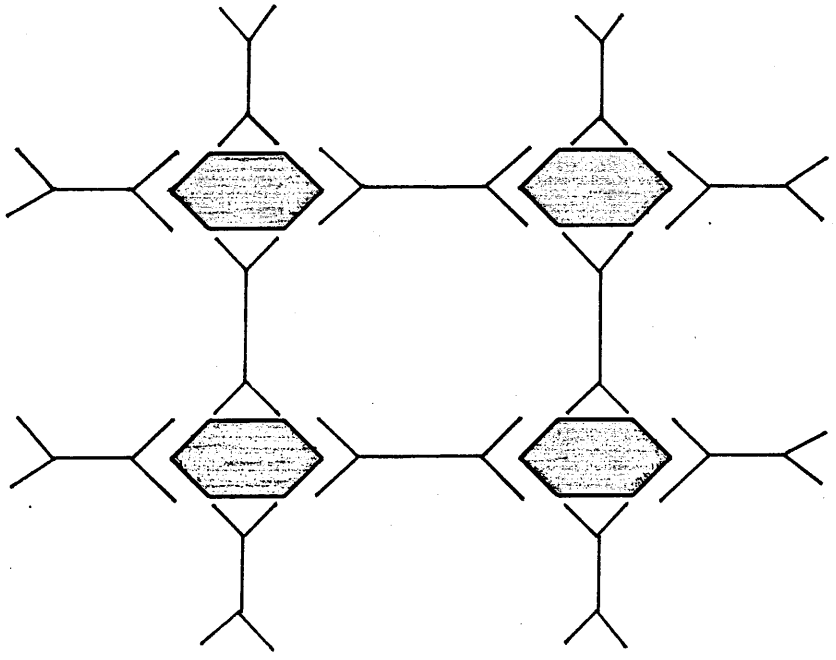
Antigens can consist of a variety of substances e.g. proteins, glycoconjugates, nucleic acids, carbohydrates or polysaccharides etc. It was assumed that the D. arnfieldi antigen to be tested for would be present in serum either in an uncomplexed (free) form or as soluble immune complexes but there was also the possibility that D. arnfieldi antigens might form insoluble complexes with antibody.

EDTA Treatment

To separate D. arnfieldi antigen from any immune complexes in serum, samples were boiled with EDTA to disrupt the bonding between antibodies and antigens. It was hoped that separation of any immune complexes into their constituent parts would leave epitopes in a form recognised by the antibody used in the ELISA as it was unlikely that any paratope of the FrR would be able to bind very successfully with large immune complexes due to steric hindrance. Binding capacity could also be reduced by in vivo formed antibodies masking epitopes rendering them less or unavailable for binding (see Figure 2.11).

If, following EDTA treatment, the optical densities (ODs) obtained with the ELISA were increased compared with the ODs obtained prior to treatment then it would imply that the D. arnfieldi antigen was present as immune complexes. However,

Figure 2.11. **Diagram of Immune Complex**



Dictyocaulus arnfieldi antigen.

Host antibody.

as the process also involves heating the sample, it is possible that treatment would expose more heat stable epitopes. If no increase in ODs were observed it could indicate either that immune complexes were absent or that the epitopes were damaged when the complexes were separated and thus were no longer recognisable by the paratopes.

Autoclave Treatment

This was carried out to destroy the tertiary structure of the D. arnfieldi antigen if it was peptide in nature. A reduction in the ODs following autoclave treatment would therefore indicate the D. arnfieldi antigen was protein in nature, however the exposure of more heat stable peptide epitopes could confuse the picture. Disruption of the tertiary structure obviously alters the topography of the antigenic entity which might reduce or eliminate the 'best fit' needed for optimal binding between antigen and antibody.

Periodate Treatment

This treatment would disrupt any terminal sugars if the D. arnfieldi antigen extract was a glycoconjugate and so bring about a reduction in ODs. In this instance periodate was used under harsh conditions which are designed to completely disrupt the structure of the saccharide unlike mild periodate treatment where only the most vulnerable bonds are cleaved. Periodate oxidation of exposed sugars oxidises vicinal dihydrols of the reducing terminal of an oligo or polysaccharide.

RESULTS

Autoclave and periodate treatment of the D. arnfieldi antigen reduced the sensitivity of detection by 19% and 27% respectively when compared with untreated antigen (Figure 2.12).

CONCLUSIONS

The reduced sensitivity following autoclave and periodate treatment indicated that approximately 20% of the epitopes were protein in nature and 30% carbohydrate.

ELISA PROCEDURE FOR D. arnfieldi ANTIGEN DETECTION

The ELISA adopted was a 96 well heterogeneous antigen capture MICRO ELISA (Figure 2.13). The same polyclonal antibody was used both to capture the antigen and to develop the reaction. This was accomplished by utilising the biotin-streptavidin system, whereby only the development antibody was biotinylated. The development of an ELISA necessitates the optimal dilution of all reagents. To this end certain recognised procedures were followed to establish the necessary parameters. Detailed results of these procedures are given in Appendices 2.1 - 2.60. To ensure reproduceability of the ELISA the following parameters were analysed:

(1) Concentration of capture antibody required to coat the plate.

(2) Concentration of development (biotinylated) antibody.

(3) Concentration of streptavidin peroxidase.

When calculating the concentration of capture antibody required to coat the plate to ensure optimal trapping of antigen,

Figure 2.12

Optical densities obtained in the D. arnfieldi ELISA with untreated, periodate treated and autoclaved D. arnfieldi antigen

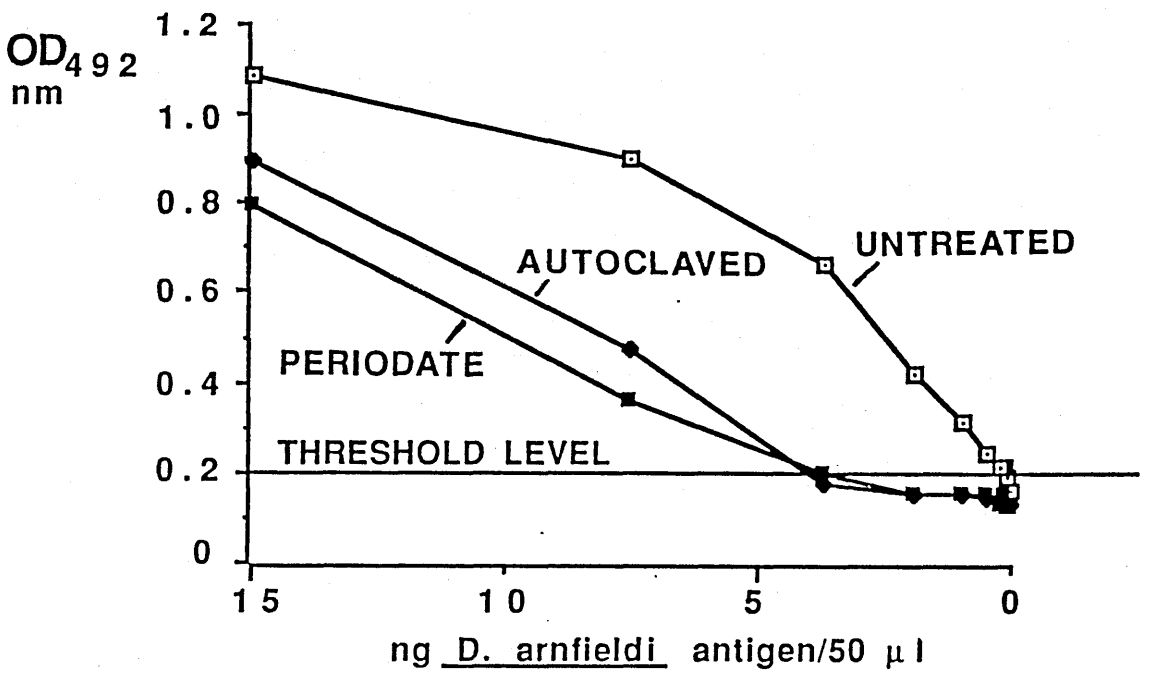
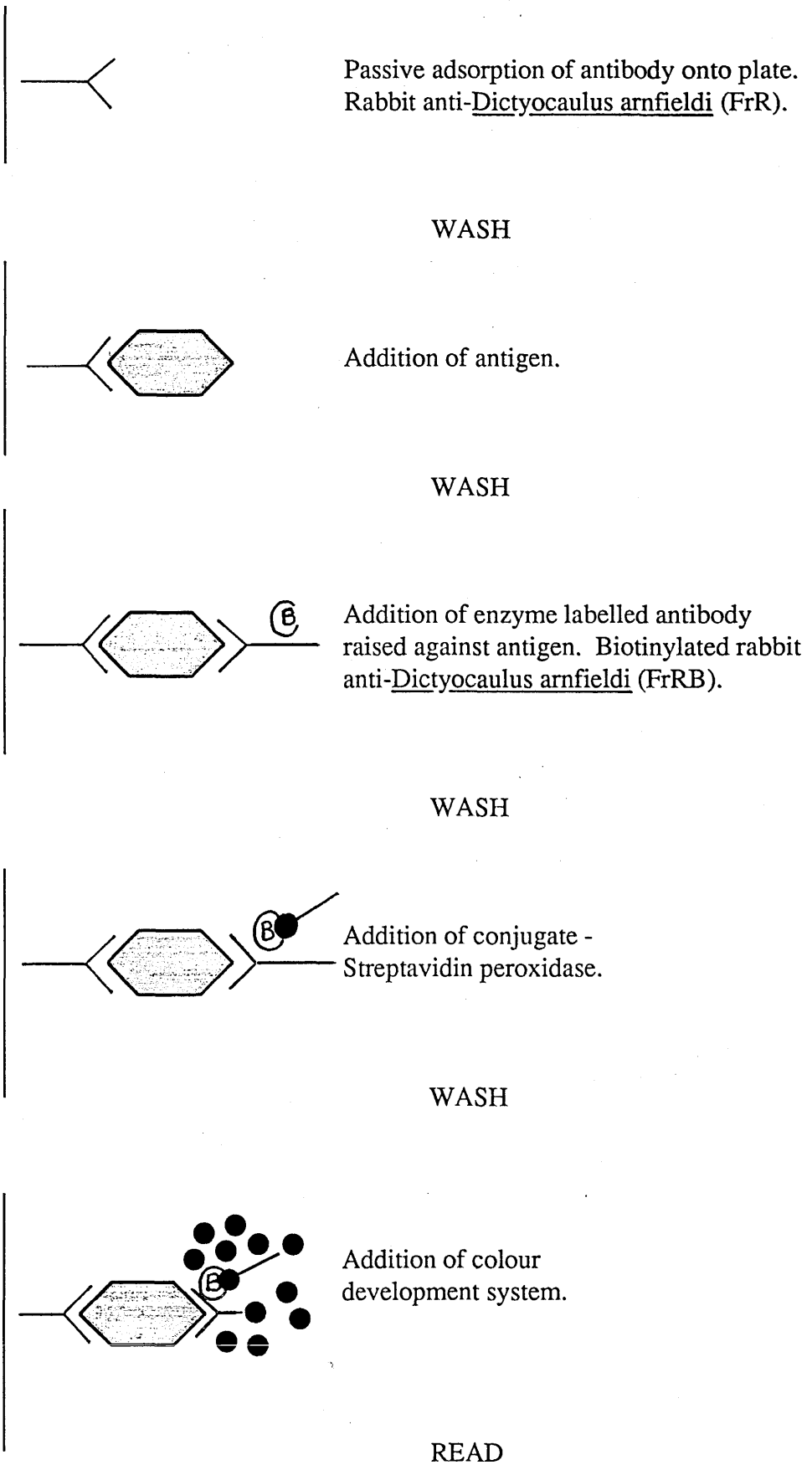


Figure 2.13. **Diagrammic representation of the ELISA for Dictyocaulus arnfieldi antigen.**



the fact that on average an ELISA well can only adsorb approximately 300 ng of IgG is taken into account (Crowther and Smith, 1986). Both antibody solutions (FrR_1 and FrR_2) were purified by salt fractionation and column chromatography and concentrated by ultrafiltration to a protein concentration of 1 mg/ml. The plate was coated with varying concentrations of antibody (1:25 -1:3200) and a standard amount of antigen solution (protein concentration 30 µg/ml) sufficient to ensure an antigen excess was used. The optimal concentration of non-biotinylated antibody for coating the plate was found to be 1:100.

Due to the scarcity of available materials it was decided to biotinylate the FrR to allow the use of the same antibody to capture antigen and develop the colorimetric reaction. Since both capture and development antibodies were raised in rabbits the problems of cross reactions between species were eliminated. The concentration of biotinylated antibody to be used was calculated by titrating various dilutions (range 1:10 - 1:1280) with the concentrations of both capture antibody and antigen being kept constant. An antibody excess is required to ensure all available binding sites on the antigen are saturated. The optimal concentration of biotinylated antibody was found to be 1:100.

The colour development system utilised in this ELISA was streptavidin peroxidase as the enzyme conjugate. The substrate contained hydrogen peroxide and the chromogen was orthophenylene diamine (O.P.D.). Initially the streptavidin peroxidase had been

titrated for use in another ELISA system and was being used at a 1:5000 dilution. When used at this dilution in the D. arnfieldi ELISA the colour development was unacceptably slow and insufficient only producing a maximum O.D. after 45 min development time of 0.085 when read at 492 nm after stopping with 2M H_2SO_4. The streptavidin peroxidase was re-titrated to 1:1000 which produced a more rapid and intense colour development giving a maximum O.D. after 30 min development time of of 0.790 at 492 nm.

In summary, the following procedure was defined for the ELISA for D. arnfieldi antigen.

(1) Coat plate with non biotinylated FrR at a dilution of 1:100 in coating buffer, adding 50 µl per well.

(2) Incubate plate for two hours at 37°C.

(3) Wash plate three times with wash buffer and tap dry.

(4) Add antigen, 50 µl per well, incubate at 37°C for 30 minutes.

(5) Wash plate three times with wash buffer and tap dry.

(6) Add biotinylated FrR (FrRB) at a dilution of 1:100 in diluting buffer, 50 µl per well. Incubate at 37°C for 30 minutes.

(7) Wash plate three times with wash buffer and tap dry.

(8) Add conjugate, streptavidin peroxidase, at a dilution of 1:1000 in diluting buffer, 50 µl per well. Incubate at 37°C for 30 minutes.

(9) Wash plate three times with wash buffer and tap dry.

(10) Add substrate/chromogen, 50 µl per well. Incubate at 37°C for 30 minutes in the dark.

(11) Read at 450 nm.

(12) Stop with 2M H_2SO_4, 50 µl per well.

(13) Read at 492 nm.

Although 30 minutes was the average time required for suitable colour development, with the colour change being easily detectable by eye, the visual assessment of stopping time was backed up by reading the plates at 450 nm. If the control positive well had an OD of 0.3 - 0.47 at 450 nm the reaction was then stopped by the addition of acid. The plates were stopped to prevent any further change in the OD after the development time. Stopping produces a change in the coloured product which changes the optimal wavelength required for measurement. It is very important for maximum accuracy that a standard volume of each reagent is added to each well especially the last two additions (substrate and acid) as the volume in the well influences the OD.

Antigen Titration

Having defined the parameters for the ELISA for D. arnfieldi antigen the level of antigen detection had to be ascertained. There was some background noise (O.D. readings not attributable to the presence of D. arnfieldi antigen) in the test due to non-specific adsorption of proteins from the antigen solution/serum samples onto any unoccupied plastic sites, i.e. spaces in the ELISA well where no FrR had bound. This background noise can be reduced by the addition of blocking agents to the buffer solutions. The blocking agents are immunologically inert

substances (for the system used) which compete with the non-specific factors, for the available binding sites of the plastic plate and hence reduce the amount of non-specific binding to the wells (Figure 2.14). Examples of blocking agents in common use are BSA (bovine serum albumin), horse serum and dried skimmed milk (Marvel, Cadbury). Another method of blocking is the use of non-ionic detergents which prevent non-specific adsorption by preventing any non-covalent and non-ionic interaction between the surface of the plastic and the proteins which would adsorb non-specifically onto the surface of the well. One such agent is Tween 20 (polyoxyethylene sorbitan monolaurate). Any agent or combination of agents used must not have any detrimental effect upon antigen, antibody or antigen/antibody interaction.

Antigen solution with a protein concentration of 30 µg/ml was used in a variety of diluents, each containing a blocking agent, to try to minimise non specific binding.

In diluting buffer - which contained 0.05% Tween 20 and 0.2 - 1% BSA - antigen appeared detectable down to dilutions of 1:1600. Below this, background reaction made it impossible to differentiate a positive reaction.

The addition of Marvel (range 1 - 5%) to the diluting buffer reduced the background noise further. The optimal concentration was a solution of 2.5% Marvel.

The goal for this ELISA was the detection of active infection in the living horse/donkey by the detection of D. arnfieldi antigen in equine serum. To reduce any non-specific

Figure 2.14. **Diagrams showing action of blocking agent**

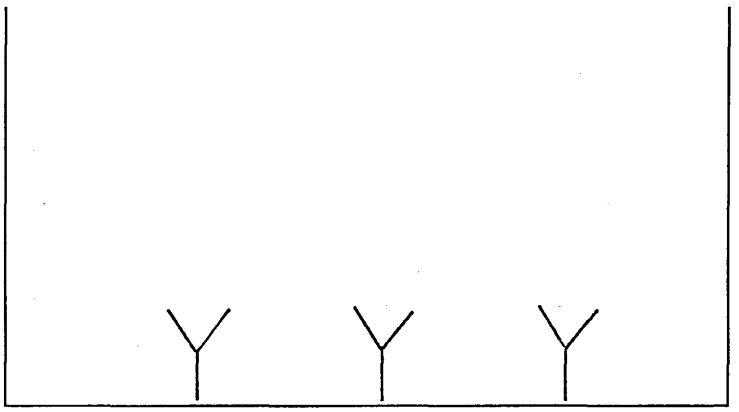
1. 

Plate coated with FrR.

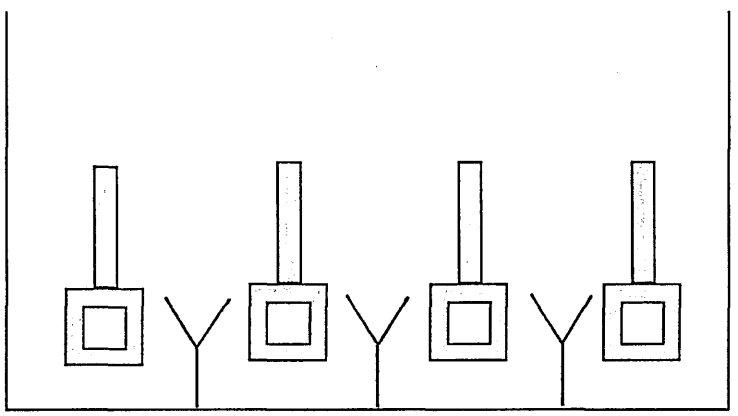
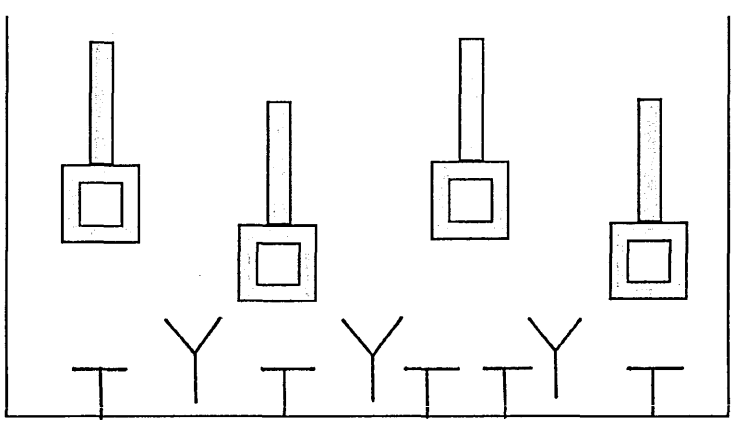
2. 

Plate coated with FrR and non-specific proteins binding.

3. 

Blocking agent (BSA, Marvel) occupying 'spare' sites on plastic and preventing non-specific proteins from binding.

reactions due to common immunological factors which could be present in horse serum, bovine serum was used as a vehicle for the antigen. The possibility that a common 'equine' component of the serum could be recognised by the FrR existed because the D. arnfieldi worms used to raise antibodies in the rabbits were recovered from donkey lungs and, despite vigorous washing, some equine blood or mucus may have been present in the final worm homogenate used to immunise the rabbits. Following detection of D. arnfieldi antigen in bovine serum at dilutions down to 1:800, antigen solution was added to inactivated horse serum (Gibco, Glasgow, Scotland). The amount of non-specific reaction did increase with the horse serum, however antigen in horse serum was definitely detectable down to dilutions of 1:80 which is a protein level of 1.5 ng. Overall antigen was detectable down to levels of 1.2 - 2.6 ng which is the equivalent of approximately 0.01 of an adult worm. This was judged to be a satisfactory sensitivity.

USE OF THE ELISA FOR DETECTION OF D. arnfieldi ANTIGEN IN EQUINE SERUM SAMPLES

Having determined the basic parameters for the ELISA for the detection of D. arnfieldi antigen and having detected antigen in spiked samples down to levels of 1.5 ng the next step was to use the test to try to detect antigen in serum samples taken from horses, ponies or donkeys which were either known or suspected to have been exposed to infection. These samples fell into four main groups.

(1) Samples from donkeys at the Donkey Sanctuary in Devon.

(2) Samples from ponies experimentally infected with D. arnfieldi.

(3) Samples from donkeys resident at Glasgow Veterinary School.

(4) Samples from clinical cases where lungworm infection was suspected.

In the ELISA, 50 µl of both neat (undiluted) serum and serum treated with EDTA prior to testing were used: Both untreated and EDTA-treated samples were tested on the same plate.

Samples taken from animals at Glasgow were aliquoted into polythene eppendorfs (Alpha Laboratories, Eastleigh, Hampshire) until required. Samples taken from animals elsewhere were posted to Glasgow and aliquoted and frozen as above: These samples tended to have thawed if they had been frozen prior to posting.

(1) Samples from the Donkey Sanctuary in Devon.

Serum samples were taken routinely from donkeys on arrival at the Sanctuary at the same time as they were given a general medical check over and had faecal samples taken for examination for parasite eggs or larvae. The results of the faecal examinations were supplied together with the serum samples. Although the history of these donkeys was mostly unknown it would be reasonable to assume all had been at grass previously.

(2) Samples from ponies experimentally infected with D. arnfieldi.

The experimentally infected mature ponies (Section 1.3) had blood samples samples taken on arrival and then at regular intervals

thus providing known pre- and post-infection samples. The experimentally infected mares and foals (Section 1.4) only had blood samples taken at necropsy thus providing a known post-infection sample.

(3) Samples from donkeys resident at Glasgow Veterinary School.

Resident donkeys provided blood samples over a three year period under a variety of conditions.

(i) Adult donkeys with patent D. arnfieldi infections either while grazing infected pasture or while housed.

(ii) Adult donkeys without patent D. arnfieldi infections either while grazing infected pasture or while housed.

(iii) Donkey foals housed from birth under worm-free conditions until turnout onto infected pasture.

(4) Samples from clinical cases where lungworm infection was suspected.

Veterinary surgeons in practice provided blood samples from cases of chronic coughing in horses and ponies. These were usually accompanied by a faecal sample. None of the horses or ponies had evidence of D. arnfieldi infection on faecal examination but samples from one outbreak of coughing were from animals known to have had grazing contact with donkeys which had patent D. arnfieldi infections.

RESULTS

(1) Samples from Donkeys at the Donkey Sanctuary in Devon.

The results from samples sent to the laboratory together with data provided by the Donkey Sanctuary are displayed in

Figure 2.15. From these it can be seen that there is little correlation between the faecal D. arnfieldi larval recoveries and the ODs achieved in the ELISA for D. arnfieldi antigen. In general the untreated serum gave marginally greater ODs than the EDTA treated samples at the same dilutions indicating an adverse effect on antigen availability by either heat or EDTA treatment. These samples from the Donkey Sanctuary were single samples taken from a large number of animals with unknown histories. As they were taken on a 'one-off' basis it was not possible to investigate any trends in D. arnfieldi antigen detection over a period of time.

(2) Samples from Animals Experimentally Infected with D. arnfieldi

(a) Mature Ponies.

The results of the ELISAs on repeated samples from individual animals used in Experiment 1) are shown in Figure 2.16.

Pony 1 and Pony 2

No larvae were recovered from the faeces of either pony and, although lesions characteristic of infection were present in the lungs of both, no D. arnfieldi were recovered. In both ponies there was no change in the ODs obtained following infection.

Pony 3 and Pony 4

No larvae were detected in the faeces of either pony but lesions characteristic of D. arnfieldi infection were present in the lungs of both. No D. arnfieldi were recovered from the lungs of Pony 4 but 16 D. arnfieldi were recovered from Pony 3 by lung

Figure 2.15

The optical densities using raw and EDTA-treated serum in the ELISA for *D. arnfieldi* together with larval counts in 50 grams of faeces (Da/50 g) from the donkeys admitted to the Donkey Sanctuary from 1986 - 1988

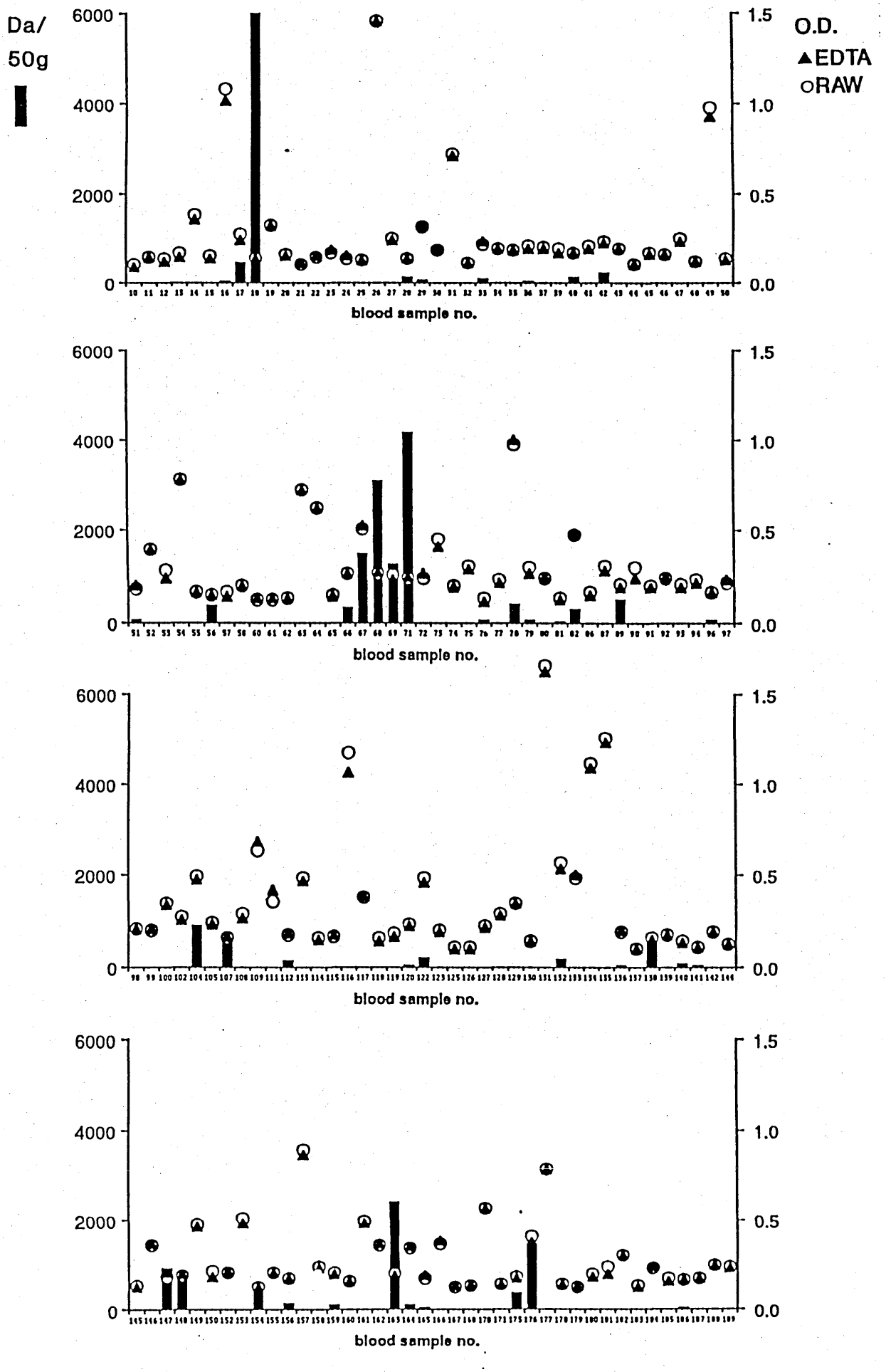

Da/
50g
O.D.
▲EDTA
○RAW
6000
4000
2000
0
1.5
1.0
0.5
0.0
blood sample no.

Figure 2.15 (Cont'd)

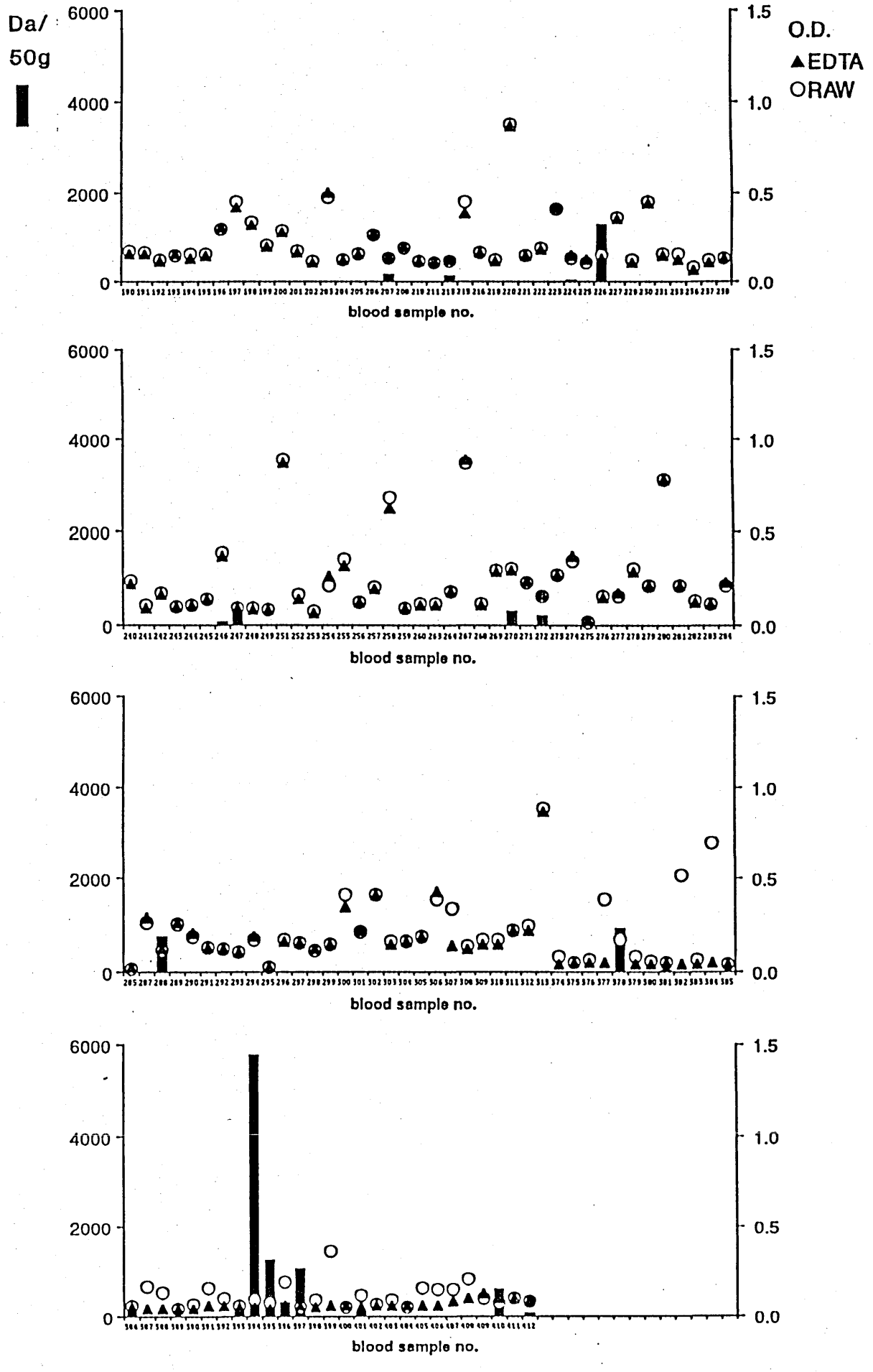
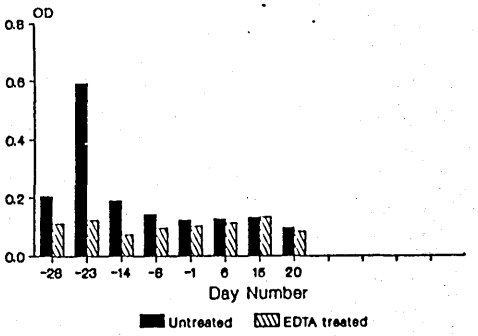


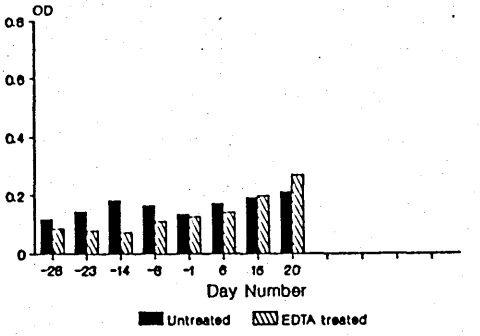
Figure 2.16

The optical densities using raw and EDTA-treated serum in the ELISA for D. arnfieldi in mature ponies experimentally infected with 2,000 (Ponies 1-8) or 5,000 (Ponies 9 and 10) D. arnfieldi L_3

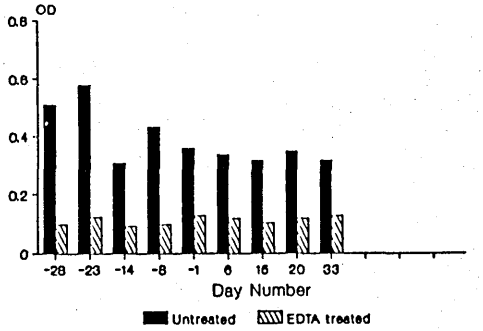
PONY 1



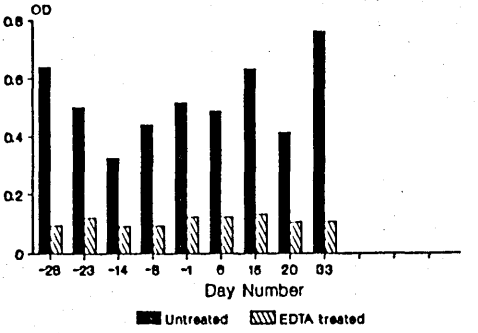
PONY 2



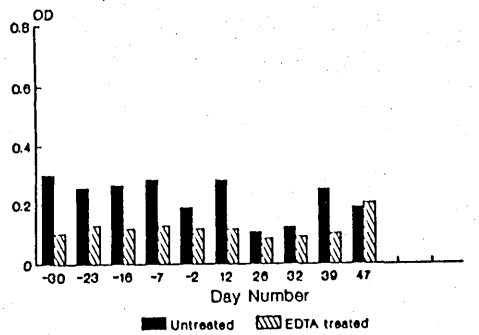
PONY 3



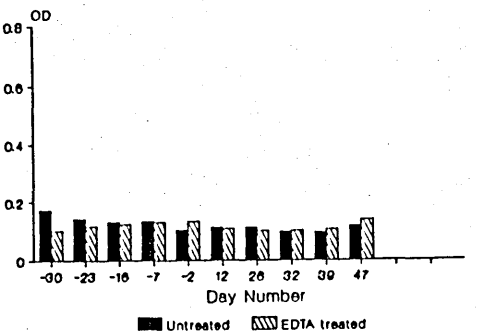
PONY 4



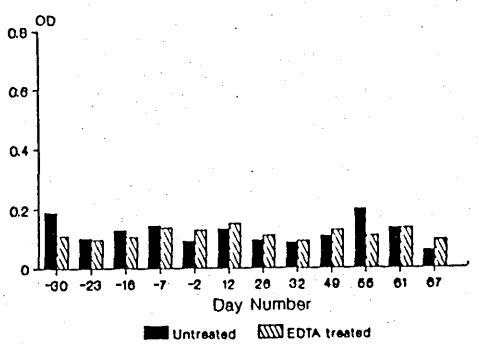
PONY 5



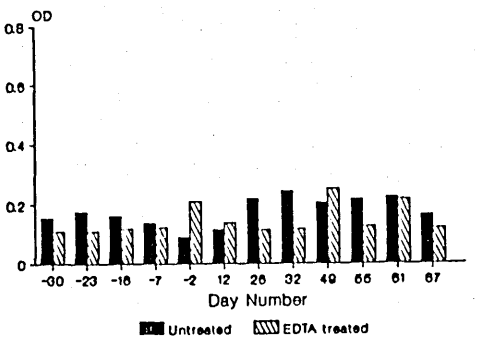
PONY 6



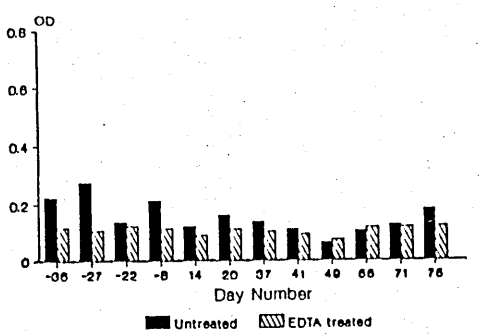
PONY 7



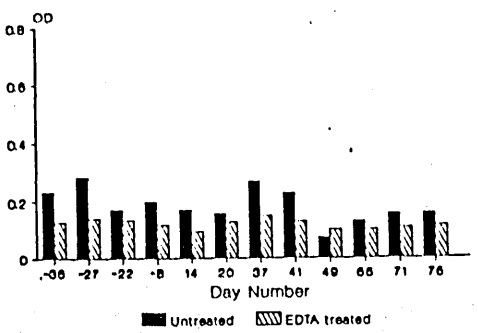
PONY 8



PONY 9



PONY 10



perfusion. There was no change in the ODs after infection in either pony.

Pony 5 and Pony 6

No larvae were detected in the faeces of either pony but 38 and 36 D. arnfieldi were recovered by lung perfusion from Ponies 5 and 6 respectively. In both ponies there was no marked change in the ODs obtained post-infection.

Pony 7 and Pony 8

No larvae were detected in the faeces of either pony and although 12 and 24 D. arnfieldi were recovered at necropsy from Ponies 7 and 8 respectively there was no appreciable change in the ODs post-infection.

Pony 9 and Pony 10

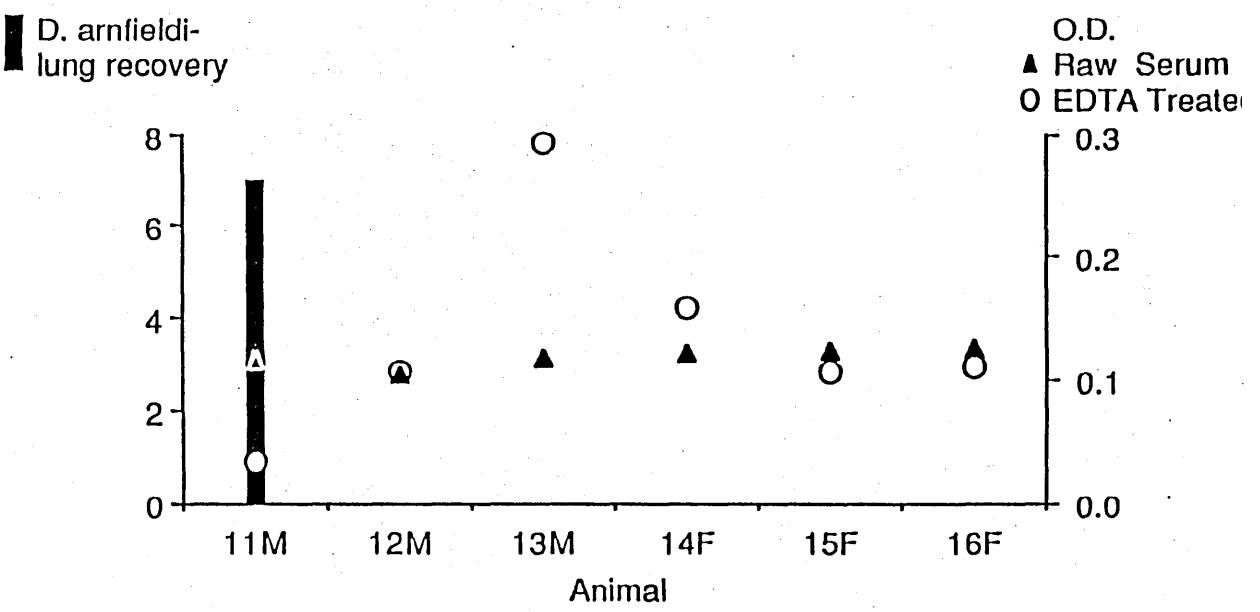
No larvae were detected in the faeces of either pony but 1 and 102 D. arnfieldi were recovered at necropsy of Ponies 9 and 10 respectively. There was no appreciable change in the ODs post-infection despite the marked coughing shown by Pony 10.

(b) Mares and Foals.

The results of the ELISA ODs of individual mares and foals are shown in Figure 2.17. Blood samples were only taken from these animals on one occasion - at necropsy 6 weeks (13M and 14F), 8 weeks (11M and 16F) and ten weeks (12M and 15F) post infection. Only two animals (11M and 15F) had D. arnfieldi recovered from their lungs. The ODs of the samples from the mare (11M) and the foal (15F) were of the same order as those of the other animals where no D. arnfieldi were recorded. There was no

Figure 2.17

The optical densities using raw and EDTA-treated serum in the ELISA for D. arnfieldi in mares and foals experimentally infected with 6-10,000 and 2,500 D. arnfieldi L_3 respectively



difference in the ODs of the mares compared to the foals. In two animals (11M and 13M) there were differences between ODs of the untreated and EDTA treated sera. With 11M the untreated serum gave an OD value of 0.1 greater than the EDTA treated serum value. In contrast 13M gave an untreated serum value of 0.15 less than the EDTA treated serum.

(3) Samples from Donkeys Resident at Glasgow Veterinary School.

Results of the ELISA ODs on repeated samples from six donkeys maintained in Glasgow for up to two years are shown in Figure 2.18.

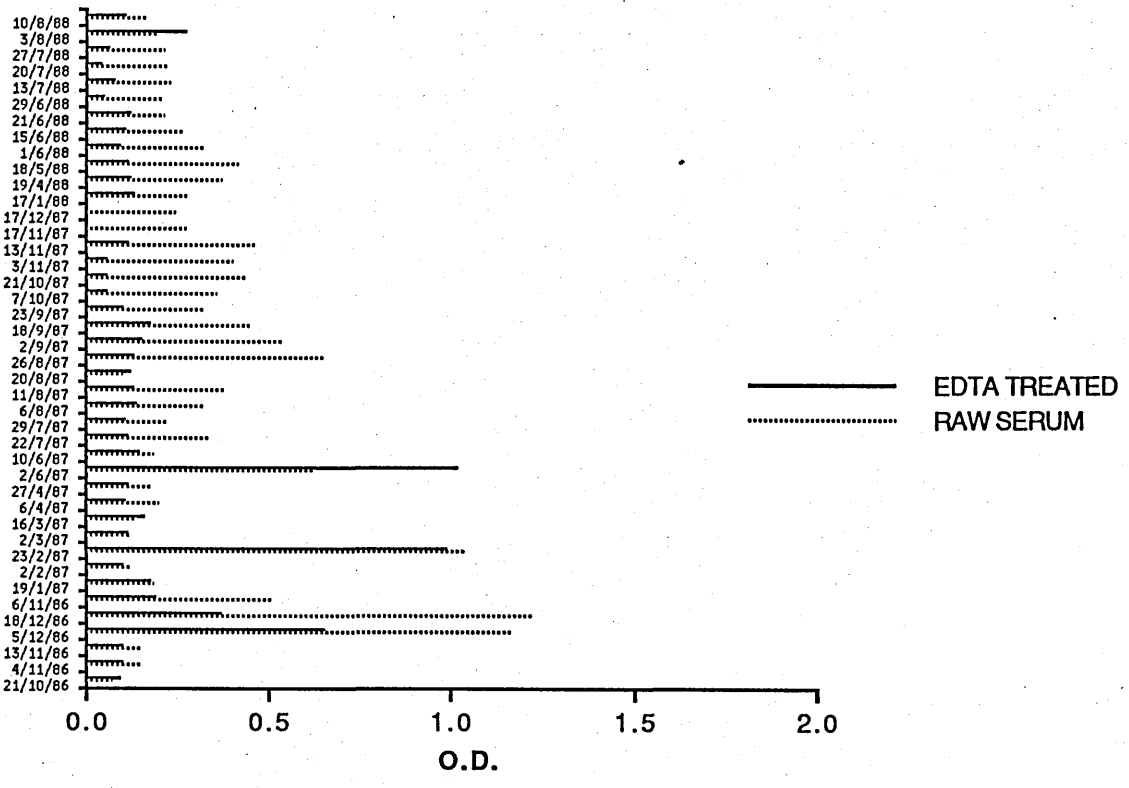
Donkey 20A was an adult mare sampled over a two year period. Throughout this time she had a patent D. arnfieldi infection with fluctuations in faecal larval counts from 10 - 220 lpg. The D. arnfieldi larval counts were greater during the grazing period of the second year. In all but one occasion the OD obtained with untreated serum was greater than with the EDTA treated serum but there was no correlation between any of the ODs and faecal larval output. Generally the ODs of the untreated serum were in the range 0.3 - 0.5 irrespective of whether the donkey was housed or grazing infected pasture.

Donkey 21A was an adult mare, again sampled over a two year period. Initially this animal had low numbers of D. arnfieldi in the faeces which, on one occasion, was associated with an ELISA OD of 0.8 with untreated serum. Only one further faecal sample was positive for lungworm. Generally the untreated serum gave slightly greater ODs than the EDTA treated serum but overall the ODs were around 0.3 with no change after turnout despite the

Figure 2.18

The optical densities using raw and EDTA-treated serum in the ELISA for D. arnfieldi together with faecal D. arnfieldi larval counts (l.p.g.) from donkeys at Glasgow Veterinary School

Donkey 20A


10/8/88
3/8/88
27/7/88
20/7/88
13/7/88
29/6/88
21/6/88
15/6/88
1/6/88
18/5/88
19/4/88
17/1/88
17/12/87
17/11/87
13/11/87
3/11/87
21/10/87
7/10/87
23/9/87
18/9/87
2/9/87
26/8/87
20/8/87
11/8/87
6/8/87
29/7/87
22/7/87
10/6/87
2/6/87
27/4/87
6/4/87
16/3/87
2/3/87
23/2/87
2/2/87
19/1/87
6/11/86
18/12/86
5/12/86
13/11/86
4/11/86
21/10/86
0.0
0.5
1.0
1.5
2.0
O.D.
EDTA TREATED
RAW SERUM

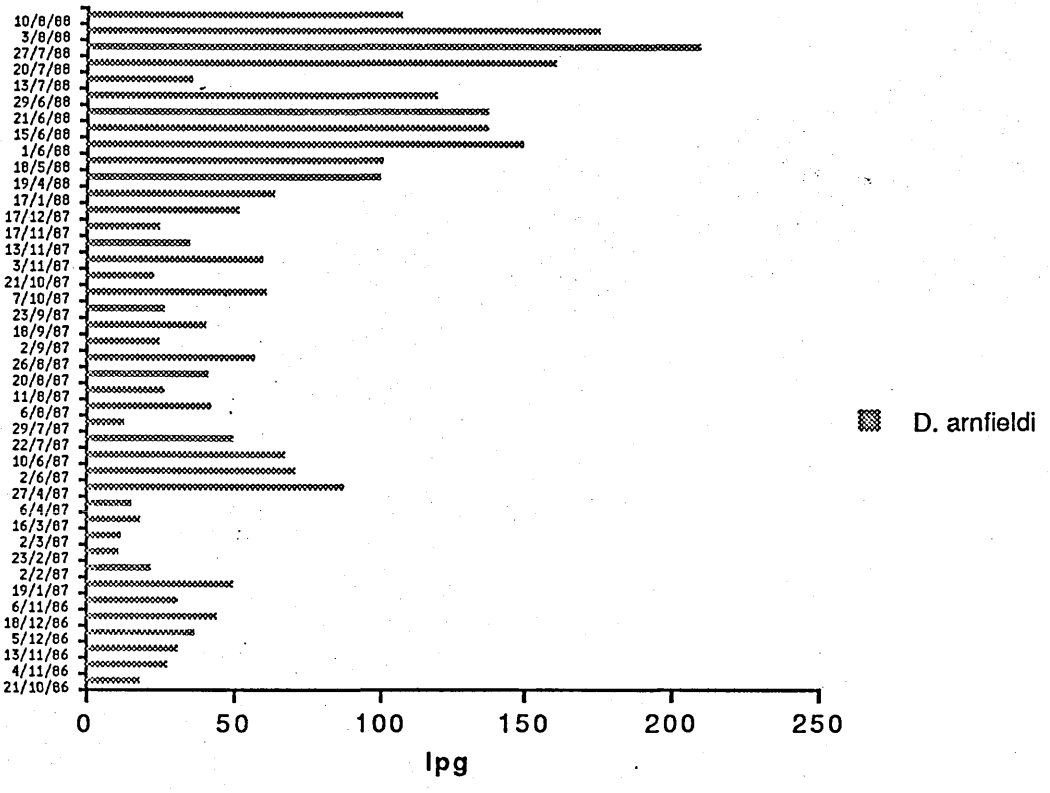
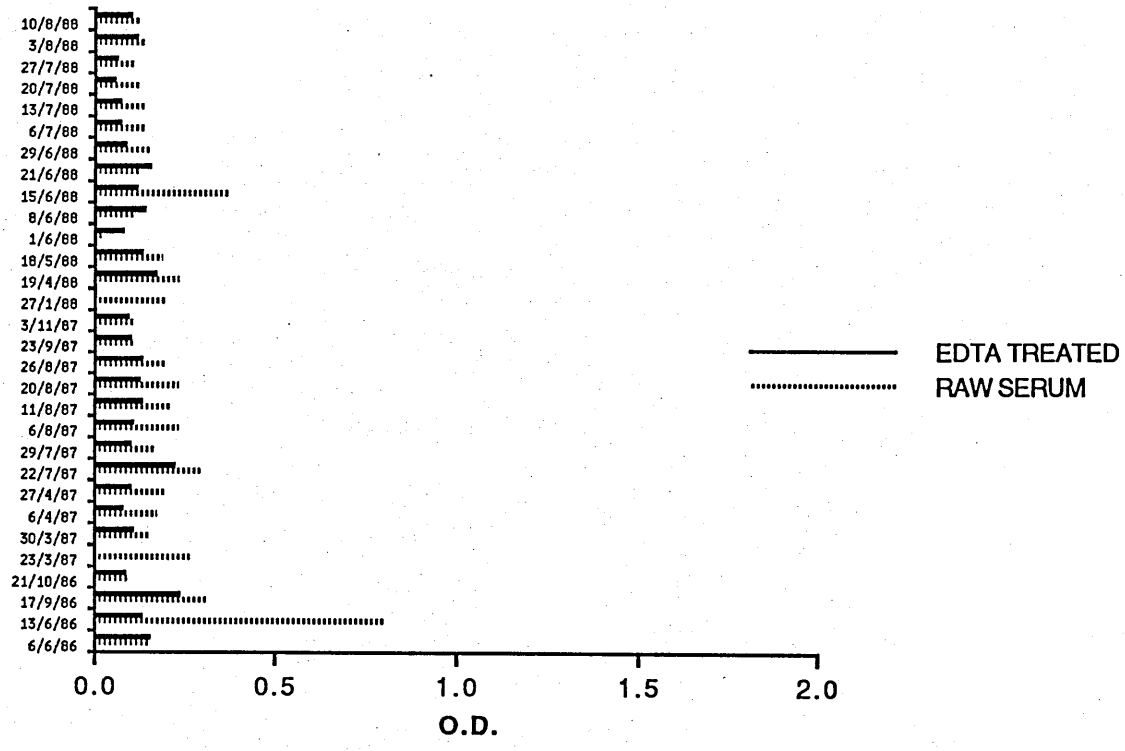

10/8/88
3/8/88
27/7/88
20/7/88
13/7/88
29/6/88
21/6/88
15/6/88
1/6/88
18/5/88
19/4/88
17/1/88
17/12/87
17/11/87
13/11/87
3/11/87
21/10/87
7/10/87
23/9/87
18/9/87
2/9/87
26/8/87
20/8/87
11/8/87
6/8/87
29/7/87
22/7/87
10/6/87
2/6/87
27/4/87
6/4/87
16/3/87
2/3/87
23/2/87
2/2/87
19/1/87
6/11/86
18/12/86
5/12/86
13/11/86
4/11/86
21/10/86
0
50
100
150
200
250
lpg
D. arnfieldi

Figure 2.18 (Cont'd)

Donkey 21A



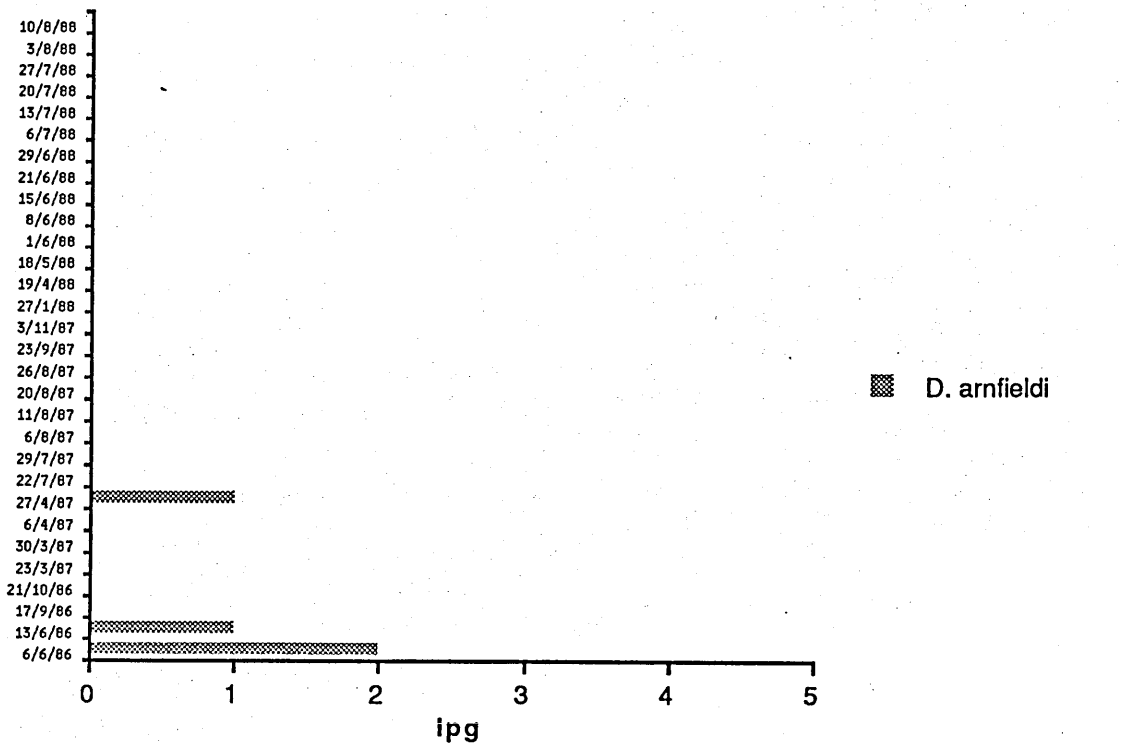
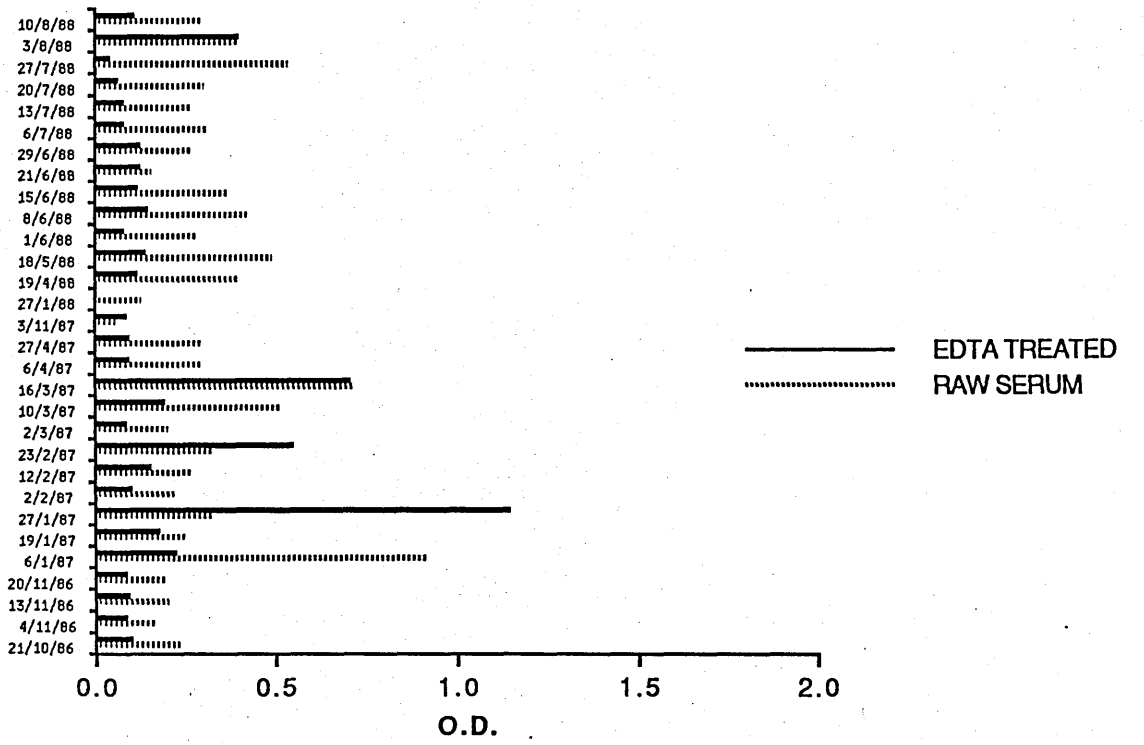


Figure 2.18 (Cont'd)

Donkey 22A



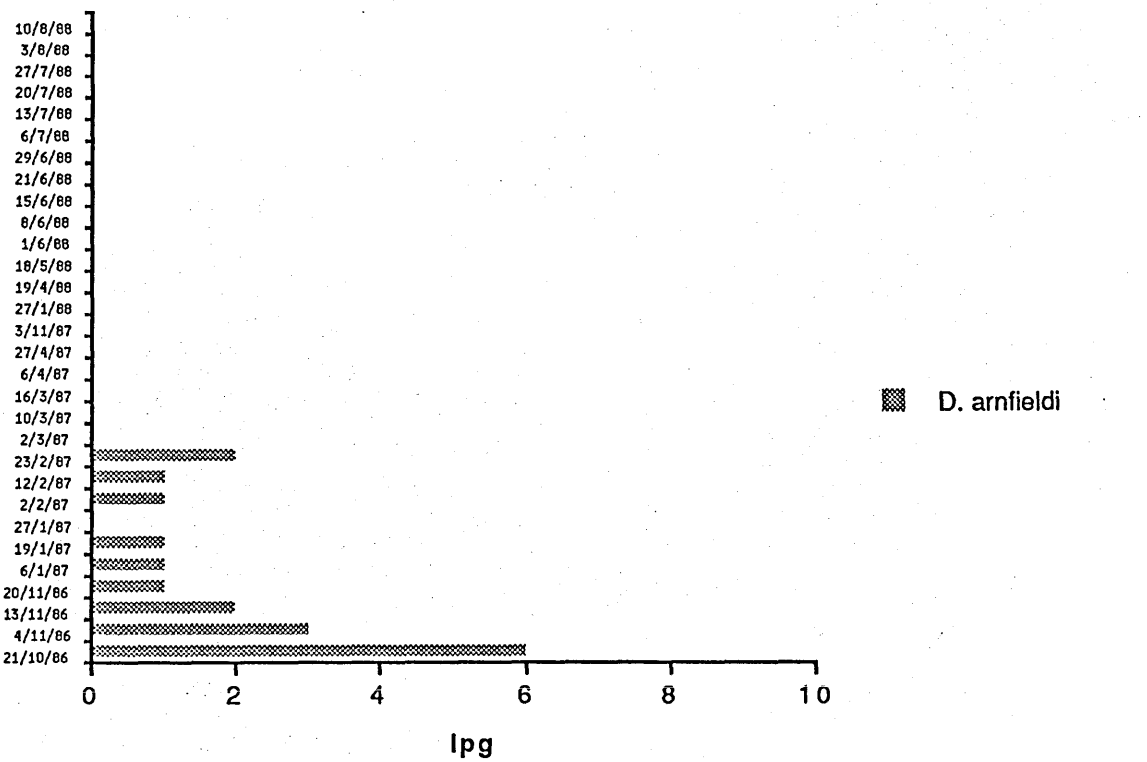


Figure 2.18 (Cont'd)

Donkey 23F

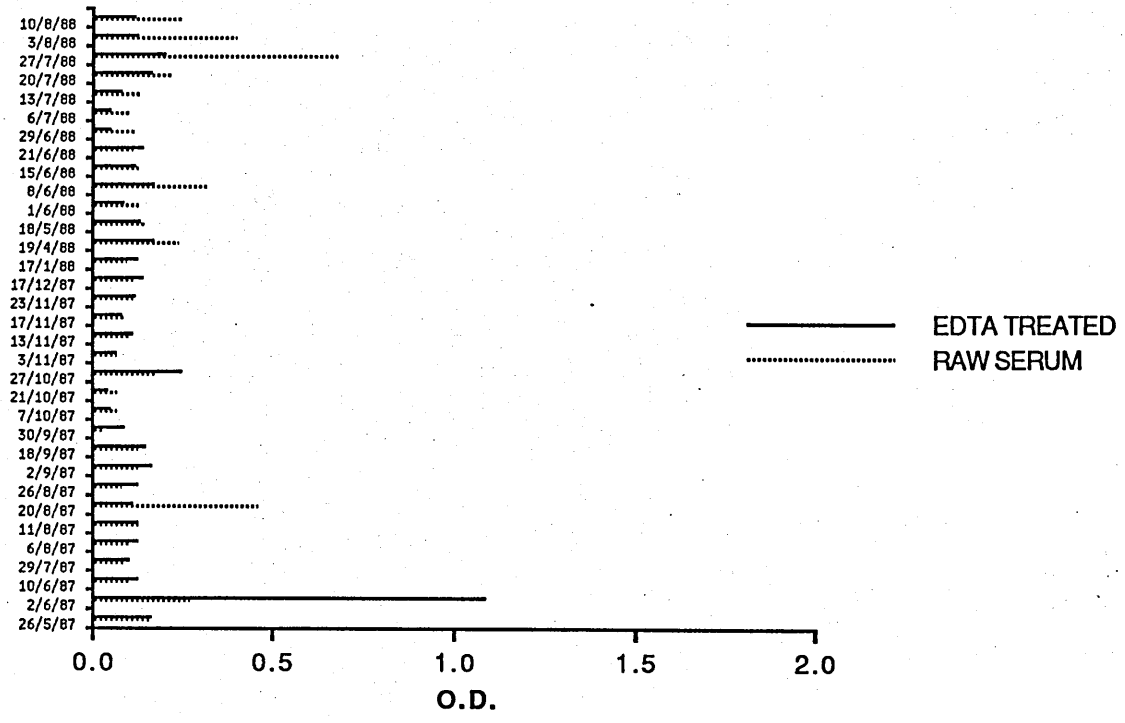


Figure 2.18 (Cont'd)

Donkey 24F

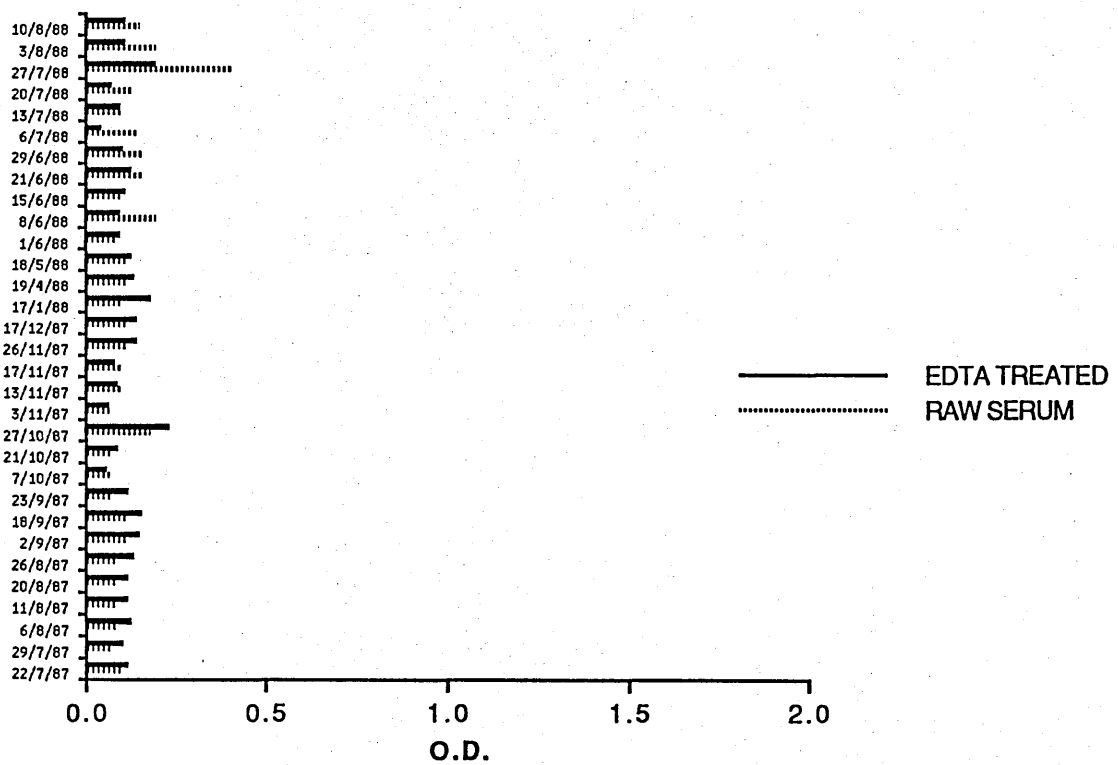
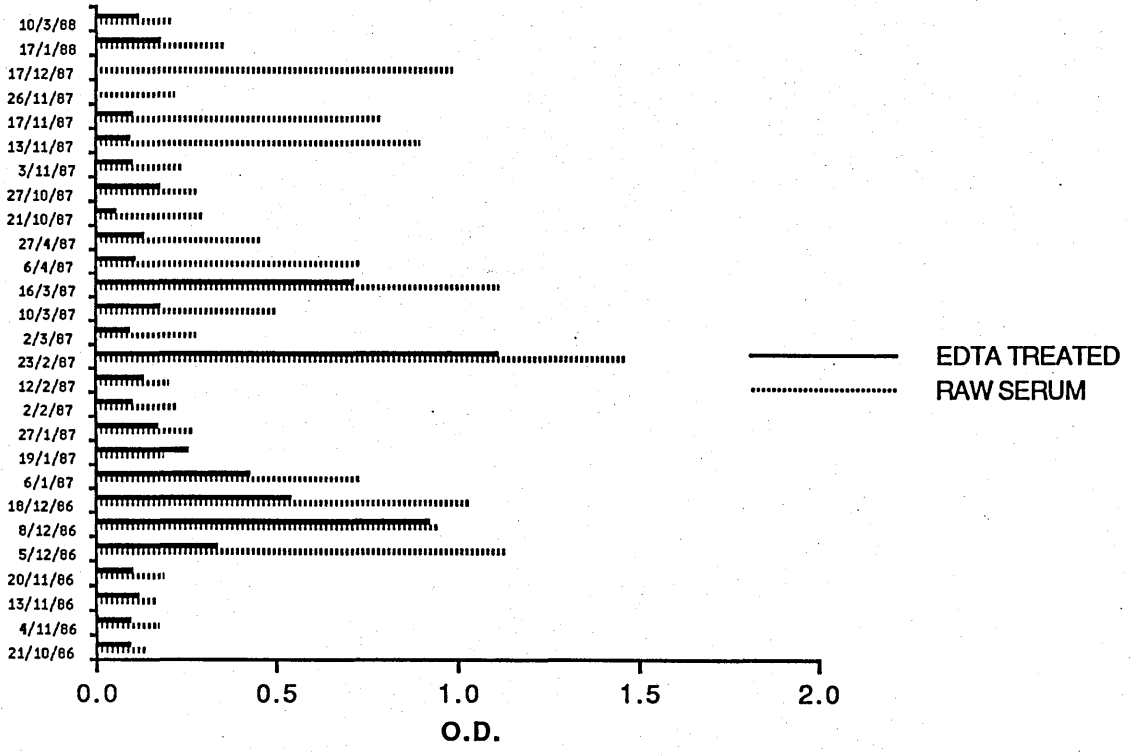
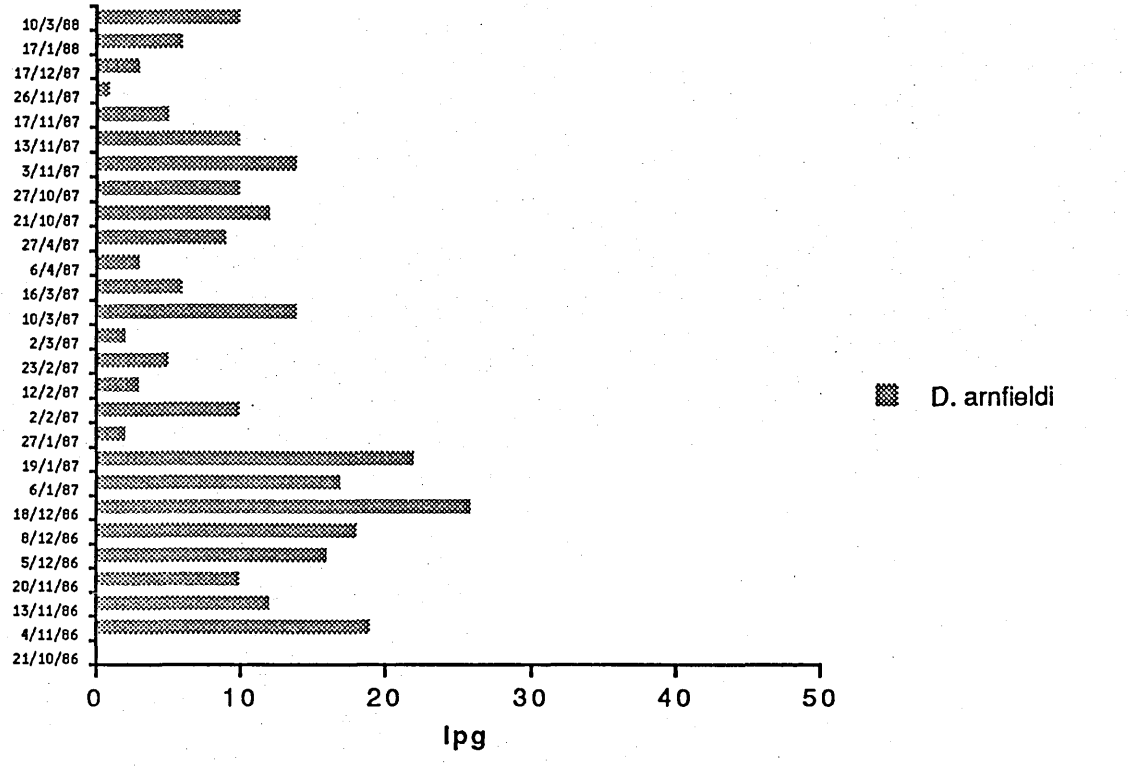


Figure 2.18 (Cont'd)

Donkey 25A





fact that this animal was grazing pasture contaminated with D. arnfieldi larvae.

Donkey 22A, like the previous animal, was an adult mare sampled over a two year period. For the first four months of the study, while housed, the mare had a patent D. arnfieldi infection (1 - 6 lpg faeces) and over this period the ODs were greater than 0.5 on a few occasions. However over the two year observation period there was no apparent correlation between ELISA ODs and either patency or exposure to infection from contaminated pasture. Although there was a slight increase in the untreated serum ODs after turnout in Spring, 1988.

Donkey 23F is the foal of donkey 21A. Donkey 21A had become non-patent for D. arnfieldi before 23F was conceived, 23F being foaled indoors and reared worm free. It is therefore very difficult to explain the OD of > 1 with the EDTA treated serum taken when the foal was only a few days old as there is no passive transfer of D. arnfieldi infection from dam to offspring. There was no increase in the ODs on turnout onto contaminated pasture along with infected donkeys in Spring, 1988. Towards the end of the grazing period (July, 1988) a slight increase in the ODs, especially with the untreated serum was shown but this began to tail off in August.

Donkey 24F was the foal of mare 20A which had a patent D. arnfieldi infection throughout this study. This foal was born and reared indoors until turned out to graze contaminated pasture with the other donkeys in April, 1988. The ODs were almost all

less than 0.3 except for one occasion in July. In general, and in contrast to the previous animals, the ODs with the EDTA treated serum were marginally greater than those obtained with untreated serum.

Donkey 25A was an adult mare which had a patent D. arnfieldi infection over a period of approximately 18 months. In contrast to Donkey 20A, which also had a persistent infection, in this animal there appeared to be some correlation between increases in D. arnfieldi larval output in faeces and the ELISA ODs and overall the ODs obtained in samples from this donkey were greater than with those obtained from the donkeys which did not have patent infections. As with most of the other donkeys, the ODs with untreated serum were greater than the ODs with the EDTA treated serum.

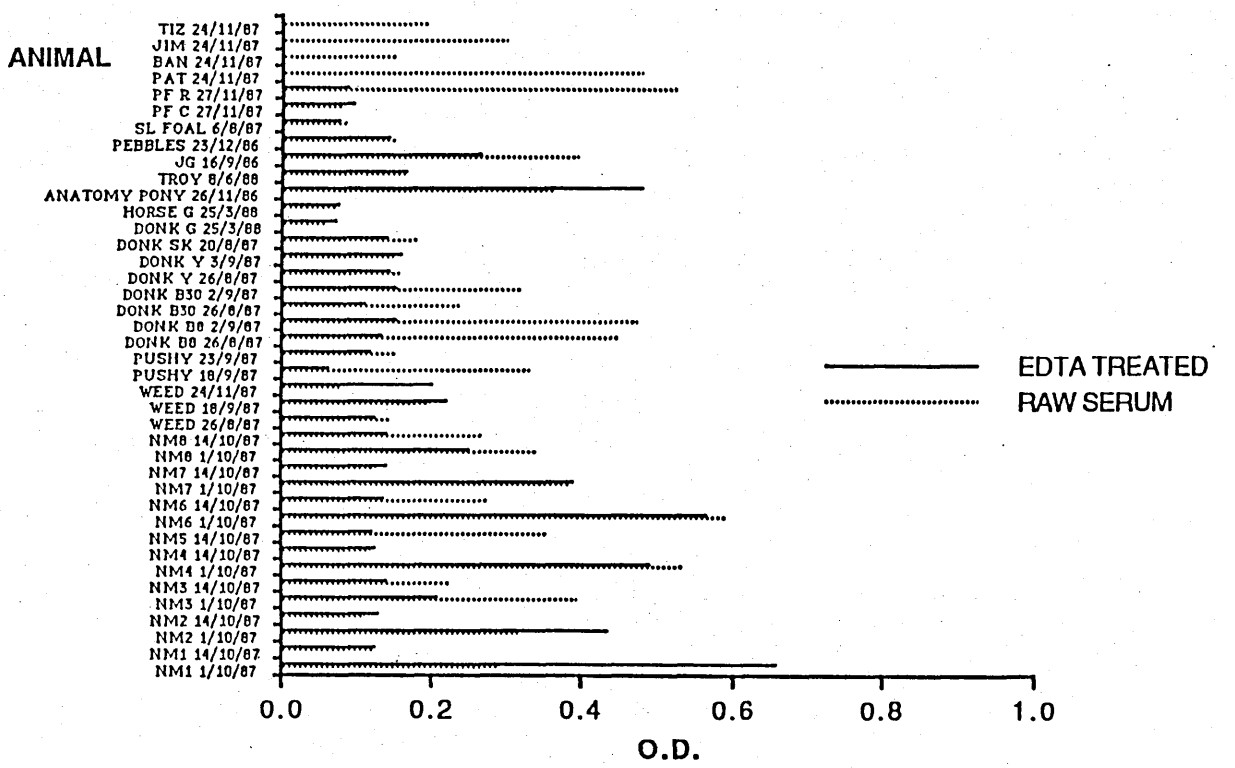
(4) Samples from horses and donkeys in some of which lungworm infection was confirmed or suspected.

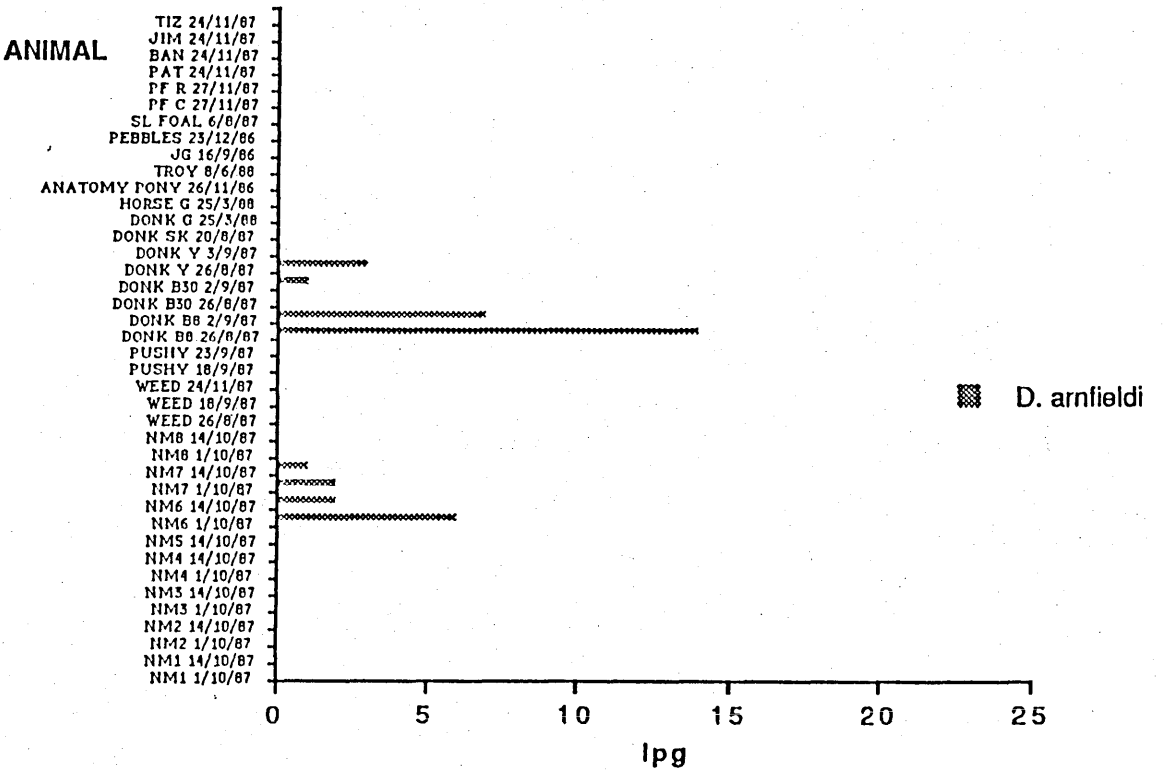
These were essentially samples taken from animals referred to Glasgow University Veterinary School or sent in by practitioners. Most had a known history which in some included a recent coughing episode. Several animals were sampled on more than one occasion. The results are illustrated in Figure 2.19.

Of the 29 animals sampled, five showed patent D. arnfieldi infections all of which were donkeys . The ODs of serum samples from these five animals varied from 0.1 - 0.6 and were not significantly different from those obtained with the sera from the other donkeys and horses included in this survey.

Figure 2.19

The optical densities using raw and EDTA-treated serum in the ELISA for *D. arnfieldi* together with faecal larval *D. arnfieldi* counts (l.p.g.) from animals referred to Glasgow Veterinary School


ANIMAL
TIZ 24/11/87
JIM 24/11/87
BAN 24/11/87
PAT 24/11/87
PF R 27/11/87
PF C 27/11/87
SL FOAL 6/8/87
PEBBLES 23/12/86
JG 16/9/86
TROY 8/6/88
ANATOMY PONY 26/11/86
HORSE G 25/3/88
DONK G 25/3/88
DONK SK 20/8/87
DONK Y 3/9/87
DONK Y 26/8/87
DONK B30 2/9/87
DONK B30 26/8/87
DONK B8 2/9/87
DONK B8 26/8/87
PUSHY 23/9/87
PUSHY 18/9/87
WEED 24/11/87
WEED 18/9/87
WEED 26/8/87
NM8 14/10/87
NM8 1/10/87
NM7 14/10/87
NM7 1/10/87
NM6 14/10/87
NM6 1/10/87
NM5 14/10/87
NM4 14/10/87
NM4 1/10/87
NM3 14/10/87
NM3 1/10/87
NM2 14/10/87
NM2 1/10/87
NM1 14/10/87
NM1 1/10/87
0.0
0.2
0.4
0.6
0.8
1.0
O.D.
EDTA TREATED
RAW SERUM


ANIMAL
TIZ 24/11/87
JIM 24/11/87
BAN 24/11/87
PAT 24/11/87
PF R 27/11/87
PF C 27/11/87
SL FOAL 6/8/87
PEBBLES 23/12/86
JG 16/9/86
TROY 8/6/88
ANATOMY PONY 26/11/86
HORSE G 25/3/88
DONK G 25/3/88
DONK SK 20/8/87
DONK Y 3/9/87
DONK Y 26/8/87
DONK B30 2/9/87
DONK B30 26/8/87
DONK B8 2/9/87
DONK B8 26/8/87
PUSHY 23/9/87
PUSHY 18/9/87
WEED 24/11/87
WEED 18/9/87
WEED 26/8/87
NM8 14/10/87
NM8 1/10/87
NM7 14/10/87
NM7 1/10/87
NM6 14/10/87
NM6 1/10/87
NM5 14/10/87
NM4 14/10/87
NM4 1/10/87
NM3 14/10/87
NM3 1/10/87
NM2 14/10/87
NM2 1/10/87
NM1 14/10/87
NM1 1/10/87
0
5
10
15
20
25
lpg
D. arnfieldi

In the case of Donkey Y whose faecal D. arnfieldi larval counts were 4 lpg and zero in samples taken one week apart, the ODs with untreated or EDTA treated serum samples were both < 0.2. In the remaining four donkeys where patent D. arnfieldi infections were confirmed (B8, B30, NM6 and NM7) the OD's varied from < 0.2 - 0.6.

No D. arnfieldi larvae were evident in the faeces of the other animals in this survey but this does not rule out the possibility of infection as retardation of development often occurs in horses/ponies. In the animals in the NM group which had been grazing with the two infected donkeys and were clinically ill prior to treatment, the initial ODs were > 0.3: All these animals showed a decrease in ODs in samples taken one week after anthelmintic treatment. The four animals for which only untreated serum was tested were horses with no known history of lungworm infection or exposure to D. arnfieldi within the preceding 18 months. Two of these animals (Jim and Pat) had ODs greater than 0.3 when read at 492 nm.

USE OF AN ELISA FOR DETECTION OF *Dictyocaulus arnfieldi* ANTIBODIES

Although the main aim of the ELISA used in this work was to diagnose current infection by detecting *D. arnfieldi* antigen, a test was also carried out on sera from animals known to have been exposed to infection to find out if it was possible to detect antibodies to *D. arnfieldi*.

The format used was a 96 well microelisa plate which was coated with *D. arnfieldi* antigen solution at varying dilutions. Serum from a mare which was known to be infected with *D. arnfieldi* (*D. arnfieldi* worms were recovered from the lungs at necropsy) was tested using FrR_1 as the positive control and serum from a naive horse foal, which had never had access to pasture, as a negative control. Following incubation and washing, the plate was developed using anti-rabbit IgG peroxidase for the FrR_1 and anti-horse Ig peroxidase (affinity isolated antibody-Sigma) for the test sample and the negative control. Following incubation and washing, the substrate and chromogen (hydrogen peroxide and OPD respectively) were added, the plate incubated in the dark for 30 minutes before reading at 450 nm prior to stopping the reaction with acid and re-reading at 492 nm.

Summary of Procedure

(1) Coat plate with *D. arnfieldi* antigen in coating buffer (50 µl per well).

(2) Incubate plate for two hours at 37°C.

(3) Wash plate three times with wash buffer and tap dry.

(4) Add antibody (sample-test or control), 50 µl per well.

(5) Incubate plate for 30 minutes at 37°C.

(6) Wash plate three times with wash buffer and tap dry.

(7) Add appropriate anti-species IgG peroxidase (1:500 in diluting buffer, 50 µl per well).

(8) Incubate plate for 30 minutes at 37°C.

(9) Wash plate three times with wash buffer and tap dry.

(10) Add substrate/chromogen, 50 µl per well.

(11) Incubate for 30 minutes at 37°C in the dark.

(12) Read at 450 nm.

(13) Stop with $2MH_2SO_4$, 50 µl per well.

(14) Read at 492 nm.

RESULTS

This test detected antibody in the serum of the mare down to dilutions of 1:320 with the plate coated with antigen at a dilution of 1:40.

Following this successful detection of *D. arnfieldi* antibody in one animal, serum samples obtained at necropsy from all the animals used in the experimental infection of mares and foals (Section 1 Experiment 2) were tested (Table 2.5 and Figure 2.20). The plate was coated with antigen at a dilution of 1:40 and a naive foal was used as a negative control. Serum was tested in a range of dilutions - undiluted, 1:10, 1:20 and 1:40. The aforementioned procedure was followed.

Table 2.5

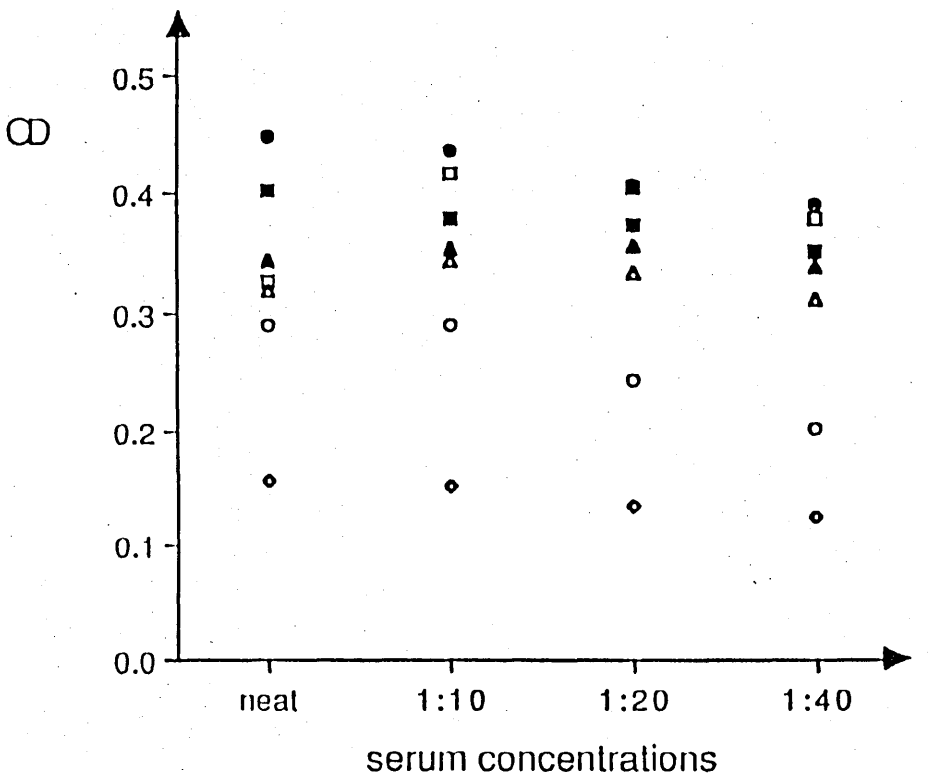
Mean Optical Densities Obtained Using the ELISA for D. arnfieldi Antibody at Varying Serum Dilutions

Animal	Mean O.D. obtained at varying serum dilutions at 492 nm				Weeks post infection
	Undiluted	1:10	1:20	1:40	
13 M	.402	.377	.373	.351	6
14 F	.327	.416	.404	.378	6
11 M	.449	.436	.408	.390	8
16 F	.290	.291	.245	.202	8
12 M	.345	.354	.356	.340	10
15 F	.319	.344	.334	.313	10
Naive Foal	.156	.151	.135	.125	-

The results of this small experiment showed that it was possible to detect serum antibodies to D. arnfieldi by an ELISA test. While refinement of the test could improve the low optical densities achieved, for reasons stated previously antigen detection is still preferable as a diagnostic test for equine lungworm infection. The slightly lower optical densities shown by two of the foals compared with the mares could be related to the generally less severe clinical respone shown by the infected foals.

Figure 2.20

Mean optical densities obtained using the ELISA for *D. arnfieldi* at varying dilutions of serum from mares and foals experimentally infected with *D. arnfieldi* (Section 1 - Experiment 2) and a *D. arnfieldi*-naive foal



Mare no. 13 ■ Foal no. 14 □ Naive foal ◇

Mare no. 11 ● Foal no. 16 ○

Mare no. 12 ▲ Foal no. 15 △

DISCUSSION OF ELISA STUDIES

Despite the successful development of tests to detect both lungworm antigen and antibody to D. arnfieldi, the results of the antigen detection assay in terms of the optical densities obtained by the ELISA did not correlate well with the parasitological findings, e.g. the detection of D. arnfieldi larvae in faeces. For example in 21 animals which were positive on faecal examination for lungworm larvae the serum ELISA ODs of 18 of these animals were > 0.3 while in the remaining three they were < 0.3. Also in a further 131 animals which were not found to be excreting lungworm larvae on faecal examination, 92 had serum ELISA ODs > 0.3 while in the remaining 39 the ODs were > 0.5.

There is no obvious explanation for these discrepancies but similar problems have been encountered with ELISA tests for Dictyocaulus viviparus (Boon, Kloosterman and Brink, 1982) and Trichinella spiralis (Saunders, Clinard, Bartlett and Saunders, 1977) although both of these tests detected antibody. The test for D. viviparus had poor specificity due to a large proportion of false positives, which included some naive calves. It was suggested that this may have been due to colostral antibodies or minimal infections acquired via hay. There was an additional problem of a low level of cross reactions with gastrointestinal nematodes. Overall however, the specificity and sensitivity of the ELISA for antibodies to D. viviparus was sufficient for herd screening for infection but not for diagnosis of infection in the individual animal. In trichinosis the ELISA was successful in

the detection of antibodies to T. spiralis in experimental infections but when used in commercial pigs at slaughter, a 15% level of false positive reactions occurred. These false positives were found to be due to non-specific serum factors which bound to components of the crude antigen preparation used in this ELISA. Another ELISA used to detect T. spiralis also produced false positives but in this case the genetic backgrounds of the pigs were considered to have some effect (van Knapen, Franchimont, Skovgaard, Guildal and Henriksen, 1984). The apparent lack of correlation in the D. arnfieldi antigen ELISA could be due simply to technical difficulties in detecting low levels of D. arnfieldi larvae in faeces. A similar explanation was given by van Knapen, Franchimont and Ruitenberg (1981), who suggested that some increases in specific antibody titres in the ELISA for trichinellosis could be accounted for by low numbers of Trichinella parasites infecting the pigs, which were not detected by classical parasitological means. Another explanation is that the D. arnfieldi ELISA was detecting non-patent infections as prepatent D. viviparus infections have given positive results by ELISA in calves (Bos, Beekman-Boneschanscher and Boon, 1986). In this cattle study a different response to larval and adult worm antigens was found as anti-larval titres appeared approximately one week earlier than anti-adult titres which appeared coincidentally with patency. However in an ELISA developed for detection of antibody to Fasciola hepatica in cattle (Burden and Hammet, 1978), antigen prepared from adult

flukes proved useful in the detection of prepatent infections. With D. arnfieldi there is the additional problem of retardation of development of the parasites which therefore do not mature to produce patent infections.

The D. arnfieldi antigen used in these studies was a crude antigen obtained from a homogenate of worms which included all lung dwelling stages and, unavoidably, some mucus and other lung secretions. An advantage of this crude antigen was that it included a wide, if not entire, range of parasite stages and secretions liable to be found in the host. The wide range of stages included in the antigen extract ensured that only those parasite antigens which occured in the host were used to raise antibodies in the rabbits. The disadvantage of the crude antigen was that there was the possibility of host tissues contaminating the worm homogenate thus the rabbits could have produced antibodies against some equine proteins as well as against D. arnfieldi antigens. This would increase the likelihood of non-specific reactions leading to false positives due to recognition, by the antibodies raised in the rabbits, of epitopes in the equine test serum. If a more specific antigen had been used to raise antibodies, a narrower spectrum of epitopes would have been detected in the ELISA which may in turn have reduced the background in the negative controls and thus eliminated some of the false positive reactions. In the development of an ELISA for the diagnosis of human hydatidosis (Farag, Bout and Capron, 1975), two antigens were used - whole hydatid fluid and purified hydatid antigens. Using the purified hydatid antigens the

overlap between infected patients and uninfected controls, which was evident when whole hydatid fluid was used as antigen, was eliminated; the ELISA values were also higher with the purified hydatid antigen. A similar situation occurred in the ELISA assay for antibodies in schistosomiasis (Huldt, Lagerquist, Phillips, Draper and Voller, 1975) where the use of a purified egg antigen produced considerably better differentiation between positive and negative samples than that achieved when using crude S. mansoni antigen. However, until it is determined which parts of the antigenic repertoire of D. arnfieldi are responsible for the immunopathogenesis of disease, it is possible that a highly purified antigen might not contain some essential epitopes and thus reduce antigen detection.

GENERAL DISCUSSION

Although D. arnfieldi is the sole equine lungworm and is worldwide in distribution, apart from the relatively detailed studies of Round (1972) there has been little research on this parasite. This is no doubt due in part to the fact that unlike the bovine lungworm D. viviparus which may cause severe disease with fatalities, the equine lungworm is considered of limited importance in horses and although known to be a common parasite of the donkey, it apparently causes few respiratory problems in this host. In horses however D. arnfieldi has been incriminated in cases of chronic coughing but confirmation of diagnosis of lungworm infection in such cases has posed major problems. This is due to the fact that the parasites do not often mature to the adult stage in horses and first-stage larvae cannot therefore be detected on faecal examination. The major thrust of the work reported in this thesis was to develop a test which would confirm the presence of an active lungworm infection by the detection of D. arnfieldi antigens and thus be a useful aid to diagnosis.

A pre-requisite for a number of studies proposed in this project was a reliable source of lungworm larvae. To this end various methods of laboratory culture of D. arnfieldi from L_1 present in faeces to infective third stage larvae (L_3) were attempted but most gave inconsistent or disappointing results. Faeces were incubated in water with sawdust, vermiculite, filter paper or charcoal; they were also spread in shallow layers on trays with and without the addition of moist sawdust, vermuculite or filter paper but none of these methods produced significant yields of L_3 and most were contaminated. Attempts to reduce this

contamination by sieving (38 μ) or filtering through milk filters at various stages in the culture process led to unacceptable larval losses. The most satisfactory method developed in this laboratory, involved the baermanisation of infected faeces in urine glasses followed by sedimentation of D. arnfieldi L_1. Larval rich fractions were then combined, fresh tap water added and several washing and sedimentation cycles carried out, to obtain a clean concentrated suspension of D. arnfieldi larvae. Once clean samples of D. arnfieldi larvae were obtained, they were incubated in shallow water in flat bottomed dishes for several weeks prior to use. This method was a modification of that used by Britt and Preston (1985) and although time-consuming and requiring a lot of space, the results were acceptable and relatively consistent.

In the initial experimental infection of mature ponies (Experiment 1) one animal showed a marked clinical response, the remainder exhibiting only intermittent coughing and slightly raised respiratory rates; this was similar to the findings of Round (1972) who attributed the lack of response to low infectivity of larvae. However the results of necropsies in Experiment 1 and in a study by Britt and Preston (1985) in 12 naturally reared Fell ponies which failed to show any significant clinical signs after infection, confirmed that D. arnfieldi worms were present in most of the ponies indicating that the lack of clinical signs was not due to failure of the experimental infections to establish. This lack of clinical response after

single or repeated experimental infections of lungworm larvae is in contrast to reports of marked coughing in horses and ponies which have been exposed to D. arnfieldi infection when grazing, usually through contact with infected donkeys. Such a situation was observed during the course of the West of Scotland survey of lungworm infection when two ponies from a group of five showed increased respiratory rates, frequent coughing and harsh lung sounds four weeks after the introduction of two D. arnfieldi infected donkeys to share the same grazing. Mackay and Urquhart (1979) also recorded increased respiratory rates, moist productive coughing and wheezes and crackles on auscultation in a group of horses which had grazed with lungworm infected donkeys for two months while in 1981, Neilson and Anderson reported chronic coughing in several horses which commenced three weeks after the arrival of an infected donkey: at necropsy of these animals D. arnfieldi were found in the bronchi. A more unusual report (Rickard and James, 1976) was the finding of D. arnfieldi larvae in the faeces of two yearlings which were showing clinical signs of pulmonary infection which did not respond to antibiotics but resolved following anthelmintic treatment.

The apparent absence of significant clinical signs in experimental infections compared with 'naturally acquired' infections is difficult to explain. One explanation could be that the majority of naturally acquired infections in horses are also sub-clinical and only a few clinical outbreaks are reported after investigation by a veterinary surgeon. Another factor which could influence the occurrence of clinical signs is

exercise. Ponies kept under experimental conditions are not usually exercised and therefore not subjected to the sustained respiratory effort required by the average pleasure horse which receives regular exercise at slow and fast paces.

Despite the mild clinical response of the ten mature ponies in Experiment 1 there were lesions suggestive of lungworm infection (Nicholls et al, 1978) in the lungs of all the animals at necropsy and the severity of these lesions was more pronounced with the increased duration of infection. The lack of a clinical response could be related to the level of experimental challenge but the pathological changes observed could have been expected to produce more obvious clinical signs. One factor mentioned previously which could have had an influence on the clinical response was the lack of exercise or stress in this group of ponies. These animals had been housed together and allowed to settle in their accommodation for several weeks before the start of the experiment. As they had access to shelter and hay ad lib there was no stress due to adverse weather conditions or competition for food, and as the pecking order had been established previously, bullying was not a problem. The concrete run out area gave the ponies ample room to walk about but not to take fast exercise which could have induced more coughing. The effect of exercise on the clinical manifestations of D. arnfieldi infection has not been examined.

The only report of severe clinical signs associated with experimental lungworm infections was in ponies which had been

reared indoors, worm-free (Round, 1972): it was suggested however, that other infectious agents might have been involved in the observed clinical response. Also this is somewhat at variance with the finding that horse foals infected with D. arnfieldi during grazing develop patent infections without clinical signs (Clayton and Duncan, 1981): in this case the level of exposure was probably important with establishment following repeated daily ingestion of low numbers of larvae in young susceptible animals.

In the experimental infection of the pony mares and foals (Experiment 2) minimal clinical signs were observed in the foals compared with the mares. There is a general belief that horse/pony foals behave more like donkeys than their adult counterparts and tend not to develop clinical disease with D. arnfieldi infection. Similar findings were reported in a field study by Clayton and Duncan (1981) when pony mares coughed while their foals remained clinically normal although all animals were kept together on infected pasture. This could have been related to differences in the level of challenge, however there are reports of horse foals developing clinical signs due to D. arnfieldi. For example in Kentucky, Lyons et al (1982) reported the case of an Arab foal which first exhibited respiratory disease when only a few months old after being pastured, with its dam, in a paddock occupied by a donkey with a patent D. anfieldi infection. This foal was clinically ill showing an increased respiratory rate, dyspnoea and a raised temperature. This was attributed to D. arnfieldi infection since

D. arnfieldi larvae were repeatedly found in the tracheal washings. Following effective anthelmintic treatment (thiabendazole 440 mg/kg) the foal recovered and became clinically normal with no lasting respiratory impairment. Poncet (1983) also reported a case of a six month-old foal which developed respiratory problems after grazing with donkeys. This foal had a spasmodic cough, raised temperature and an increased respiratory rate. Unlike the foal in Kentucky this foal was found to be passing D. arnfieldi larvae in its faeces. Again, following effective anthelmintic treatment, the foal recovered.

The pathological changes in the lungs of the infected foals in Experiment 2 were much less severe than those observed in the infected mares or in the mature ponies in Experiment 1. This could be related simply to the lower dose of larvae used to infect the foals but it is also possible that the young animals could be showing a diminished immunological response to infection and, as much of the pathology associated with infection is thought to be related to the immune response to D. arnfieldi, this resulted in fewer lesions and a minimal clinical reaction.

The latest reported survey of D. arnfieldi in the U.K. equine population was conducted by Round in 1972. He found an overall prevalence of infection in donkeys of 73%. Our survey of 700 donkeys admitted to the Donkey Sanctuary in Devon showed a considerable decline in the prevalence of infection to 34%. In recent years the widespread use of new and improved anthelmintics has pobably played a key role in the decrease in the numbers of

infected donkeys in U.K. and in other parts of the world (Boersema and Kalis, 1978; Pandey, 1980; Heil, 1983).

Over the course of these studies there was the opportunity to examine faecal samples from donkey mares under a variety of conditions e.g. while housed, during grazing, during pregnancy and lactation and after weaning. None of these factors exerted any noticeable influence on the faecal larval output of these donkeys. This is in contrast to the seasonal peaks of infection, in autumn and winter, recorded in donkeys in Morocco by Pandey (1980) but similar to the findings of Round (1976) who showed that although there were fluctuations in the faecal larval output of individual donkeys, these were of minor significance and were not apparently influenced by season of the year or any other factors. One donkey, which had a patent infection at the start of the observations described here subsequently became non-patent and never resumed patency despite almost constant contact with the other infected donkeys. Although Pandey (1980) thought patency was maintained due to reinfection and there was no age immunity, the continued lack of patency in adult donkeys exposed to reinfection after "self cure" or effective anthelmintic treatment (Round, 1976) suggests that some animals develop an effective immunity. It is possible, however, that these donkeys could be behaving like some horses and harbouring non-patent infections since heavy lungworm burdens have been found in donkeys which had no demonstrable larvae in their faeces (Pandey, 1980). Another possible explanation for discrepancies between lungworm burdens and faecal larval recoveries is that the present

parasitological methods of faecal examinations for D. arnfieldi L_1 are fairly crude and that many infections therefore go undetected. To resolve the question of the extent of non-patent infections in donkeys a large number of lungs from animals with known histories would need to be examined at necropsy.

One of the main problems in studies of D. arnfieldi in horses arises from the individual nature of the host. With D. viviparus the problem has a herd basis and it is therefore possible to study large numbers of animals of a similar age kept under identical management conditions. In contrast studies on D. arnfieldi tend to involve small groups of animals of widely different ages and breeds kept under varied management conditions. Again there is little doubt that a great deal of information regarding D. arnfieldi infection in the horse could be gained by routine examination of lungs at necropsy. This would increase our knowledge of various aspects of D. arnfieldi infection and could contribute information which would be useful in its control in the equine population.

The experimental and field studies reported in Section 1 provided some information on the prevalence, pathogenesis and clinical response to D. arnfieldi infection. They were also designed to provide material for use in the development of a diagnostic test for equine lungworm infection and in Section 2 the procedures adopted in the establishment of ELISAs to detect D. arnfieldi antigens and antibodies are described. Using antibodies raised in rabbits to all stages of D. arnfieldi found

at necropsy of a naturally infected animal, ELISAs were successfully developed which detected both lungworm antigens and antibodies. Unfortunately the ELISA for D. arnfieldi antigen gave variable results in animals of known lungworm status including a large proportion of false positive reactions in animals with no known exposure to infection. This indicated that further refinements of the test were required to achieve a diagnostic test for current infection.

It is possible that a system using excretory/secretory (ES) antigens rather than somatic/surface antigens would have been more specific as ES antigens form the first host/parasite interface. For example in an ELISA for the detection of swine trichinosis (Gamble, Anderson, Graham and Murrell, 1983) the nature of the antigen used in the test was considered the most important determinant in the occurrence of false positive reactions. The use of ES antigens in this ELISA eliminated all positive reactivity in the sera of farm-raised pigs. This increase in specificity was due to the lack, in the ES fraction, of a number of high molecular weight proteins present in the crude extract which gave rise to non-specific reactions. An ELISA using Toxocara ES antigens (de Savigny, Voller and Woodruff, 1979) was highly sensitive in the detection of toxocaral antibodies and it was predicted that any antibody responses to parasite ES antigens would be better indicators of active or recent Toxocara infection than responses to somatic antigens. If an animal has specific antibodies to ES antigens, the parasite must have been present recently in a viable form

within the animal as only live parasites produce ES antigens. Excretory/secretory antigens were not used in the ELISA for D. arnfieldi as it was found impossible to maintain D. arnfieldi parasites for any length of time in culture. No such culture problems are presently encountered with Toxocara and Ascaris and ES antigens of the L_2 stage as well as the lung stages (L_3/L_4) have been compared with the body fluid of adult Ascaris for antigenicity. Antibody responses to body fluid were inferior to those against ES antigens which were found to be both stage specific and targets of considerable host antibody response (Kennedy and Qureshi, 1986). As there is systemic distribution of the ES components in the host this makes them ideal candidates for the detection of antigen in sites distant from the infection site. The origin of these ES antigens is unclear but they are likely to be a composite of surface secretions and excretions of the parasite as well as fluids released by the parasite during moulting.

In this study antiserum was raised in rabbits by injecting D. arnfieldi homogenate in Freund's complete adjuvant, subcutaneously at multiple sites. Due to the limited availability of worm antigen, the rabbits only received a second dose of homogenate also in Freund's complete adjuvant which had been premixed and stored at $-20^{o}C$ for four weeks. A more usual immunisation schedule would have been to give the second dose of D. arnfieldi homogenate in Freund's incomplete adjuvant followed at a later date with a boost of D. arnfieldi antigen only. After

this final boost the rabbits would then have been 'test bled', to determine from a rise in titre, the best time to bleed out to obtain the maximum yield of high affinity and avidity antibodies. Due to the scarcity of antigen together with the fact that a suitable test for D. arnfieldi antibody was not available at that time, it was not possible to test bleed the immunised rabbits to determine their optimal response in terms of high avidity antibody. With the antibody test developed during the course of these studies and assuming a continued availability of D. arnfieldi antigen, it would now be possible to test bleed the rabbits to determine the peak response.

It is also the case that had more refined D. arnfieldi antigens been used to immunise the rabbits, the antibody responses might have been more specific as they would have been directed against fewer and more defined epitopes.

Antibody responses are usually directed against the most immunogenic worm components and, as mentioned previously, it is most likely that maximum antibody responses are directed against worm excretions and secretions rather than somatic components. If these 'target' antigens could be isolated and used, a more avid antibody with a higher affinity could be raised which would enhance the sensitivity and specificity of the assay. Part of the importance of ES antigens is that they are continually produced/renewed by the living worm hence giving a greater biomass. As D. arnfieldi migrate from the intestine to the lungs, they are likely to continually shed ES antigens and each 'depot' of ES antigen is equivalent to a microimmunisation of the

host. Only after the death of the parasite are the somatic antigens exposed to form the second host parasite interface. Obviously, the quantities of somatic antigen are finite and cannot be renewed.

While the assay for D. arnfieldi did not always correlate with the detection of D. arnfieldi larvae in faeces, the ELISA which was developed was robust and the results, with samples of known antigen content were reproducible. It is vital in any ELISA that the binding involved is irreversible or that there is minimal leeching and desorption of antigen/antibody from the plate or antigen from antibody especially in the presence of detergents. This was achieved in the D. arnfieldi ELISAs described in this thesis.

ELISAs with greater affinities are produced utilising the blocking properties of detergents. By incorporating detergents in the buffers, antibodies with low affinities are prevented from binding in the assay, hence only those antibodies with the greatest affinity bind which increases the specificity of an assay. With further refinements, a reduction of the optical densities of negative samples could be achieved and thus any overlap between positive and negative samples is reduced if not eliminated. Other favourable aspects of ELISAs are their simplicity and the rapidity with which results can be obtained. For example the ELISA for Trichinella spiralis in pigs (Ruitenberg, Steerenberg, Brosi and Buys, 1976) enables a spot diagnosis to be carried out at the slaughterhouse. Also large

numbers of samples can be tested in one day. This has been demonstrated using a semiautomated ELISA for Toxoplasma gondii when 4,000 samples were tested daily (Walls, Bullock and English, 1977). Other advantages are the small quantities of sera required which are relatively cheap and the fact that expensive equipment is not required for the test.

Although most ELISAs used in human and veterinary medicine are for antibody detection e.g. Malaria, African Trypanosomiasis, Chagas disease, Amoebiasis, Toxoplasmosis, Babesiosis, Leishmaniasis, Schistosomiasis, Hydatid disease, Trichinosis and Onchocerciasis (Voller, Bartlett and Bidwell, 1976; 1978), in view of the nature of D. arnfieldi infection in grazing horses and donkeys, an antibody detection test would only indicate exposure, either previous or current, to D. arnfieldi. To be of use diagnostically, a quantitative antibody test using a minimum of two successive samples would be required to determine whether there was a rising titre indicating current infection. Such a quantitative test would obviously be more complicated than a simple positive/negative test for antigen. Unlike the situation with D. viviparus, infections with D. arnfieldi may be longstanding before investigations are initiated. It is unlikely therefore that antibody profiles would show the classic rising titre associated with the acute stage of infection. Another factor complicating the diagnosis of D. arnfieldi infection is that it is usually an individual rather than a herd problem so ELISAs which are adequate for herd or population diagnosis e.g. in bovine dictyocauliasis (Boon et al, 1982) or trypanosomiasis

(Ruitenberg and Buys, 1977; Luckins, 1977), are inadequate in the case of D. arnfieldi. The lack of a rising titre is further compounded by the lack of multiplication of lungworms within the host which in turns affects the host's immune response. Also in the case of D. arnfieldi it is likely that antibody production will have reached a plateau by the time of testing which makes interpretation of results difficult.

While the detection of antigen would be confirmation of a current, active infection, there are problems concerning antigen detection. It would be expected that free antigen would be most abundant during the acute phase of infection or reinfection and, in the ELISA for the detection of toxoplasmosis (van Knapen and Panggabenn, 1977), it was only possible to detect circulating antigen during the short period of the active phase of infection. In animals chronically infected with D. arnfieldi the amount of free circulating antigen is likely to be very small and present as immune complexes which, in this case would hinder antigen detection. However in an ELISA for Toxoplasma antigen (van Knapen, Panggabean and van Leusden, 1985), circulating immune complexes could be detected coincidently with antibodies and remained detectable for several weeks. In this case the detection of immune complexes represented an amplification of the detection of free circulating antigen as both followed the same pattern although the levels of free Toxoplasma antigen were too low to be detectable by the ELISA.

Apart from any technical limitations of the test, the discrepancies between the D. arnfieldi serological and parasitological results could be due to a number of factors. It is possible that, especially with the samples sent by post, freezing and thawing had an adverse effect on the D. arnfieldi antigen. In an ELISA for detection of Entamoeba histolytica in faecal specimens (Ungar, Yolken and Quinn, 1985), it was found that the antigenicity of the specimens varied with repeated freezing and thawing. Freezing and thawing can disrupt the bonding of antigens which are either polypeptide or carbohydrate in nature. Also if samples are transported in plastic tubes some antigen may bind to the tubes thus reducing the total amount of antigen available to react in the ELISA.

In the animals where no necropsies were performed and the only data available were the faecal larval counts, the possible existence of non-patent D. arnfieldi infections could account for the varied positive optical densities associated with negative faecal larval counts. Positive serological results linked with negative parasitological results could also be due to the detection of blood borne immune components shortly after initial infection and before the detection of larvae in faeces which are produced by sexually mature adult D. arnfieldi in the lungs. This development from infective larva to adult parasite takes a minimum of 12 weeks, hence the ELISA could be detecting prepatent D. arnfieldi infection. In the case for the ELISA test for Trichinella spiralis in pigs (Ruitenberg, Steerenberg, Brosi and Buys, 1974) positive results are obtained prior to the

demonstration of larvae in the muscles.

A more specific test for D. arnfieldi infection in horses might be developed using monoclonal antibodies (Mabs). By their nature, Mabs have a precise and predefined paratope which ideally reacts with an individual epitope. At the present time it is not known which of the epitopes involved in the immunogenic response to D. arnfieldi would be the most potent and specific to choose for use in a detection test utilising Mabs. A more novel technique, which may be feasible to develop as a detection assay for D. arnfieldi is the use of single domain antibodies (Dabs). These are very small regions of the heavy chain of the IgG molecule which contain the configurations necessary for binding antigens of high affinity with only a 10% reduction in binding compared with that obtained using the whole molecule (Austin, 1989; Anon, 1989). Dabs possess the same ability as Mabs to recognise specific epitopes of antigens in a mixture but they have the advantage in that they can be produced in bulk from bacteria within a few days while large quantities of Mabs require extensive tissue culture which is slow and expensive (Ward, 1989; Wilkes, 1989).

Finally variations in the degree of antigenicity of different antigens may have an indirect effect on the success of an ELISA. In the case of D. arnfieldi, if the antigens are highly immunogenic, a strong host antibody response might be initiated which would remove worm antigens from the circulation very rapidly thus interfering with their detection. On the other

hand, if D. arnfieldi antigens are only weakly immunogenic, a lesser response might be mounted by the host leaving antigens in the circulation for a longer period which would facilitate their detection by both Mabs or Dabs. In practice however, various stages of D. arnfieldi and their secretions are likely to be composed of a range of antigens with differing antigenicities. Characterisation of these antigens together with refinement and development of the basic D. arnfieldi ELISA described here should make it possible to produce an accurate, reliable and specific diagnostic test for equine lungworm infection.

REFERENCES

Akramovskii, M.N. (1952a). Life Cycle of the Causative Agent of Horse Dictyocauliasis. Uchen Zap Vitebs Vet. Inst. 11, 117-124.

Akramovskii, M.N. (1952b). Dictyocaulus in Horses. Veterinariya 29, 44.

Anderson, S. and Fogh, J. (1981). Prevalence of Lungworm, Dictyocaulus arnfieldi in donkeys in Denmark. Nordisk Veterinaer Medicin 33 9/11, 484-491.

Anon (1989). Single Domain Antibodies. Lancet Editorial, p. 1370.

Austin, P. (1989). Will dAbs Challenge MAbs? Nature 341, 484-485.

Boersema, J.H. and Kalis, C.H.J. (1978). Occurrence of Dictyocaulus arnfieldi Infection among Donkeys in The Netherlands. Tijdschrift voor Diergeneeskunde 103 19, 998-1001.

Boon, J.H., Kloosterman, A. and Brink, R. van den (1982). The incidence of Dictyocaulus viviparus Infections in Cattle in The Netherlands. I. The Enzyme Linked Immunosorbent Assay as a Diagnostic Tool. The Veterinary Quarterly 4, 155-160.

Borovkova, A.M. (1948). Epizootiology of Dictyocaulus arnfieldi in Horses. Veterinariya 25, 13-14.

Bos, H.J., Beekman-Boneschanscher, J. and Boon, J.H. (1986). Use of ELISA to Assess Lungworm Infection in Calves. Veterinary Record 118, 153-156.

Britt, D.P. and Preston, J.M. (1985). Efficacy of Ivermectin against Dictyocaulus arnfieldi in Ponies. Veterinary Record 116, 343-345.

Burden, D.J. and Hammet, N.C. (1978). Microplate Enzyme Linked Immunosorbent Assay for Antibody to Fasciola hepatica in Cattle. Veterinary Record 103, 158.

Clayton, H.M. (?). Lung Parasites. Current Therapy in Equine Medicine 520-522.

Clayton, H.M. and Duncan, J.L. (1981). Natural Infection with Dictyocaulus arnfieldi in Pony and Donkey Foals. Research in Veterinary Science 31 3, 278-280.

Clayton, H.M. and Murphy, J. (1980). The Coughing Horse. In Practice 25-34.

Clayton, H.M. and Neave, R.M.S. (1979). Efficacy of Mebendazole against Dictyocaulus arnfieldi in the Donkey. Veterinary Record 104 25, 571-572.

Clayton, H.M. and Trawford, A.F. (1981). Anthelmintic Control of Lungworm in Donkeys. Equine Veteterinary Journal 23 (3), 192-194.

Cobbold, T.S. (1884). New Parasites from the Horse and Ass. Veterinarian, London (673) 57 4.s. (349), v 30, 4-7.

Crowther, J. and Smith, H.V. (1986). Use of Enzyme Linked Immunosorbent Assays in Disease Diagnosis, November 1986. FAO/IAEA Regional Training Course on Radio- and Enzyme Immunoassay Techniques in Animal Reproduction and Disease Diagnosis.

de Savigny, D.H., Voller, A. and Woodruff, A.W. (1979). Toxocariasis: Serological Diagnosis by Enzyme Immunoassay. Journal of Clinical Pathology 32, 284-288.

Duwel, D. (1983). Panacur for the Treatment of Lungworm in Horses. Personal Communication.

Enigk, K. and Dey-Hazra, A. (1976). Febendazole Treatment of Helminth Infections in Wild Animals and Birds. Kleintierpraxis 21 4, 133-140.

Enigk, K. and Weingartner, G. (1973). Distribution and Treatment of Dictyocaulosis in Solipeds. Deutsche Tierarztliche Wochenschrift 80, no. 7, 145-148.

Farag, H., Bout, D. and Capron, A. (1975). Specific Immunodiagnosis of Human Hydatidosis by the Enzyme Linked Immunosorbent Assay (ELISA). Boimedicine 23, 276-278.

Fischer, J., Deegan, E. and Lieske, R. (1982). Bronchoscopic Detection of a Patent Lungworm Infection in Horses. Tierarztl. Praktik 10, 219-224.

Gamble, H.R., Anderson, W.R., Graham, C.E. and Murrell, K.D. (1983). Diagnosis of Swine Trichinosis by Enzyme Linked Immunosorbent Assay (ELISA) Using an Excretory Secretory Antigen. Veterinary Parasitology 13, 349-361.

Gordon, H. McL. and Whitlock, H.V. (1939). A New Technique for Counting Nematode Eggs in Sheep Faeces. Journal of Council for Science and Industry Research, Australia, 12-50.

Heil, H.G. (1983). Intestinal Parasite Fauna and Dictyocaulus arnfieldi: Infection of Donkeys in Hesson and Seasonal Variations in Infection Rates and Intensities. Dissertation, Justus-Liebig-Universitatt, Giessen, FRG.

Henriksen, S.A. (1965). An Improved Technique for the Examination of Faeces for Larvae of Lungworms. Nord. Vet. Med. 17, 446-454.

Huldt, G., Lagerquist, B., Phillips, T., Draper, C.C. and Voller, A. (1975). Detection of Antibodies in Schistosomiasis by Enzyme Linked Immunosorbent Assay (ELISA). Annals of Tropical Medicine and Parasitology 69 (4), 483-488.

Inderbitzen, F. (1976). Experimentall erzengte entwieklungshemmung von Dictyocaulus viviparus des Rhindes. Ph.D. Thesis, Institud fur Parasitologie der Universitat Zurich (Switzerland).

Jarrett, W.F.H., Jennings, F.W., McIntyre, W.I.M., Mulligan, W. and Urquhart, G.M. (1957). The Natural History of Parasitic Bronchitis with Notes on Prophylaxis and Treatment. Veterinary Record 69, 1329-1336.

Kennedy, M.W. and Qureshi, F. (1986). Stage-Specific Secreted Antigens of the Parasitic Larval Stages of the Nematode Ascaris. Immunology 58, 515-522.

Knapen, F. van, Franchimont, J.H. and Ruitenberg, E.J. (1981). (On behalf of the members of the EEC Working Group on Trichinellosis). In C.W. Kimm, E.J. Ruitenberg and J.S. Teppema (Editors), Trichinellosis. Reedbooks, Windsor, Berks. pp. 399-404.

Knapen, F. van, Franchimont, J.H., Skovgaard, N., Guildal, J. and Henriksen, S.A. (1984). Husbandry, Parasitic and Other Diseases as Factors in the Reliability of the ELISA for Detection of Trichinellosis in Pigs. Veterinary Parasitology 16, 17-22.

Knapen, F. van and Panggabean, S.O. (1977). Detection of Circulating Antigen During Acute Infections with Toxoplasma gondii by Enzyme Linked Immunosorbent Assay. Journal of Clinical Microbiology 6 (6), 545-547.

Knapen, F. van, Panggabean, S.O. and Leusden, J. van (1985). Demonstration of Toxoplasma Antigen Containing Complexes in Active Toxoplasmosis. Journal of Clinical Microbioloty 22 (4), 645-650.

Kates, K.C., Colglazier, M.L. and Enzie, F.D. (1975). Oxibendazole: Critical Anthelmintic Trials in Equids. Veterinary Record 97, 23, 442-444.

Koulikov, N.S. (1935). Dictyocauleuses du Cheval et Methode de Diagnostic chez L'animal Vivant. Trudy Voes Inst. Gel'mint 1, 82-84.

Luckins, A.G. (1977). Detection of Antibodies in Trypanosome Infected Cattle by Means of a Microplate Enzyme Linked Immunosorbent Assay. Tropical Animal Health and Production 9, 53-62.

Lyons, E.T., Drudge, J.H., Zygmont, S.M., Twehues, J.L., Downing, R.G. and Sutton, H.H. (1982). Clinical History of Lungworm Disease in a Foal. Veterinary Medicine and Small Animal Clinician 77, 10, 1533-1537.

Lyons, E.T., Tolliver, S.C., Drudge, J.H., Swerczek, T.W. and Crowe, M.W. (1985). Parasites in Lungs of dead Equids in Kentucky: Emphasis on Dictyocaulus arnfieldi. American Journal of Veterinary Research 46 (4), 924-927.

MacKay, R.J. and Urquhart, K.A. (1979). An Outbreak of Eosinophilic Bronchitis in Horses Possibly Associated with Dictyocaulus arnfieldi Infection. Equine Veterinary Journal 11, 110-112.

Michel, J.F. (1968). Experiments on the loss Dictyocaulus filaria from the Lungs of Infected Sheep. Reduction in Worm Numbers before Patency. Folia Parasitologica 15, 309-316.

Nicholls, J.M., Clayton, H.M., Duncan, J.L. and Buntain, B. (1979). Lungworm (Dictyocaulus arnfieldi) Infection in Donkeys. Veterinary Record 104, 567-570.

Nicholls, J.M., Duncan, J.L. and Greig, W. (1978). Lungworm, Dictyocaulus arnfieldi, Infection in the Horse. Veterinary Record, March, 216-217.

Nielsen, K. and Anderson, S. (1981). Lungworms in Horses, Probably Transmitted by a Donkey. Dansk Veterinaertidsskrift 64, 4, 124-126.

Pandey, V.S. (1980). Epidemiological Observations on Lungworm Dictyocaulus arnfieldi in Donkeys from Morocco. Journal of Helminthology 54, 275-279.

Parfitt, J.W. and Sinclair, I.J. (1967). Cross Resistance to Dictyocaulus viviparus Produced by Dictyocaulus filaria Infections in Calves. Research in Veterinary Science 8, 6-13.

Pirie, M., Pirie, H.M., Cranston, S. and Wright, N.G. (1990). An Untrastructural Study of the Equine Lower Respiratory Tract. Equine Veterinary Journal 22 (5), 338-342.

Poncet, P. (1983). A Case of Pulmonary Parasitosis in a Foal. Schweiz Arch Teirheilk 125, 97-102.

Ribeiro, H.S., Larangeira, N.L. and Paiva, F. (1979). Prevalence of Dictyocaulus arnfieldi (Cobbald, 1884) Raillet and Henry 1907 in Horses of the Pantaneira Breed of the Pocone Region, Mato Grosso, Brazil. Arg. Inst. Bid., Sao Paulo 46 (3/4), 107-110.

Rickard, M.D. and James, D.E. (1976). Treatment of Lungworm Infestation in Horses. Australian Veterinary Journal 52, 487-488.

Round, M.C. (1972). A Study of the Natural History of Lungworm Infection of Equidae. Titles of Dissertations, University of Cambridge, 1972-1972.

Round, M. (1976). Lungworm Infection (Dictyocaulus arnfieldi) of Horses and Donkeys. Veterinary Record 99, 393-395.

Rouse, B.T. and Angulo, A.B. (1970). A Method for the Collection of Nasal Secretions from the Horse and Cow. Research in Veterinary Science 11, 98-99.

Ruitenberg, E.J. and Buys, J. (1977). Application of the Enzyme Linked Immunosorbent Assay (ELISA) for the Serodiagnosis of Human African Trypanosomiasis (Sleeping Sickness). The American Journal of Tropical Medicine and Hygiene 26 (1), 31-36.

Ruitenberg, E.J., Steerenberg, P.A., Brosi, B.J.M. and Buys, J. Serodiagnosis of Trichinella spiralis Infections in Pigs by ELISA. Bulletin World Health Organisation 51, 108-109.

Ruitenberg, E.J., Steerenberg, P.A., Brosi, B.J.M. and Buys, J. (1976). Reliability of the ELISA for the Serodiagnosis of Trichinella spiralis Infections in Conventionally Raised Pigs. Journal of Immunological Methods 10, 67-83.

Saunders, G.C., Clinard, E.H., Bartlett, M.L. and Saunders, W.M. (1977). Application of the Indirect Enzyme-Labelled Antibody Microtest to the Detection and Surveillance of Animal Diseases. Journal of Infectious Disease 136 (Suppl.), S258-S266.

Tolliver, S.C., Lyons, E.T. and Drudge, H.J. (1985). Species of Small Strongyles and Other Internal Parasites Recovered from Donkeys at Necropsy in Kentucky. Proceedings of Helminthology Society of Washington 52 (2), 260-265.

Ungar, E.L.P., Yolken, R.H. and Quinn, T.C. (1985). Use of a Monoclonal Antibody in an Enzyme Immunoassay for the Detection of Entamoeba histolytica in Fecal Specimens. American Journal of Tropical Medicine and Hygiene 34 (3), 465-472.

Urch, D.L. and Allen, W.R. (1980). Studies on Fenbendazole for Treating Lung and Intestinal Parasites in Horses and Donkeys. Equine Veterinary Journal 12 (2), 74-77.

Voller, A., Bartlett, A. and Bidwell, D.E. (1976). Enzyme Immunoassays for Parasitic Diseases. Transactions of the Royal Society of Tropical Medicine and Hygiene 70 (2), 98-106.

Voller, A., Bartlett, A. and Bidwell, D.E. (1978). The Use of the Enzyme Linked Immunosorbent Assay in the Serology of Viral and Parasitic Diseases. Scandanavian Journal of Immunology 8 (Suppl.7), 125-129.

Walls, K.W., Bullock, S.L. and English, D.K. (1977). Use of the Enzyme Linked Immunosorbent Assay (ELISA) and its Microadaptation for the Serodiagnosis of Toxoplasmosis. Journal of Clinical Microbiology 5 (3), 273-277.

Ward, G.S., Gussow, D., Griffiths, A.D., Jones, P.T. and Winter, G. (1989). Binding Activities of a Repertoire of Single Immunoglobulin Variable Domains Secreted from Escherichia coli. Nature 341, 544-546.

Wetzel, R. and Enigk, K. (1938). Zur Biologie von Dictyocaulus arnfieldi dem Lungenwurm der Einhufer. I Mitteilung z. Mitteilung. Arch. Wissensch. Prakt. Tier. 73, 94-105 and 106-114.

Wilkes, D. (1989). An alternative to Monoclonal Antibodies. An Alternative to Monoclonal Antibodies. Laboratory Equipment Digest, 17-18.

APPENDICES

Appendix 1.1

Individual Body Weights in Kilogrammes of Mature Ponies Experimentally Infected with *D. arnfieldi*

Day Post Infection	Pony number 1	2	3	4	5	6	7	8	9	10
0	158	188	150	158	168	123	117	115	157	180
7	166	189	157	163	167	121	119	114	160	188
14	165	190	154	162	165	125	121	111	149	181
21			156	161	169	126	116	110	146	174
28			153	164	170	129	117	115	152	163
35					170	126	114	113	149	159
42					148	125	109	114	149	161
49							110	95	151	160
56							107	94	149	158
63									150	160

Appendix 1.2

Packed Cell Volumes of Mature Ponies Experimentally Infected with D.arnfieldi

Day Post Infection	Pony number 1	2	3	4	5	6	7	8	9	10
0	26	29	28	32	25	31	28	26	27	26
7	28	28	28	30	30	28	27	26	27	26
14	28	29	29	30	26	27	26	24	24	27
21			33	28	27	27	38	26	28	33
28			35	31	28	28	29	27	27	30
35					25	26	24	29	28	28
42					23	27	27	31	28	32
49							27	30	29	29
56							31	27	29	26
63									33	29

Appendix 1.3

Respiratory Rates of Mature Ponies Experimentally Infected with D. arnfieldi

Day Post Infection	Pony number 1	2	3	4	5	6	7	8	9	10
0	20	18	15	18	12	15	12	12	15	15
1	24	15	15	18	9	12	12	12	12	15
2	18	18	15	21	9	15	12	12	15	18
3	21	15	18	18	15	15	12	12	12	15
4	18	15	15	21	9	12	12	12	15	15
5	15	12	18	18	12	15	15	18	15	15
6	18	21	15	18	9	9	18	12	12	15
7	18	18	18	15	12 c	15	12	9	15	15
8	15	21	15	18	15	15	12	12	12	15
9	18	18	15	21	12	15	15	18	18	15
10	15	21	18	18	12 c	12	15	19	15	18
11	18	21	18	18	15	12	12	15	18	18
12	15	15	18	18	15	15	12	18	15	18
13	18	15	15	15	15	15	12	18	15	18
14	15	18	18	15	12	12	15	9	15	18
15	15	15	18	18	15	12	15	12	12	15
16	21	15	18	18	15 c	15	18	15	12	18
17	15	18	18	15	15 c	15	18 c	12	15	21
18	18	15	21	18	12	15	24	9	12	36
19	24	12	18	21	12 c	18	15 c	18	15	39
20	24	12	21	24	15	18	15 c	18	21	42
21			18	18	12	12	15	15	12	33
22			12	12	15	15	15 c	18	12	33
23			12	12	12	12	18 c	15	15	33
24			12	12	12	12	18 c	15 c	12	30
25			9	12	12	15	21 c	15	9	21
26			20	12 c	15	18	21 c	18 c	15	24
27			12	15	15	15	18	18	18	27
28			12 c	12	15	15	27 c	15	15	30
29			15	18	15 c	18	27 c	15	18	27
30			12	18	15	15	24 c	18	18	30
31			15	18	15	15	24 c	15	18	27
32			12 c	12 hr	18 c	18	21 c	21	21	24
33			12	15	15	18	24 c	18	21 c	21
34			15 c	15	18	15	15 c	21	30	26
35					12	18 c	18	18	18	21
36					15	18 c	15	18	15	12
37					15	18	18 c	21	15	18
38					12	15	15	18	18	27

c - cough
hr - harsh respirations

Appendix 1.3 (Cont'd)

Day Post Infection	Pony number 1	2	3	4	5	6	7	8	9	10
39					18	18	18 c	21	15	33 c
40					21	21	18	18 c	30	24
41					15	18	15	18	24 c	21
42					15	21	15 c	18 c	24	30
43					12	18	18	18 c	27	24 c
44					15	15	15	15	27 c	24 c
45					12	12	18 c	12	24	21 c
46					15	15	12	18	21	21
47							18	21	24	30
48							15 c	15	24 c	30
49							12	18	27 c	27 c
50							12 c	15	27	24
51							15	18	24	24 c
52							18	15	24 c	27
53							18	18	21 c	24
54							15	15	27	27 c
55							18	18	21	27
56							15	12	27	33
57							15	15	21 c	33 c
58							18	12	24 c	30 c
59							15	12	27	30
60							21	15	21 c	27
61									27	27 c
62									24 c	27 c
63									24 c	27
64									27 c	30
65									21	30 c
66									24	27
67									24	30
68									21	27

Appendix 1.4

Respiratory Rates of Mares and Foals Experimentally Infected with *D. arnfieldi*

Day Post Infection	Mare 13	Foal 14	Mare 11	Foal 16	Mare 12	Foal 15
0	12	12	12	12	12	12
1	12	12	12	12	12	12
2	12	12	12	12	15	12
3	12	12	12	12	12	12
4	12	12	12	12	12	12
5	12	12	12	15	12	12
6	12	12	15	12	12	15
7	12	12	12	12	12	12
8	15	12	12	15	12	12
9	12	15	12	12	15	12
10	15	15	12	12	12	12
11	15	15	12	15	12	15
12	18	12	12	15	12	15
13	15	15	12	12	15	15
14	21	15	15	15	15	18
15	15	12	12	12	12	18
16	15	15	12	15	13	15
17	18	15	12	12	12	18
18	18	15	12	15	15	18
19	18	15	12	15	15	18
20	21	15	12	15	15	18
21	21	15	12	15	15	15
22	18	15	15	12	15	18
23	18	15	12	12	12	15
24	18	12	12	12	15	15
25	18	15	15	12	15	18
26	21	15	12	15	15	18
27	21	12	12	12	12	15
28	18	15	15	15	12	15
29	18	12	12	12	15	12
30	18	12	15	12	15	15
31	18	15	12	12	15	12
32	18	15	12	12	15	15
33	21	12	12	12	12	15
34	18	15	15C	15	18C	15
35	15	12	15	12	21	15C
36	18	15	18C	15	18	15
37	18	12	18C	12	15	15
38	15	15	18	12	15	12
39	18	12	18C	12	18C	12
40	21	15	18	15	18C	12
41	18	12	18C	12	15C	12

Appendix 1.4 (Cont'd)

Day Post Infection	Mare 13	Foal 14	Mare 11	Foal 16	Mare 12	Foal 15
42	15	12	18	12	18C	15
43			15	12	18	12
44			18C	12	15C	15
45			18C	12	15C	12
46			15	12	18	12
47			18	12	15	15
48			15	15	18C	12
49			15	12	15C	12
50			15C	12	18C	12
51			15C	15	18C	12
52			18	12	18C	12
53			18	12	18C	12
54			15	15	15	15
55			18	12	15C	12
56			15	12	15	12
57					18C	15
58					15	12
59					15C	12
60					18C	15
61					15	12
62					18	12
63					18C	12
64					18	12
65					18C	12
66					18C	15
67					15C	15
68					15C	12
69					15C	12
70					15C	12

Appendix 1.5

Parasitological Data of Donkey Sanctuary Donkeys 86 - 88

Donkeys Aged 0 - 2 Years

Donkey Number	Lungworm/50g	Strongyles e.p.g.
2609	-ve	-ve
2610	42	408
2647	-ve	596
2679	-ve	-ve
2683	-ve	1
2707	5	160
2713	-ve	-ve
2735	131	151
2736	12	1620
2737	-ve	8
2748	-ve	98
2762	-ve	38
2764	332	252
2859	-ve	-ve
2864	-ve	-ve
2887	-ve	45
2933	12	10
2954	-ve	-ve
2967	-ve	-ve
2989	5	62
3012	56	252
3015	-ve	509
3028	52	357
3066	-ve	-ve
3075	-ve	236
3090	-ve	123
3109	1484	321
3118	-ve	158
3119	-ve	440
3123	74	60
3165	-ve	1
3190	-ve	-ve
3201	-ve	1
3295	-ve	25
3354	5	47
3364	385	210
3420	-ve	8
3470	-ve	17
3533	-ve	-ve
3545	1230	2
3547	10146	23

Appendix 1.5 (Cont'd)

Donkeys Aged 3 - 5 Years

Donkey Number	Lungworm (50g)	Strongyles e.p.g.
2578	-ve	-ve
2603	23	550
2620	342	467
2645	-ve	434
2663	-ve	-ve
2668	-ve	10
2680	-ve	69
2681	219	419
2687	43	274
2689	81	61
2696	153	501
2703	-ve	-ve
2712	128	16
2725	-ve	260
2727	-ve	316
2732	-ve	35
2733	-ve	-ve
2765	131	202
2771	-ve	42
2790	66	14
2810	-ve	112
2822	-ve	-ve
2831	16	20
2832	-ve	356
2865	-ve	91
2883	44	1
2910	-ve	1
2918	6	69
2919	-ve	64
2920	52	195
2934	-ve	66
2935	-ve	236
2936	-ve	320
2947	94	61
2950	-ve	88
2959	67	65
3004	55	102
3006	-ve	7
3011	3642	76
3034	-ve	53
3051	-ve	105

Appendix 1.5 (Cont'd)

Donkeys Aged 3 - 5 Years

Donkey	3 - 5 yrs Lungworm (50g)	Stronglyes e.p.g.
3060	-ve	1
3110	3067	512
3114	4158	371
3122	390	106
3124	-ve	574
3131	-ve	137
3135	-ve	5
3138	-ve	-ve
3149	-ve	385
3151	883	1935
3152	6	192
3156	-ve	122
3170	36	61
3180	-ve	6
3181	-ve	2
3189	-ve	-ve
3194	-ve	1
3209	-ve	1
3232	118	307
3242	-ve	-ve
3280	-ve	2
3281	-ve	604
3282	-ve	267
3294	-ve	40
3296	3	11
3337	-ve	-ve
3350	-ve	34
3353	-ve	79
3384	-ve	1
3426	-ve	391
3435	-ve	109
3456	-ve	-ve
3255	-ve	36
3548	-ve	140
3560	-ve	3
3590	297	30
3598	-ve	-ve

Appendix 1.5 (Cont'd)

Donkeys Aged 6 - 10 Years

Donkey Number	Lungworm (50g)	Strongyles e.p.g.
2577	-ve	35
2579	-ve	-ve
2581	1	232
2584	25	131
2591	-ve	6
2594	136	242
2598	-ve	240
2600	36	470
2607	152	115
2608	1	-ve
2615	56	381
2617	-ve	596
2622	-ve	-ve
2623	-ve	-ve
2625	532	139
2627	139	208
2646	182	142
2653	-ve	194
2664	-ve	-ve
2676	-ve	758
2690	26	149
2693	4	216
2702	-ve	27
2714	-ve	15
2716	215	27
2720	9	502
2728	93	501
2730	-ve	2496
2740	3	181
2742	100	130
2749	-ve	58
2750	-ve	-ve
2751	-ve	1394
2775	-ve	1
2788	-ve	-ve
2798	-ve	-ve
2799	-ve	106
2800	-ve	95
2807	134	176
2808	19	112

Appendix 1.5 (Cont'd)

Donkeys Aged 6 - 10 Years

Donkey Number	Lungworm (50g)	Stronglyes e.p.g.
2809	-ve	109
2812	-ve	104
2817	44	37
2818	79	142
2821	-ve	-ve
2827	-ve	-ve
2830	-ve	22
2837	-ve	224
2843	2	28
2848	-ve	56
2856	-ve	11
2860	11	25
2861	13	121
2866	-ve	-ve
2874	91	25
2877	-ve	226
2889	-ve	572
2905	-ve	76
2932	9	28
2949	15	97
2956	138	99
2961	1	95
2966	-ve	-ve
2998	22	27
2999	434	23
3003	-ve	167
3008	42	556
3013	62	132
3014	-ve	502
3027	16	135
3031	330	167
3035	-ve	11
3050	-ve	109
3054	97	323
3081	-ve	178
3107	317	248
3134	494	260
3136	-ve	107
3142	-ve	-ve
3154	496	86
3173	1099	49
3182	-ve	-ve
3184	-ve	21
3188	-ve	-ve

Appendix 1.5 (Cont'd)

Donkeys Aged 6 - 10 Years

Donkey Number	Lungworm (50g)	Stronglyes e.p.g.
3200	24	20
3202	474	321
3207	-ve	600
3210	-ve	1
3220	-ve	6
3230	-ve	339
3243	2401	126
3269	1484	69
3273	-ve	-ve
3290	-ve	75
3292	-ve	105
3293	-ve	5
3297	7	214
3336	5	113
3358	10	12
3376	283	313
3377	-ve	5
3382	-ve	-ve
3430	-ve	239
3434	-ve	97
3478	-ve	18
3167	-ve	310
3291	-ve	3
3360	-ve	2
3588	-ve	151
3592	-ve	-ve
3605	-ve	4
3608	-ve	-ve
3610	-ve	-ve
3611	-ve	-ve
3621	-ve	126
3629	74	100
3630	-ve	2520

Appendix 1.5 (Cont'd)

Donkeys Aged 11 - 15 Years

Donkey Number	Lungworm (50g)	Strongyles e.p.g.
2574	-ve	-ve
2580	-ve	-ve
2583	-ve	168
2585	-ve	43
2586	-ve	363
2587	-ve	355
2595	1	224
2597	128	2125
2605	58	365
2613	26	252
2639	3	14
2643	78	308
2648	-ve	45
2651	490	120
2665	113	219
2669	-ve	-ve
2695	-ve	37
2697	17	4
2698	-ve	47
2706	50	176
2726	-ve	701
2747	40	1580
2757	-ve	565
2758	-ve	803
2763	819	283
2766	-ve	-ve
2767	-ve	283
2782	33	1480
2784	14	8
2785	-ve	222
2789	-ve	325
2826	2	52
2828	34	35
2829	169	50
2833	-ve	200
2834	-ve	80
2835	376	5
2851	-ve	5
2852	62	727
2857	-ve	192
2858	-ve	-ve

Appendix 1.5 (Cont'd)

Donkeys Aged 11 - 15 Years

Donkey Number	Lungworm (50g)	Stronglyes e.p.g.
2862	29	10
2863	-ve	361
2875	-ve	-ve
2876	-ve	152
2878	-ve	41
2881	32	129
2886	54	76
2891	-ve	515
2897	1	259
2911	-ve	-ve
2939	217	365
2940	134	73
2952	-ve	265
2953	52	212
2968	-ve	-ve
2975	-ve	90
2983	4	359
2986	-ve	-ve
2987	-ve	96
2988	-ve	133
3005	927	347
3010	-ve	25
3024	-ve	78
3029	1	7
3039	352	17
3061	-ve	-ve
3071	-ve	17
3074	1	7
3078	-ve	33
3079	-ve	63
3092	358	454
3139	-ve	-ve
3147	2	135
3155	-ve	100
3159	140	285
3163	-ve	1
3169	16	34
3185	-ve	71
3191	-ve	-ve
3192	-ve	19
3193	-ve	-ve
3195	-ve	2
3196	158	2
3198	-ve	131

Appendix 1.5 (Cont'd)

Donkeys Aged 11 - 15 Years

Donkey Number	Lungworm (50g)	Strongyles e.p.g.
3217	-ve	131
3218	688	252
3229	453	277
3237	-ve	-ve
3240	-ve	-ve
3245	25	161
3246	-ve	3
3284	-ve	-ve
3289	-ve	-ve
3323	2	49
3330	-ve	-ve
3342	-ve	29
3370	-ve	387
3379	-ve	45
3477	3	57
3494	-ve	-ve
3505	-ve	2
3525	-ve	169
3530	-ve	8
3535	-ve	12
3536	-ve	-ve
3546	300	69
3554	52	62
3556	-ve	55
3569	-ve	-ve
3572	-ve	23
3575	-ve	1
3579	66	62
3582	-ve	254
3591	-ve	-ve
3596	7	117
3597	-ve	1
3599	145	163
3600	145	163
3613	-ve	123
3614	186	230
3616	5	232
3617	-ve	60
3641	-ve	141

Appendix 1.5 (Cont'd)

Donkeys Aged 16 - 20 Years

Donkey Number	Lungworm (50g)	Stronglyes e.p.g.
2571	-ve	21
2596	-ve	128
2599	16	547
2601	1	349
2604	-ve	556
2611	-ve	211
2626	42	10
2628	-ve	143
2629	-ve	31
2630	-ve	16
2631	-ve	12
2644	-ve	238
2649	-ve	11
2650	408	30
2652	-ve	-ve
2656	-ve	72
2667	2	277
2672	-ve	-ve
2677	8	12
2678	104	-ve
2692	3	540
2708	-ve	10
2710	-ve	37
2724	-ve	120
2729	-ve	654
2752	-ve	195
2753	54	196
2755	-ve	746
2756	-ve	600
2774	-ve	5
2776	-ve	1
2783	-ve	242
2787	-ve	5
2791	-ve	452
2794	6	686
2806	2	-ve
2816	-ve	50
2824	4	81
2836	-ve	15
2838	414	896
2844	5	598

Appendix 1.5 (Cont'd)

Donkeys Aged 16 - 20 Years

Donkey Number	Lungworm (50g)	Strongyles e.p.g.
2885	20	136
2888	5	122
2902	-ve	1
2904	-ve	24
2907	-ve	173
2909	-ve	-ve
2913	-ve	21
2917	7	53
2941	-ve	27
2943	-ve	140
2951	-ve	204
2964	39	135
2972	257	60
2974	-ve	89
3007	104	42
3030	49	80
3037	-ve	367
3040	847	127
3043	-ve	317
3058	-ve	17
3059	-ve	53
3072	-ve	46
3080	-ve	80
3085	-ve	18
3088	-ve	42
3091	6	345
3096	-ve	134
3098	-ve	2
3099	-ve	36
3111	1238	231
3125	23	150
3127	296	351
3158	15	186
3162	-ve	3
3168	1	88
3183	-ve	1
3205	41	204
3233	-ve	13
3238	1	210
3239	107	34
3247	-ve	136
3271	-ve	138
3287	-ve	4
3310	-ve	-ve

Appendix 1.5 (Cont'd)

Donkeys Aged 16 - 20 Years

Donkey Number	Lungworm (50g)	Stronglyes e.p.g.
3319	-ve	137
3326	-ve	31
3328	147	115
3333	-ve	135
3338	6	91
3339	-ve	145
3348	-ve	-ve
3359	4	6
3367	-ve	-ve
3380	184	10
3386	-ve	24
3421	-ve	49
3448	-ve	122
3449	-ve	3
3514	-ve	24
3521	-ve	95
3527	-ve	-ve
3528	-ve	-ve
3532	-ve	-ve
3537	-ve	890
3543	5785	87
3549	-ve	76
3561	-ve	3
3578	-ve	-ve
3584	-ve	52
3585	-ve	2
3594	-ve	-ve
3602	-ve	192
3609	-ve	-ve
3612	-ve	191
3635	-ve	6
3498	-ve	1

Appendix 1.5 (Cont'd)

Donkeys Aged 21 - 25 Years

Donkey Number	Lungworm (50g)	Stronglyes e.p.g.
2585	-ve	82
2662	130	25
2670	-ve	-ve
2675	-ve	696
2682	-ve	374
2704	-ve	-ve
2705	40	320
2773	14	120
2779	-ve	1
2780	-ve	3
2786	-ve	-ve
2792	-ve	231
2805	-ve	18
2825	-ve	238
2850	44	65
2871	-ve	1
2882	163	140
2900	5	216
2908	25	40
2916	-ve	0
2921	-ve	-ve
2922	-ve	22
2937	-ve	23
2955	-ve	-ve
2965	-ve	-ve
2976	288	26
2992	3	415
3026	70	124
3055	14	20
3065	26460	245
3076	-ve	-ve
3087	61	528
3089	-ve	223
3097	-ve	118
3116	-ve	422
3141	63	545
3144	-ve	105
3197	6	26
3203	-ve	56
3221	-ve	151
3244	110	116

Appendix 1.5 (Cont'd)

Donkeys Aged 21 - 25 Years

Donkey Number	Lungworm (50g)	Strongyles e.p.g.
3248	-ve	-ve
3252	-ve	46
3257	-ve	90
3258	347	-ve
3265	-ve	31
3270	-ve	548
3279	-ve	-ve
3286	-ve	175
3331	-ve	-ve
3355	-ve	51
3371	-ve	237
3372	-ve	161
3422	26	108
3452	-ve	72
3467	-ve	2
3473	-ve	198
3506	-ve	448
3516	-ve	-ve
3526	34	182
3529	-ve	3
3534	221	557
3541	254	321
3553	-ve	454
3555	-ve	65
3558	-ve	1
3573	584	53
3577	-ve	15
3583	-ve	10
3606	118	62
3622	-ve	82
3623	-ve	31
3625	-ve	125
3633	-ve	281
3634	239	52

Appendix 1.5 (Cont'd)

Donkeys Aged 26 Years or More

Donkey Number	Lungworm (50g)	Strongyles e.p.g.
2575	-ve	-ve
2576	1	317
2593	-ve	-ve
2602	12	195
2606	-ve	198
2612	2	216
2616	7	93
2618	36	89
2619	101	385
2621	3	679
2624	27	198
2640	-ve	54
2661	136	79
2666	7	602
2691	-ve	82
2699	-ve	78
2721	27	263
2722	-ve	251
2731	-ve	1506
2745	-ve	10
2746	-ve	12
2761	1	15
2772	8	190
2815	-ve	12
2849	44	1212
2867	-ve	315
2868	1	398
3869	13	125
2893	-ve	5
2914	-ve	-ve
2915	-ve	24
2924	-ve	12
2925	-ve	16
2927	-ve	270
2938	-ve	186
2942	2	73
2944	-ve	2
2946	46	678
2963	6000+	236
2970	-ve	49
2971	-ve	3

Appendix 1.5 (Cont'd)

Donkeys Aged 26 Years of More

Donkey Number	Lungworm (50g)	Stronglyes e.p.g.
2984	-ve	109
2991	7	237
3001	-ve	-ve
3006	-ve	16
3038	-ve	196
3042	-ve	1
3052	-ve	72
3053	-ve	265
3063	-ve	338
3064	-ve	991
3077	-ve	3
3093	-ve	26
3100	-ve	149
3102	-ve	51
3115	-ve	30
3120	58	1053
3121	2	981
3132	-ve	1
3137	-ve	101
3143	-ve	15
3166	2	318
3199	-ve	49
3204	51	26
3211	-ve	109
3223	-ve	5
3228	-ve	6
3241	-ve	24
3253	-ve	-ve
3272	6	75
3278	-ve	-ve
3283	44	165
3285	-ve	1
3288	-ve	5
3298	-ve	250
3299	-ve	413
3300	-ve	48
3301	-ve	47
3322	161	181
3325	-ve	30
3335	3	377
3340	-ve	65
3341	1271	158
3343	10	107
3347	-ve	-ve

Appendix 1.5 (Cont'd)

Donkeys Aged 26 Years or More

Donkey Number	Lungworm (50g)	Stronglyes e.p.g.
3357	-ve	73
3362	-ve	380
3363	65	114
3366	-ve	-ve
3369	-ve	1
3373	-ve	17
3375	-ve	23
3385	-ve	191
3418	-ve	28
3431	-ve	328
3446	-ve	101
3447	766	-ve
3453	-ve	126
3455	-ve	-ve
3466	-ve	533
3469	-ve	-ve
3492	-ve	-ve
3493	-ve	-ve
3495	-ve	-ve
3515	-ve	164
3517	929	107
3518	-ve	583
3540	-ve	353
3568	-ve	116
3576	-ve	-ve
3581	450	11
3593	-ve	-ve
3601	5000	455
3603	-ve	423
3604	-ve	513
3607	-ve	79
3618	-ve	60
3631	-ve	373
3636	2040	57
3637	-ve	183
3639	-ve	132

Appendix 1.5 (Cont'd)

Donkeys of Unknown Age

Donkey Number	Lungworm (50g)	Strongyles e.p.g.
2637	-ve	171
2671	-ve	121
2711	-ve	15
2811	-ve	82
2993	-ve	-ve
3009	-ve	85
3082	-ve	3
3083	-ve	245
3164	-ve	1
3175	-ve	-ve
3176	-ve	-ve
3179	-ve	26
3471	-ve	98
3472	-ve	143
3483	-ve	-ve
3484	-ve	-ve
3489	2	81
3497	-ve	-ve
3465	-ve	203
3538	-ve	112
3539	-ve	313
3574	-ve	2
3619	-ve	8
3628	-ve	590
3632	627	181

Appendix 2.1

ELISA - Titration of *D. arnfieldi* Antibody and also *D. arnfieldi* with R_1B and R_2B Using Streptavadin Peroxidase at 1:5000

All volumes 50 µl/well.

1. Coat plate antibody.
   Initial dilution 1:50 column 2. Doubling dilutions →
   Incubate 37°C 2 hr.
   Wash

2. Add *D. arnfieldi* antigen 1:1000. Columns 4-11. Doubling dilutions ↓
   Incubate 37°C 30 min.
   Wash.

3. Add biotinylated antibody - plate 1 R_1B)) 1:100. Columns 3-10.
   plate 2 R_2B)
   Incubate 37°C 30 min.
   Wash.

4. Add streptavadin peroxidase 1:5000. Columns 2-9.
   Incubate 37°C 30 min.
   Wash.

5. Add substrate. O.P.D. all wells.
   Incubate in dark 37°C 40 min.

6. Stop with acid. Read 492 nm.

Appendix 2.1 (Cont'd)

Plate 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	9.991	9.993	0.020	0.043	0.050	0.045	0.031	0.059	0.056	0.048	0.033	0.030
B	9.985	0.046	0.006	0.095	0.037	0.026	0.072	0.015	0.066	0.057	9.996	9.998
C	9.986	0.009	0.017	0.028	0.031	0.033	0.014	0.016	0.022	0.016	9.995	0.030
D	0.056	0.005	0.026	0.034	0.085	0.027	0.031	0.037	0.025	0.018	0.022	0.031
E	0.004	0.011	0.039	0.044	0.037	0.028	0.019	0.023	0.036	0.018	0.025	0.009
F	0.015	0.000	0.037	0.081	0.057	0.030	0.018	0.046	0.060	0.018	0.015	0.009
G	0.025	9.988	0.047	0.048	0.041	0.035	0.018	0.026	0.035	0.034	9.998	0.012
H	0.001	1.417	0.048	0.033	0.035	0.022	0.020	0.024	0.029	0.017	0.012	0.010

Plate 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0.014	0.084	0.073	0.069	0.044	0.026	0.032	0.026	0.017	0.019	0.066
B	0	0.001	0.081	0.060	0.051	0.028	0.006	0.002	9.994	0.027	0.032	0.004
C	0.001	0.007	0.077	0.062	0.039	0.029	0.024	0.043	0.059	0.018	0.003	0.021
D	0.001	0.014	0.069	0.049	0.051	0.038	0.020	0.019	0.015	0.002	0.048	0.015
E	9.999	0.023	0.068	0.046	0.047	0.046	0.032	0.023	0.013	0.012	0.066	0.020
F	0	0.002	0.120	0.029	0.028	0.024	0.023	0.014	0.012	0.008	0.007	0.010
G	0	0.013	0.056	0.036	0.052	0.030	0.027	0.031	0.003	0.021	0.038	0.018
H	0	0.004	0.049	0.048	0.041	0.026	0.022	0.035	0.013	0.016	0.013	0.020

Appendix 2.2

ELISA - Titration of D. arnfieldi Antigen with R_2B Using Streptavadin Peroxidase of 1:1000

All volumes 50 µl/well.

1. Coat plate D. arnfieldi antigen.
   Initial dilution 1:100 Column 2. Doubling dilutions →
   Incubate 37°C 2 hr.
   Wash.

2. Add biotinylated antibody (R_2B). Columns 4-11. Doubling dilutions ↓
   Incubate 37°C 30 min.
   Wash.

3. Add Streptavadin peroxidase 1:1000 columns 3-10.
   Incubate 37°C 30 min.
   Wash.

4. Add substrate O.P.D. all wells.
   Incubate in dark 37°C. 1 hr 15 min.

5. Stop with acid. Read 492 nm.

Appendix 2.2 (Cont'd)

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0.001	9.982	0.790	0.765	0.747	0.670	0.671	0.728	0.637	9.974	9.975
B	0	9.993	0.006	0.687	0.562	0.533	0.463	0.487	0.503	0.477	9.996	9.995
C	9.999	0	0.034	0.495	0.398	0.371	0.283	0.291	0.321	0.295	0.019	0.005
D	9.998	0.004	0.017	0.336	0.228	0.195	0.182	0.175	0.177	0.164	0.018	0
E	0	9.984	0.009	0.207	0.116	0.134	0.085	0.078	0.114	0.103	0.014	9.984
F	0	9.997	0.028	0.137	0.121	0.103	0.076	0.070	0.093	0.091	0.034	0.029
G	0	0.027	0.059	0.113	0.088	0.082	0.077	0.076	0.086	0.081	0.049	0.027
H	0	0.007	0.066	0.077	0.051	0.054	0.037	0.064	0.054	0.053	0.046	0.013

Appendix 2.3

ELISA - Titration of *D. arnfieldi* R_2B Using Streptavadin Peroxidase at 1:500

All volumes 50 µl/well.

1. Coat plate *D. arnfieldi* antigen starting column 1.
   Initial dilution 1:100 (300 ng/well). Doubling dilutions →
   Incubate 37°C 2 hr.
   Wash.

2. Add biotinylated antibody R_2B. Columns 2-9.
   Initial dilution 1:50. Doubling dilutions ↓
   Incubate 37°C 30 min.
   Wash.

3. Add Streptavadin peroxidase. 1:500 all wells.
   Incubate 37°C 30 min.
   Wash.

4. Add substrate. O.P.D. all wells.
   Incubate 37°C in dark 40 min.

5. Stop with acid. Read 492 nm.

Appendix 2.3 (Cont'd)

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0.716	0.562	0.467	0.387	0.391	0.364	0.328	0.350	0.007	9.995	9.998
B	0	0.487	0.328	0.263	0.221	0.191	0.188	0.184	0.219	0.008	0	0
C	0.001	0.264	0.203	0.161	0.135	0.115	0.108	0.095	0.104	0.011	0.006	0.011
D	9.999	0.158	0.097	0.076	0.075	0.053	0.069	0.042	0.046	0.001	9.995	0.002
E	0.001	0.082	0.050	0.041	0.047	0.030	0.047	0.026	0.031	0	0.004	0.014
F	0	0.044	0.031	0.029	0.047	0.028	0.046	0.020	0.025	0.009	0.018	0.028
G	0	0.024	0.019	0.020	0.035	0.020	0.039	0.009	0.014	0.018	0.022	0.024
H	9.999	0.013	0.008	0.013	0.023	0.006	0.035	0.010	0.009	0.005	0.017	0.020

Appendix 2.4

ELISA - Titration of D. arnfieldi Antigen both Rotated and Non-Rotated R_2B Using Streptavadin Peroxidase at 1:500

All volumes 50 µl/well.

1. Coat plates D. arnfieldi antigen excluding column 12.
   Initial dilution 1:50. Doubling dilutions →
   Incubate 37°C 2hr.
   Wash.

2. Add biotinylated antibody R_2B. Plate 1 - rotated. R_2B.
   Plate 2 - non-rotated. R_2B.
   Initial dilution 1:50. Doubling dilutions ↓ Columns 2-11.
   Incubate 37°C 30 min.
   Wash.

3. Add Streptavadin peroxidase 1:500 columns 1-10.
   Incubate 30 min.
   Wash.

4. Add substrate O.P.D. all wells.
   Incubate 37°C in dark 1 hr.

5. Stop with acid. Read 492 nm.

Appendix 2.4 (Cont'd)

Plate 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.237	1.073	0.953	0.813	0.749	0.664	0.601	0.596	0.038	0.036	0.005	0.003
B	0.932	0.764	0.648	0.533	0.430	0.390	0.354	0.338	0.030	0.039	0.021	0.008
C	0.533	0.491	0.396	0.340	0.289	0.263	0.213	0.218	0.034	0.035	0.018	0.004
D	0.308	0.262	0.243	0.196	0.177	0.154	0.135	0.131	0.030	0.027	0.014	0.001
E	0.179	0.164	0.145	0.124	0.121	0.101	0.080	0.080	0.025	0.021	0.011	0
F	0.105	0.093	0.090	0.080	0.065	0.063	0.054	0.052	0.020	0.014	0.009	0.002
G	0.061	0.055	0.060	0.046	0.037	0.089	0.028	0.029	0.014	0.008	0.004	0
H	9.975	9.986	9.968	9.959	9.957	9.959	9.947	9.947	9.938	9.938	9.924	9.927

Plate 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.102	1.106	0.805	0.588	0.480	0.371	0.356	0.337	0.009	9.991	9.993	0
B	0.893	0.724	0.535	0.402	0.315	0.225	0.198	0.200	0.009	0.006	9.994	0.001
C	0.523	0.407	0.311	0.231	0.166	0.143	0.115	0.101	0.011	0.010	9.993	9.999
D	0.288	0.245	0.190	0.154	0.112	0.080	0.071	0.069	0.018	0.009	9.998	0
E	0.154	0.127	0.108	0.085	0.064	0.059	0.043	0.042	0.013	0.008	0	0
F	0.083	0.063	0.060	0.047	0.041	0.034	0.025	0.019	0.009	0.006	9.992	0
G	0.074	0.059	0.038	0.038	0.051	0.027	0.027	0.030	0.023	0.017	0.003	0.001
H	0.043	0.026	0.027	0.030	0.022	0.021	0.013	0.018	0.011	0.008	9.995	0

Appendix 2.5

ELISA - Titration of *D. arnfieldi* Antigen with Rotated R_1B Using Streptavadin Peroxidase at 1:500

All volumes 50 µl/well.

1. Coat plate *D. arnfieldi* antigen columns 3-12.
   Initial dilution 1:50. Doubling dilutions.→
   Incubate 37°C 2 hr.
   Wash.

2. Add biotinylated (rotated) antibody R_1B columns 3-10.
   Initial dilution 1:50. Doubling dilutions. ↓
   Incubate 37°C 30 min.
   Wash.

3. Add Streptavadin peroxidase 1:500 all wells.
   Incubate 37°C 30 min.
   Wash.

4. Add substrate. O.P.D. all wells.
   Incubate 37°C in dark 1 hr 10 min.

5. Stop with acid. Read 492 nm.

Appendix 2.5 (Cont'd)

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	9.998	1.268	1.223	0.916	0.640	0.405	0.231	0.201	0.155	9.996	0.039
B	0	9.986	1.222	1.036	0.737	0.485	0.273	0.188	0.115	0.053	0.012	9.987
C	0	0.001	0.853	0.694	0.514	0.337	0.212	0.098	0.076	0.048	0.026	0.018
D	0	0.003	0.495	0.407	0.295	0.217	0.157	0.089	0.047	0.024	0.004	0.017
E	0.001	0.015	0.253	0.227	0.165	0.130	0.066	0.057	0.047	0.021	9.992	0.011
F	0	0.020	0.133	0.125	0.098	0.081	0.057	0.043	0.076	0.012	0.029	0.025
G	0	0.020	0.085	0.078	0.067	0.068	0.048	0.048	0.030	0.027	0.023	0.027
H	0.002	0.006	0.039	0.039	0.034	0.013	0.021	0.016	0.007	9.994	0.005	0.016

Appendix 2.6

ELISA - Titration of *D. arnfieldi* Antigen with Both R_1B and R_2B Using Streptavidin Peroxidase at 1:1000

All volumes 50 µl/well.

1. Coat plate *D. arnfieldi* antigen columns 3-12.
   Initial dilution 1:50. Doubling dilutions. →
   Incubate 37°C 2 hr.
   Wash.

2. Add biotinylated antibody, columns 3-10. Plate 1 R_1B.
   Plate 2 R_2B.
   Initial dilution 1:50. Doubling dilutions. ↓
   Incubate 37°C 30 min.
   Wash.

3. Add Streptavadin peroxidase 1:1000 all wells.
   Incubate 37°C 30 min.
   Wash.

4. Add substrate. O.P.D. all wells.
   Incubate 37°C in dark 1 hr.

5. Stop with acid. Read 492 nm.

Appendix 2.6 (Cont'd)

Plate 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	9.999	0.003	1.494	1.329	0.972	0.613	0.352	0.229	0.193	0.137	9.993	0.019
B	0	0.021	1.357	1.122	0.813	0.515	0.287	0.167	0.110	0.095	0.011	0.017
C	0.003	0.033	0.940	0.739	0.543	0.356	0.191	0.110	0.080	0.063	0.014	0.037
D	0	9.999	0.544	0.428	0.352	0.217	0.099	0.064	0.038	0.015	0.010	0
E	9.999	9.981	0.278	0.196	0.161	0.113	0.029	0.019	0.006	0.006	9.986	9.973
F	9.997	9.980	0.152	0.105	0.102	0.055	9.993	9.981	0.002	0.018	9.990	0
G	9.997	9.975	0.053	0.019	0.024	0.017	9.957	9.974	9.983	0.002	9.983	9.983
H	0.001	9.996	0.034	0.048	0.040	0.018	9.996	9.983	0.017	0.003	9.991	0.005

Plate 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0.105	1.349	1.332	1.090	1.101	0.928	0.828	0.851	0.850	0.011	0.023
B	0	0.021	1.066	1.021	0.763	0.709	0.575	0.525	0.539	0.538	0.008	0.014
C	0.001	0.004	0.689	0.558	0.415	0.416	0.316	0.325	0.299	0.289	0.005	0.010
D	0	0.032	0.374	0.349	0.256	0.252	0.200	0.238	0.189	1.181	0.008	0.064
E	0	9.995	0.014	0.149	0.089	0.112	0.068	0.076	0.085	0.079	9.992	0.023
F	0.001	0.035	0.082	0.094	0.052	0.070	0.012	0.054	0.039	0.053	0.039	0.016
G	0	0.040	0.030	0.063	0.017	0.037	0.017	0.028	0.023	0.022	9.996	0.031
H	0	0.026	0.005	0.045	0.007	0.016	0.042	0.025	0.017	0.009	0.013	0.042

Appendix 2.7

ELISA - Titration of *D. arnfieldi* Antigen with R_1B Using Streptavadin Peroxidase at 1:1000

All volumes 50 µl/well.

1. Coat plate *D. arnfieldi* antigen columns 3-12.
   Initial dilution 1:50. Doubling dilutions.→
   Incubate 37°C 2 hr.
   Wash.

2. Add biotinylated antibody (R_1B). Columns 3-10
   Initial dilution 1:50. Doubling dilutions. ↓
   Incubate 37°C 30 min.
   Wash.

3. Add Streptavadin peroxidase 1:1000 all wells.
   Incubate 37°C 30 min.
   Wash.

4. Add substrate O.P.D. all wells.
   Incubate 37°C in dark 1 hr.

5. Stop with acid. Read 492 nm.

Appendix 2.7 (Cont'd)

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	9.993	1.143	0.905	0.542	0.328	0.156	0.100	0.062	0.064	0.004	0.019
B	9.999	9.993	0.835	0.623	0.369	0.223	0.100	0.051	0.027	0.027	9.994	9.995
C	0	0.002	0.491	0.387	0.243	0.166	0.081	0.054	0.033	0.032	0.003	0.012
D	0	0.007	0.239	0.205	0.148	0.091	0.039	0.034	0.022	0.021	0.009	0.008
E	0	0.004	0.129	0.107	0.081	0.053	0.022	0.020	0.017	0.023	0.010	0.007
F	9.999	9.991	0.082	0.045	0.046	0.032	0.004	0.007	0.008	0.017	0.005	0.008
G	9.999	0.026	0.039	0.028	0.049	0.043	0.011	0.023	0.022	0.033	0.019	0.023
H	9.999	9.987	0.010	0.015	0.018	0.017	9.993	9.998	0.005	0.016	0.024	0.009

Appendix 2.8

ELISA - Titration of D. arnfieldi Antigen with both R_1B and R_2B Using Streptavadin Peroxidase at 1:1000

All volumes 50 µl/well.

1. Coat plate D. arnfieldi antigen columns 3-12.
   Initial dilution 1:50. Doubling dilutions.→
   Incubate 37°C 2 hr.
   Wash.

2. Add biotinylated antibody columns 3-10. Plate 1 R_1B.
   Plate 2 R_2B.
   Initial dilution 1:25. Doubling dilutions. ↓
   Incubate 37°C 30 min.
   Wash.

3. Add Streptavadin peroxidase 1:1000 all wells.
   Incubate 37°C 30 min.
   Wash.

4. Add substrate. O.P.D. all wells.
   Incubate 37°C in dark 1 hr.

5. Stop with acid. Read 492 nm.

Appendix 2.8 (Cont'd)

Plate 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	9.994	1.395	1.249	1.012	0.665	0.393	0.269	0.196	0.148	9.993	9.991
B	0	9.975	1.458	1.308	0.956	0.590	0.293	0.199	0.117	0.071	9.987	9.986
C	0	9.977	1.364	1.158	0.856	0.509	0.265	0.145	0.091	0.049	0.028	0
D	0	0.010	0.918	0.838	0.560	0.353	0.207	0.113	0.060	0.095	0.014	0.005
E	0.001	0.003	0.543	0.460	0.320	0.201	0.141	0.088	0.061	0.073	0.017	0.022
F	9.999	0.003	0.277	0.245	0.174	0.106	0.090	0.067	0.055	0.047	0.032	0.033
G	0	9.984	0.128	0.126	0.090	0.060	0.060	0.071	0.036	0.044	0.030	0.021
H	0	9.981	0.069	0.062	0.056	0.024	0.068	0.057	0.026	0.086	0.013	0.008

Plate 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0.004	1.431	1.525	1.534	1.460	1.371	1.241	1.243	1.115	0.012	0.020
B	0	9.992	1.470	1.626	1.300	1.110	0.898	0.754	0.680	0.682	0.037	0.002
C	0	0.004	1.293	1.255	1.031	0.805	0.616	0.500	0.435	0.377	0.021	0.052
D	0	0.006	1.013	0.790	0.610	0.476	0.385	0.311	0.245	0.241	0.002	9.998
E	0	9.997	0.541	0.432	0.306	0.247	0.192	0.172	0.148	0.130	0.045	0.028
F	0.001	9.984	0.236	0.202	0.142	0.122	0.080	0.080	0.079	0.082	0.027	0.006
G	0	0.031	0.238	0.142	0.107	0.092	0.062	0.079	0.058	0.051	0.058	0.072
H	0	0.010	0.086	0.076	0.047	0.037	0.033	0.035	0.024	0.023	0.037	0.026

Appendix 2.9

ELISA - Titration of both R_1 and R_2 with D. arnfieldi Antigen and both R_1B and R_2B Using Streptavadin Peroxidase at 1:1000

All volumes 50 µl/well.

1. Coat plate antibody columns 3-10. Plate 1 R_1
   Plate 2 R_2
   Initial dilution 1:25. Doubling dilutions.→
   Incubate 37°C 2 hr.
   Wash.

2. Add D. arnfieldi antigen columns 3-12.
   Initial dilution 1:50. Doubling dilutions.↓
   Incubate 37°C 30 min.
   Wash.

3. Add biotinylated antibody columns 3-10. Plate 1 R_1B.
   Plate 2 R_2B.
   Initial dilution 1:25. Doubling dilutions.→
   Incubate 37°C 30 min.
   Wash.

4. Add Streptavadin peroxidase 1:1000 all wells.
   Incubate 37°C 30 min.
   Wash.

5. Add substrate O.P.D. all wells.
   Incubate 37°C in dark 40 min.

6. Stop with acid. Read 492 nm.

Appendix 2.9 (Cont'd)

Plate 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0.002	1.095	0.886	0.663	0.484	0.388	0.293	0.270	0.247	0.024	0.040
B	0.001	0.003	0.683	0.544	0.365	0.293	0.217	0.175	0.145	0.137	0.067	0.021
C	0.001	0	0.406	0.316	0.217	0.164	0.133	0.106	0.101	1.102	0.015	0.051
D	0	0	0.267	0.215	0.180	0.123	0.112	0.089	0.074	0.070	0.019	0.047
E	0.001	0.002	0.182	0.143	0.119	0.099	0.084	0.086	0.078	0.071	0.032	0.060
F	0.001	9.995	0.088	0.066	0.066	0.060	0.071	0.057	0.059	0.062	0.061	0.030
G	0.001	0.009	0.036	0.046	0.053	0.048	0.054	0.064	0.053	0.053	0.420	0.043
H	0.001	0.006	0.030	0.040	0.044	0.045	0.053	0.060	0.041	0.070	0.044	0.047

Plate 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	9.981	1.436	1.370	1.173	1.048	0.843	0.783	0.721	0.705	9.993	9.996
B	0	9.981	1.311	1.172	1.920	0.809	0.691	0.605	0.538	0.505	9.998	9.992
C	0	9.995	1.085	0.889	0.702	0.607	0.486	0.435	0.414	0.413	0.013	0.031
D	0	9.976	1.627	0.457	0.372	0.326	0.273	0.262	0.247	0.229	0.023	9.986
E	0.002	9.984	0.243	0.203	0.183	0.187	0.157	0.156	0.167	0.158	0.003	0.029
F	0.003	9.998	0.132	0.111	0.110	0.114	0.092	0.109	0.103	0.098	0.016	0.017
G	0.005	9.973	0.049	0.049	0.062	0.075	0.048	0.069	0.069	0.073	9.999	0.004
H	0.003	0.010	0.059	0.061	0.070	0.069	0.058	0.063	0.063	0.061	0.040	0.031

Appendix 2.10

ELISA - Titration of both R_1 and R_2 with D. arnfieldi Antigen and both R_1B and R_2B Using Streptavadin Peroxidase at 1:1000

All volumes 50 µl/well.

1. Coat plates with antibody starting column 2. Plate 1 R_1.
   Plate 2 R_2
   Initial dilution 1:25. Doubling dilutions →
   Incubate 37°C 2 hr.
   Wash.

2. Add D. arnfieldi antigen starting column 2.
   Initial dilution 1:50. Doubling dilutions. ↓
   Incubate 37°C 30 min.
   Wash.

3. Add biotinylated antibody 1:50 all wells. Plate 1 R_1B.
   Plate 2 R_2B.
   Incubate 37°C 30 min.
   Wash.

4. Add Streptavadin peroxidase 1:1000 all wells.
   Incubate 37°C 30 min.
   Wash.

5. Add substrate O.P.D.
   Incubate 37°C in dark 30 min.

6. Stop with acid. Read 492 nm.

Appendix 2.10 (Cont'd)

Plate 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	9.999	1.007	0.786	0.567	0.402	0.302	0.202	0.178	0.141	0.123	0.114	0.141
B	0	0.914	0.623	0.422	0.352	0.217	0.155	0.128	0.119	0.115	0.095	0.112
C	0	0.742	0.512	0.369	0.280	0.224	0.148	0.126	0.121	0.127	0.114	0.126
D	0	0.609	0.489	0.332	0.257	0.191	0.124	0.115	0.116	0.098	0.097	0.113
E	0.001	0.655	0.529	0.350	0.278	0.251	0.149	0.159	0.135	0.132	0.127	0.135
F	0	0.568	0.481	0.348	0.293	0.244	0.154	0.183	0.176	0.169	0.159	0.191
G	9.999	0.374	0.329	0.236	0.224	0.202	0.181	0.158	0.162	0.155	0.166	0.172
H	0.001	0.267	0.228	0.178	0.163	0.136	0.125	0.111	0.126	0.111	0.137	0.145

Plate 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.004	1.157	0.990	0.745	0.624	0.429	0.327	0.284	0.202	0.196	0.182	0.198
B	0.002	1.180	1.032	0.816	0.619	0.452	0.334	0.313	0.245	0.206	0.207	0.200
C	0.002	1.098	0.971	0.828	0.641	0.554	0.476	0.405	0.355	0.297	0.316	0.260
D	0.001	0.990	0.811	0.669	0.520	0.406	0.379	0.349	0.321	0.307	0.301	0.284
E	9.999	0.710	0.558	0.465	0.354	0.324	0.284	0.295	0.268	0.310	0.253	0.232
F	0.001	0.579	0.425	0.361	0.284	0.283	0.299	0.263	0.290	0.303	0.263	0.249
G	0.004	0.474	0.333	0.314	0.264	0.242	0.255	0.228	0.230	0.260	0.209	0.221
H	0.001	0.372	0.293	0.279	0.251	0.222	0.227	0.231	0.223	0.266	0.198	0.240

Appendix 2.11

ELISA - Titration of R_2 with D. arnfieldi Antigen in Bovine Serum and R_2B Using Streptavadin Peroxidase at 1:1000

All volumes 50 µl/well.

1. Coat plate antibody - R_2.
   Initial dilution 1:50. Doubling dilutions. →
   Incubate 37°C 2 hr.
   Wash.

2. Add D. arnfieldi antigen in bovine serum. Starting column 2.
   Initial dilution 1:5. Doubling dilutions. ↓
   Incubate 37°C 30 min.
   Wash.

3. Add biotinylated antibody (R_2B) 1:50. All wells.
   Incubate 37°C 30 min.
   Wash.

4. Add streptavadin peroxidase 1:1000. All wells.
   Incubate 37°C 30 min.
   Wash.

5. Add substrate O.P.D. All wells.
   Incubate 37°C in dark 30 min.

6. Stop with acid. Read 492 nm.

Appendix 2.11 (Cont'd)

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	1.417	1.385	1.374	1.280	1.168	1.032	0.918	0.865	0.790	0.852	0.854
B	0	1.456	1.441	1.338	1.150	1.012	0.836	0.730	0.658	0.676	0.650	0.643
C	0	1.141	1.071	0.873	0.702	0.599	0.465	0.469	0.451	0.441	0.420	0.445
D	9.999	0.533	0.442	0.379	0.306	0.304	0.219	0.247	0.235	0.242	0.262	0.253
E	0	0.200	0.169	0.155	0.136	0.155	0.119	0.149	0.121	0.146	0.169	0.145
F	0	0.088	0.086	0.073	0.073	0.088	0.089	0.107	0.083	0.102	0.110	0.110
G	9.999	0.054	0.052	0.054	0.054	0.070	0.061	0.096	0.058	0.084	0.107	0.079
H	0	0.034	0.041	0.042	0.042	0.058	0.063	0.094	0.057	0.067	0.081	0.067

Appendix 2.12

ELISA - Titration of R_2 and *D. arnfieldi* Antigen in Bovine Serum with R_2B Using Streptavadin Peroxidase at 1:1000

All volumes 50 µl/well.

1. Coat plate antibody - R_2 - Not column 1. 1:50.
   Incubate 37°C 2 hr.
   Wash.

2. Add *D. arnfieldi* Antigen in bovine serum. Starting neat in column 2.
   Doubling dilutions →
   Incubate 37°C 30 min.
   Wash.

3. Add biotinylated antibody - R_2B. 1:50. All wells.
   Incubate 37°C 30 min.
   Wash.

4. Add streptavadin peroxidase 1:1000. All wells.
   Incubate 37°C 30 min.
   Wash.

5. Add substrate O.P.D. All wells.
   Incubate 37°C in dark 30 min.

6. Stop with acid. Read 492 nm.

Appendix 2.12 (Cont'd)

	1	2	3	4	5	6	7	8	9	10	11	12
A	9.999	1.315	1.312	1.170	1.210	1.172	1.059	0.971	0.871	0.814	0.770	0.793
B	9.999	1.283	1.298	1.304	1.204	1.119	1.085	0.953	0.845	0.813	0.730	0.792
C	0	1.258	1.254	1.268	1.188	1.160	1.076	0.950	0.849	0.794	0.743	0.790
D	0.001	1.149	1.246	1.269	1.213	1.103	0.997	0.916	9.847	0.775	0.719	0.738
E	0	1.129	1.165	1.174	1.114	1.039	0.943	0.852	0.790	0.785	0.688	0.698
F	9.999	1.111	1.145	1.076	1.055	1.051	0.965	0.859	0.778	0.736	0.668	0.729
G	9.999	1.281	1.117	1.148	1.078	1.036	0.955	0.855	0.749	0.760	0.709	0.664
H	9.999	1.306	1.114	1.171	1.116	1.049	0.986	0.886	0.805	0.791	0.658	0.461

Appendix 2.13

ELISA - Titration of R_2 with D. arnfieldi Antigen in Bovine Serum and R_2B Using Streptavadin Peroxidase at 1:1000

All volumes 50 µl/well.

1. Coat plate antibody - R_2 - 1:100 starting column 2.
   Incubate 37°C 2 hr.
   Wash.

2. Add D. arnfieldi antigen in bovine serum. Columns 2 - 11.
   Initial dilution - neat column 2. Doubling dilutions →
   Incubate 37°C 30 min.
   Wash.

3. Add biotinylated antibody - R_2B. 1:100. All wells.
   Incubate 37°C 30 min.
   Wash.

4. Add streptavadin peroxidase 1:1000. All wells.
   Incubate 37°C 30 min.
   Wash.

5. Add substrate O.P.D. All wells.
   Incubate 37°C in dark 45 min.

6. Stop with acid. Read 492 nm.

Appendix 2.13 (Cont'd)

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0.430	0.320	0.165	0.081	0.008	0.017	9.996	0.009	0.047	9.965	9.983
B	0.001	0.454	0.344	0.205	0.117	0.048	0.036	0.015	9.989	9.997	9.982	0.017
C	0.001	0.478	0.349	0.222	0.121	0.058	0.039	0.025	0.026	0.024	9.978	0.017
D	0.002	0.462	0.345	0.231	0.142	0.082	0.061	0.043	0.030	0.018	9.999	0.038
E	0.001	0.434	0.361	0.210	0.126	0.072	0.052	0.045	0.020	0.067	0.042	0.030
F	0.001	0.418	0.366	0.211	0.105	0.077	0.044	0.047	0.009	0.034	0.002	0.051
G	0.002	0.452	0.350	0.270	0.113	0.083	0.079	0.057	0.040	0.045	0.051	0.080
H	0.002	0.418	0.407	0.285	0.147	0.097	0.083	0.074	0.059	0.050	0.065	0.155

Appendix 2.14

ELISA - Titration of R_2 with *D. arnfieldi* Antigen and R_2B Using Streptavadin Peroxidase at 1:1000 or TMB

All volumes 50 µl/well.

1. Coat plate antibody - R_2 - 1:100. Starting column 2.
   Incubate 37°C 2 hr.
   Wash.

2. Add *D. arnfieldi* antigen. Columns 2-11.
   Initial dilution 1:100. Doubling dilutions →
   Incubate 37°C 30 min.
   Wash.

3. Add biotinylated antibody - R_2B 1:100. Columns 2-12.
   Incubate 37°C 30 min.
   Wash.

4. Add streptavadin peroxidase 1:1000. All wells - plate 1 only.
   Incubate 37°C 30 min.
   Wash.

5. Add substrate. All wells.
   Plate 1 - O.P.D. Incubate 37°C in dark 30 min.
   Stop with acid. Read 492 nm.
   Plate 2 - T.M.B. Incubate 37°C 15 min.
   Read 450 nm.

Appendix 2.14 (Cont'd)

Plate 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	9.999	1.034	0.891	0.706	0.462	0.305	0.296	0.214	0.283	0.248	0.164	0.026
B	0	1.099	0.861	0.612	0.381	0.286	0.243	0.221	0.257	0.180	0.175	0.017
C	0.001	1.157	0.950	0.551	0.350	0.228	0.230	0.161	0.136	0.147	0.115	9.978
D	0	1.039	0.958	0.729	0.530	0.356	0.244	0.205	0.228	0.199	0.166	0.039
E	0.002	1.161	1.010	0.642	0.445	0.362	0.257	0.229	0.197	0.182	0.172	0.042
F	0	1.082	0.807	0.653	0.409	0.343	0.226	0.203	0.221	0.208	0.178	0.050
G	0.001	1.199	0.898	0.672	0.424	0.049	0.259	0.251	0.223	0.236	0.186	0.050
H	0.001	0.984	0.895	0.796	0.450	0.341	0.262	0.249	0.241	0.232	0.186	0.101
Mean		1.090	0.910	0.670	0.430	0.320	0.250	0.220	0.220	0.200	0.170	0.041

Plate 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0.900	0.897	0.046	0.842	0.751	0.618	0.585	0.629	0.567	0.524	9.986
B	0	0.657	0.629	0.486	0.379	0.356	0.333	0.347	0.288	0.364	0.508	0.005
C	0.001	0.543	0.496	0.340	0.242	0.227	0.183	0.196	0.180	0.193	0.200	0.013
D	9.999	0.454	0.490	0.333	0.206	0.141	0.151	0.147	0.165	0.203	0.236	0.007
E	0.001	0.398	0.429	0.279	0.180	0.197	0.214	0.165	0.226	0.279	0.251	0.008
F	0	0.489	0.457	0.357	0.256	0.254	0.264	0.240	0.339	0.334	0.404	9.999
G	0	0.626	0.729	0.574	0.394	0.456	0.506	0.382	0.385	0.389	0.356	9.999
H	9.999	0.954	1.018	0.948	0.799	0.692	0.593	0.515	0.467	0.402	0.433	0.002

Appendix 2.15

ELISA - Titration of R_2 with both Untreated and EDTA-Treated *D. arnfieldi* Antigen and R_2B Using Streptavadin Peroxidase at 1:1000

All volumes 50 µl/well.

1. Coat plate antibody - R_2 - 1:100. Columns 2-12.
   Incubate 37°C 2 hr.
   Wash.

2. Add *D. arnfieldi* antigen. Starting column 2 (neat).
   Doubling dilutions →
   Plate 1 - non-boiled Plate 2 - boiled with EDTA.
   Incubate 37°C 30 min.
   Wash.

3. Add biotinylated antibody - R_2B - 1:100. All wells.
   Incubate 37°C 30 min.
   Wash.

4. Add streptavadin peroxidase 1:1000. All wells.
   Incubate 37°C 30 min.
   Wash.

5. Add substrate O.P.D.
   Incubate 37°C in dark 40 min.

6. Stop with acid. Read 492 nm.

Appendix 2.15 (Cont'd)

Plate 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0.689	0.527	0.564	0.460	0.517	0.475	0.444	0.483	0.477	0.439	0.542
B	0.002	0.738	0.577	0.593	0.481	0.560	0.503	0.490	0.512	0.467	0.477	0.539
C	0	0.739	0.553	0.604	0.494	0.529	0.521	0.521	0.542	0.495	0.469	0.526
D	0.003	0.670	0.560	0.551	0.502	0.523	0.513	0.498	0.516	0.457	0.484	0.529
E	0	0.712	0.582	0.557	0.518	0.523	0.470	0.482	0.504	0.504	0.585	0.520
F	0	0.679	0.679	0.556	0.546	0.530	0.517	0.489	0.596	0.539	0.552	0.471
G	9.999	0.604	0.505	0.579	0.513	0.456	0.446	0.461	0.515	0.476	0.472	0.493
H	0.001	0.641	0.587	0.531	0.528	0.496	0.466	0.499	0.565	0.524	0.525	0.403

Plate 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	9.999	0.574	0.510	0.456	0.471	0.471	0.490	0.460	0.478	0.468	0.432	0.539
B	0	0.576	0.516	0.485	0.482	0.462	0.491	0.455	0.457	0.460	0.487	0.498
C	9.999	0.687	0.624	0.623	0.562	0.552	0.556	0.496	0.498	0.498	0.489	0.537
D	0	0.824	0.790	0.719	0.649	0.657	0.567	0.584	0.557	0.587	0.581	0.583
E	9.999	0.754	0.778	0.711	0.682	0.618	0.638	0.594	0.561	0.579	0.595	0.578
F	0	0.692	0.661	0.647	0.612	0.609	0.596	0.574	0.547	0.562	0.592	0.567
G	0	0.697	0.659	0.643	0.586	0.614	0.618	0.579	0.564	0.555	0.588	0.565
H	0	0.757	0.643	0.608	0.606	0.565	0.604	0.576	0.580	0.563	0.581	0.552

Appendix 2.16

ELISA - Titration of R_2 with both Boiled and Non-Boiled *D. arnfieldi* Antigen and R_2B Using Streptavadin Peroxidase at 1:1000 and 1:5000

All volumes 50 µl/well.

1. Coat plate antibody - R_2 - 1:100. Columns 2-12.
   Incubate 37°C 2 hr.
   Wash.

2. Add *D. arnfieldi* antigen. Rows A - D non-boiled.
   Rows E - H boiled.
   Initial dilution neat - Doubling dilutions →
   Starting columns 2-6 and 7-12.
   Incubate 37°C 40 min.
   Wash.

3. Add biotinylated antibody - R_2B 1:50. Columns 2-11.
   Incubate 37°C 40 min.
   Wash.

4. Add streptavadin peroxidase. Columns 2-6 1:1000.
   Columns 7-11 1.5000.
   Incubate 37°C 40 min.
   Wash.

5. Add substrate O.P.D. All wells.
   Incubate 37°C in dark 40 min.

6. Stop with acid. Read 492 nm.

Appendix 2.16 (Cont'd)

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0.735	0.829	0.607	0.813	0.771	0.367	0.355	0.348	0.337	0.310	0.036
B	0.001	0.622	0.771	0.550	0.668	0.671	0.344	0.345	0.336	0.322	0.330	0.042
C	0	0.616	0.654	0.484	0.586	0.607	0.370	0.316	0.325	0.329	0.333	0.046
D	0.001	0.537	0.662	0.536	0.553	0.560	0.327	0.286	0.305	0.305	0.299	0.041
E	0.001	0.401	0.618	0.493	0.537	0.597	0.253	0.257	0.251	0.244	0.249	0.062
F	0.001	0.318	0.559	0.441	0.485	0.434	0.260	0.219	0.162	0.161	0.174	0.055
G	0	0.285	0.509	0.400	0.434	0.243	0.190	0.116	0.105	0.103	0.108	0.065
H	0	0.236	0.362	0.271	0.260	0.090	0.117	0.098	0.088	0.088	0.108	0.052

Appendix 2.17

ELISA - Titration of R_2 with both Bovine Serum and Bovine Serum Plus *D. arnfieldi* Antigen and R_2B Using Streptavadin Peroxidase at 1:1000

All volumes 50 µl/well.

1. Coat plate antibody - R_2 - 1:100. Columns 2-12.
   Incubate 37°C 2 hr.
   Wash.
2. Add. Rows A, B, C, D bovine serum.
   Column 2 - Initial dilution neat.
   Doubling dilutions →
   Rows E, F, G, H *D. arnfieldi* antigen in bovine serum.
   Column 2 - Initial dilution 1:100.
   Doubling dilutions →
   Incubate 37°C 40 min.
   Wash.
3. Add biotinylated antibody - R_2B 1:100.
   Rows A - D Columns 2-6
   Rows E - H Columns 2-12
   Incubate 37°C 40 min.
   Wash.
4. Add streptavadin peroxidase 1:1000. Rows A - D Columns 2-9
   Rows E - H Columns 2-12
   Incubate 37°C 40 min.
   Wash.
5. Add substrate. O.P.D. All wells.
   Incubate 37°C in dark 40 min.
6. Stop with acid. Read 492 nm.

Appendix 2.17 (Cont'd)

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0.531	0.557	0.488	0.327	0.255	0.116	0.135	0.109	9.984	9.999	9.998
B	0	0.568	0.572	0.513	0.456	0.379	0.135	0.154	0.151	0.003	0.015	0.019
C	0.001	0.552	0.514	0.495	0.481	0.594	0.146	0.153	0.142	0.010	0.034	0.022
D	0	0.533	0.510	0.472	0.505	0.580	0.137	0.152	0.139	0.003	0.013	0.015
E	0.001	1.012	0.919	0.759	0.563	0.502	0.461	0.450	0.448	0.426	0.453	0.481
F	0.001	0.984	0.862	0.689	0.539	0.498	0.470	0.444	0.436	0.424	0.455	0.462
G	0.002	0.915	0.829	0.694	0.547	0.504	0.449	0.450	0.439	0.425	0.450	0.487
H	0.001	1.002	0.804	0.704	0.547	0.475	0.437	0.431	0.447	0.429	0.455	0.496

Appendix 2.18

ELISA - Titration of R_2 with both Bovine Serum and Bovine Serum Plus *D. arnfieldi* Antigen and R_2B Using Streptavadin Peroxidase at 1:1000

All volumes 50 µl/well.

1. Coat plate antibody - R_2 - 1:100. Columns 2-12.
   Incubate 37°C 2 hr.
   Wash.
2. Rows A - D. Add bovine serum.
   Starting column 2 - Initial dilution neat.
   Doubling dilutions → in 5% marvel.
   Rows E - H. Add *D. arnfieldi* antigen in bovine serum
   Initial dilution column 2 1:100. Doubling dilutions → in 5% marvel.
   Incubate 37°C 40 min.
   Wash.
3. Add biotinylated antibody - R_2B - 1:100.
   Rows A - D Columns 2-6.
   Rows E - H Columns 2-12.
   Incubate 37°C 40 min.
   Wash.
4. Add streptavadin peroxidase 1:1000.
   Rows A - D Columns 2-9.
   Rows E - H Columns 2-12.
   Incubate 37°C 40 min.
   Wash.
5. Add substrate. O.P.D. All wells.
   Incubate 37°C in dark 1 hr.
6. Stop with acid. Read 492 nm.

Appendix 2.18 (Cont'd)

	1	2	3	4	5	6	7	8	9	10	11	12
A	9.999	0.086	0.085	0.057	0.028	0.024	9.999	9.995	0.025	9.988	9.984	9.991
B	0	0.101	0.093	0.055	0.051	0.044	0.023	0.028	0.061	0.021	0.005	0.009
C	9.999	0.106	0.078	0.044	0.054	0.035	0.015	0.015	0.026	0.003	0.004	0.039
D	9.999	0.113	0.189	0.069	0.065	0.042	0.023	0.009	0.026	9.998	9.996	9.996
E	9.999	0.185	0.269	0.116	0.081	0.066	0.078	0.081	0.088	0.072	0.052	0.043
F	0	0.374	0.314	0.192	0.124	0.084	0.067	0.082	0.090	0.074	0.385	0.152
G	0.001	0.293	0.299	0.156	0.124	0.097	0.080	0.072	0.067	0.081	0.059	0.039
H	0	0.347	0.279	0.167	0.130	0.090	0.070	0.062	0.064	0.062	0.051	0.016

Appendix 2.19

ELISA - Titration of R_2 with *D. arnfieldi* in Serum and Serum Only Using both 2.5% and 5% Marval as Diluting Agents and R_2B Using Streptavadin Peroxidase at 1:1000

All volumes 50 µl/well.

1. Coat plate antibody - R_2 - 1:100. All wells.

   Incubate 37°C 2 hr.

   Wash.

2. Add *D. arnfieldi* antigen. Starting column 2.

   Doubling dilutions → in marvel to Column 11.

   Rows A and B. Initial dilution 1:10 in serum. 2.5% marvel.

   Rows C and D. Serum only. Initial dilution neat. 2.5% marvel.

   Rows E and F. Initial dilution 1:10 in serum. 5% marvel.

   Rows G and H. Serum only. Initial dilution neat. 5% marvel.

   Incubate 37°C 30 min.

   Wash.

3. Add biotinylated antibody - R_2B. 1:100.

   Rows A, B, E and F. Columns 2-11.

   Rows C, D, G and H. Columns 2-6.

   Incubate 37°C 30 min.

   Wash.

4. Add streptavadin peroxidase. 1:1000.

   Rows A, B, E and F. Columns 2-12.

   Rows C, D, G and H. Columns 2-9.

   Incubate 37°C 30 min.

   Wash.

Appendix 2.19 (Cont'd)

5. Add substrate. O.P.D. All wells.
   Incubate 37°C in dark 30 min.
6. Stop with acid. Read 492 nm.

Appendix 2.19 (Cont'd)

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	1.155	0.918	0.633	0.457	0.313	0.235	0.204	0.166	0.095	0.091	0.002
B	0	1.168	0.933	0.673	0.499	0.360	0.267	0.261	0.238	0.161	0.106	0.021
C	0	0.472	0.290	0.247	0.236	0.210	0.064	0.073	0.075	0.047	0.022	0.026
D	0	0.470	0.320	0.240	0.214	0.201	0.064	0.071	0.073	0.032	0.018	0.041
E	0	1.108	0.859	0.593	0.489	0.352	0.289	0.262	0.229	0.211	0.217	0.040
F	9.999	1.083	0.845	0.563	0.468	0.348	0.270	0.244	0.210	0.201	0.222	0.046
G	0	0.437	0.273	0.214	0.240	0.217	0.075	0.088	0.074	0.047	0.041	0.049
H	9.999	0.386	0.244	0.186	0.221	0.222	0.061	0.089	0.065	0.069	0.041	0.050

Appendix 2.20

ELISA - Titration of R_2 with D. arnfieldi Antigen Plus Serum, Serum and Marvel Alone Using Marval as a Diluting Agent and R_2B Using Streptavadin Peroxidase at 1:1000

All volumes 50 µl/well.

1. Coat plate antibody - R_2 - 1:100.
   Incubate 37°C 2 hr.
   Wash.
2. Rows A and B. Add D. arnfieldi in serum.
   Initial dilution Column 2 1:10 in serum.
   Doubling dilutions in 2.5% marvel:→
   Rows C and D. Add serum. Initial dilution column 2 neat.
   Doubling dilutions in 2.5% marvel:→
   Rows E and F. Add marvel. Start column 3.
   Incubate 37°C 30 min.
   Wash.
3. Add biotinylated antibody - R_2B - 1:100. Columns 2-12.
   Incubate 37°C 30 min.
   Wash.
4. Add streptavadin peroxidase. 1:1000. All wells.
   Incubate 37°C 30 min.
   Wash.
5. Add substrate. O.P.D. All wells.
   Incubate 37°C in dark 30 min.
6. Stop with acid. Read 492 nm.

Appendix 2.20 (Cont'd)

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.002	0.843	0.653	0.500	0.364	0.301	0.258	0.216	0.210	0.205	0.199	0.206
B	0.001	0.857	0.669	0.493	0.374	0.295	0.266	0.227	0.210	0.214	0.222	0.248
C	0.003	0.348	0.258	0.235	0.213	0.222	0.218	0.199	0.202	0.228	0.209	0.216
D	0.002	0.366	0.268	0.240	0.225	0.227	0.223	0.200	0.207	0.247	0.230	0.220
E	0.001	0.256	0.223	0.227	0.218	0.223	0.229	0.214	0.224	0.256	0.254	0.238
F	0.001	0.299	0.223	0.245	0.229	0.231	0.240	0.224	0.226	0.258	0.275	0.255
G	-	-	-	-	-	-	-	-	-	-	-	-
H	-	-	-	-	-	-	-	-	-	-	-	-

Appendix 2.21

ELISA - Titration of R_2 with Duplicate Serum Samples from Infected Animals and R_2B Using Streptavadin Peroxidase at 1:1000

All volumes 50 µl/well.

1. Coat plates antibody - R_2 - 1:100.

   Incubate 37°C 2 hr.

   Wash.

2. Add antigen - Plate 1.

   Row A. Columns 2-10. *D. arnfieldi* antigen in bovine serum. Initial dilution 1:10. Doubling dilutions →

Rows B - H.	Columns 2 and 3	1:2	Serum samples from infected animals. Dilutions in 2.5% marvel. Two wells for each sample at each dilution
	Columns 4 and 5	1:4	
	Columns 6 and 7	1:8	
Rows B - H.	Columns 8 and 9	1:2	Serum samples from infected animals
(Not Row F)	Columns 10 and 11	1:4	
	Columns 12 and 2 and 3 (Plate 2)	1:8	

   Row H (Plate 1) Columns 8-12 no sample added.

   Plate 2 Row A - Columns 2-10 - as plate 1

Rows B - D.	Columns 4 and 5	1:2	Serum samples from infected animals as before
	Columns 6 and 7	1:4	
	Columns 8,9,10	1:8	

   Incubate 37°C 30 min.

   Wash.

3. Add biotinylated antibody - R_2B - 1:100.

   All wells bar column 10 plate 2.

   Incubate 37°C 30 min.

   Wash.

Appendix 2.21 (Cont'd)

4. Add streptavadin peroxidase 1:1000. All wells bar column 12 Rows E - H plate 1.

   Incubate 37°C 30 min.

   Wash.

5. Add substrate. O.P.D. All wells bar column 12 Rows A - D plate 1.

   Incubate 37°C in dark 30 min.

6. Stop with acid. Read 492 nm.

Appendix 2.21 (Cont'd)

Plate 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0.663	0.474	0.437	0.298	0.212	0.163	0.116	0.130	0.094	0.100	0.005
B	0.001	0.129	0.117	0.110	0.111	0.105	0.122	0.124	0.128	0.121	0.115	0.026
C	0	0.128	0.133	0.106	0.122	0.105	0.112	0.116	0.143	0.128	0.108	0.018
D	0	0.119	0.136	0.118	0.122	0.112	0.128	0.171	0.161	0.146	0.156	0.051
E	0.001	0.158	0.164	0.127	0.132	0.125	0.132	0.156	0.156	0.135	0.136	0.043
F	0	0.118	0.125	0.115	0.129	0.129	0.131	0.140	0.038	0.121	0.134	0.040
G	0	0.106	0.113	0.116	0.123	0.122	0.139	0.237	0.218	0.132	0.133	0.043
H	0.001	0.126	0.139	0.137	0.137	0.082	0.150	0.132	0.141	0.133	0.197	0.083

Plate 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0.646	0.564	0.271	0.206	0.165	0.122	0.105	0.118	0.072	0.079	0.079
B	9.999	0.084	0.086	0.095	0.085	0.082	0.076	0.072	0.066	0.015	0.280	0.013
C	0	0.083	0.081	0.081	0.083	0.079	0.083	0.086	0.082	0.014	0.012	0.010
D	9.999	0.096	0.101	0.169	0.190	0.117	0.114	0.103	0.104	0.019	0.050	0.018
E	0.002	0.099	0.087	-	-	-	-	-	-	-	-	-
F	9.999	0.096	0.100	-	-	-	-	-	-	-	-	-
G	0	0.100	0.095	-	-	-	-	-	-	-	-	-
H	9.999	0.082	0.087	-	-	-	-	-	-	-	-	-

Appendix 2.22

ELISA - Titration of R_2 with Duplicate Serum Samples from Infected Animals and R_2B Using Streptavadin Peroxidase at 1:1000

All volumes 50 µl/well.

1. Coat plates antibody R_2 1:100. All wells.

   Incubate 37°C 2 hr.

   Wash.

2. All dilutions in 2.5% marvel.

   Row A. Starting column 2 (neat). Add *D. arnfieldi* antigen.

   EDTA treated. Doubling dilutions → to column 11.

   Row B. Starting column 2 (neat). Add *D. arnfieldi* antigen.

   Doubling dilutions → to column 11.

Plate 1.	Rows C - H.	Columns 2-3	(neat)	Serum samples from infected animals. Two wells for each sample at each dilution
		Columns 4-5	1:2	
		Columns 6-7	1:4	
		Columns 8-9	(neat)	As above
		Columns 10-11	1:2	
Plate 2.	Rows C - H.	Columns 2-3	1:4	
	Rows C - F.	Columns 4-5	(neat)	As above
		Columns 6-7	1:2	
		Columns 8-9	1:4	

   Incubate 37°C 30 min.

   Wash.

Appendix 2.22 (Cont'd)

3. Add biotinylated antibody - R_2B 1:100. All wells.
   Incubate 37°C 30 min.
   Wash.

4. Add streptavadin peroxidase 1:1000. All wells.
   Incubate 37°C 30 min.
   Wash.

5. Add substrate. O.P.D. All wells.
   Incubate 37°C in dark 40 min.

6. Stop with acid. Read 492 nm.

Appendix 2.22 (Cont'd)

Plate 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0.036	0.038	0.038	0.058	0.041	0.046	0.041	0.027	0.032	0.037	0.055
B	0	0.064	0.075	0.055	0.086	0.078	0.069	0.071	0.040	0.039	0.043	0.064
C	0	0.086	0.125	0.092	0.099	0.113	0.139	0.146	0.099	0.069	0.073	0.101
D	9.999	0.105	0.147	0.104	0.101	0.152	0.159	0.167	0.133	0.107	0.121	0.149
E	0.001	0.101	0.149	0.129	0.076	0.156	0.169	0.187	0.137	0.110	0.130	0.157
F	0	0.092	0.133	0.093	0.076	0.171	0.207	0.206	0.155	0.121	0.157	0.215
G	0.001	0.062	0.098	0.065	0.062	0.154	0.201	0.228	0.199	0.146	0.171	0.227
H	0	0.069	0.337	0.126	0.094	0.176	0.196	0.231	0.188	0.154	0.186	0.221

Plate 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0.013	0.001	0.002	0	0.077	0.047	0.007	0.007	0.011	0.008	0.013
B	0	0.224	0.115	0.030	0.024	0.080	0.064	0.033	0.019	0.020	0.019	0.029
C	0	0.083	0.080	0.071	0.083	0.003	0.010	0.064	0.041	-	-	-
D	0.001	0.061	0.068	0.069	0.092	9.966	9.983	0.072	0.036	-	-	-
E	0	0.121	0.131	0.152	0.160	0.007	0.017	0.187	0.188	-	-	-
F	0.001	0.140	0.121	0.190	0.204	0.014	0.022	0.211	0.214	-	-	-
G	0	0.148	0.181	-	-	-	-	-	-	-	-	-
H	0.001	0.163	0.173	-	-	-	-	-	-	-	-	-

Appendix 2.23

ELISA - Titration of R_2 with Serum from Possibly Infected Animals with R_2B Using Streptavadin Peroxidase at 1:1000

All volumes 50 µl/well.

1. Coat plates antibody (R_2) 1:100. All wells.

   Incubate 37°C. 2 hr.

   Wash.

2. All dilutions in 2.5% Marvel.

   Row A. Starting Column 2. Add D. arnfieldi antigen (initial dilution 1:10 in PBS). Doubling dilutions to Column 9.→

   Row B. Starting Column 2. Add D. arnfieldi antigen (initial dilution 1:10 in horse serum). Doubling dilutions to Column 9.→

   Samples from possibly infected animals added as listed:-

   Plate 1, rows C-H, columns 2-7 - one animal per row.

   Plate 1, rows C-H, columns 8-12 and Plate 2, rows C-H, columns 2-3 - one animal per row.

   Plate 2, rows C-H, columns 4-8 - one animal per row.

   Incubate 37°C, 30 min.

   Wash.

3. Add biotinylated antibody (R_2B) 1:100. All wells.

   Incubate 37°C, 30 min.

   Wash.

4. Add streptavadin peroxidase 1:1000. All wells.

   Incubate 37°C, 30 min.

   Wash.

Appendix 2.23 (Cont'd)

5. Add substrate O.P.D. All wells.
   Incubate 37°C in dark, 30 min.
6. Stop with acid.
   Read 492 nm.

Appendix 2.23 (Cont'd)

Plate 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	1.176	1.148	0.887	0.676	0.545	0.510	0.490	0.435	0.091	0.197	0.029
B	9.999	0.793	0.733	0.576	0.479	0.405	0.390	0.420	0.429	0.006	0.205	0.017
C	0	0.363	0.356	0.386	0.354	0.353	0.355	0.330	0.303	0.404	0.395	0.012
D	0	0.377	0.297	0.328	0.316	0.298	0.345	0.309	0.306	0.343	0.359	0.012
E	0	0.237	0.283	0.293	0.278	0.263	0.306	0.255	0.266	0.274	0.299	0.041
F	9.999	0.193	0.227	0.270	0.207	0.228	0.266	0.234	0.224	0.256	0.273	0.036
G	9.998	0.125	0.126	0.192	0.152	0.193	0.215	0.275	0.241	0.227	0.220	0.013
H	0	0.183	0.106	0.146	0.122	0.155	0.155	0.184	0.159	0.158	0.171	0.121

Plate 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	1.393	1.300	0.405	0.347	0.461	0.454	0.449	0.002	0.350	0.416	0.024
B	0.001	0.804	0.822	0.283	0.237	0.335	0.412	0.444	0.034	0.351	0.432	0.016
C	0	0.405	0.423	0.304	0.331	0.434	0.466	0.493	0.025	0.023	0.025	0.028
D	0	0.352	0.419	0.243	0.305	0.372	0.392	0.422	0.014	0.016	0.024	0.018
E	9.999	0.311	0.307	0.182	0.209	0.288	0.358	0.381	0.019	0.021	0.024	0.039
F	0	0.257	0.351	0.184	0.206	0.283	0.335	0.391	0.021	0.024	0.030	0.032
G	9.999	0.195	0.286	0.685	0.445	0.719	0.747	0.662	0.039	0.134	0.155	0.047
H	0	0.126	0.153	0.698	0.597	0.792	0.611	0.656	0.015	0.121	0.133	0.040

Appendix 2.24

ELISA - Titration of R_2 with *D. arnfieldi* Antigen, Following Various Treatments and with Dilutions made in Marvel and Diluting Buffer, with R_2 Using Streptavadin Peroxidase at 1:1000

All volumes 50 µl/well.

1. Coat plate antibody (R_2) 1:100. All wells.

   Incubate 37°C. 2 hr.

   Wash.

2. Starting column 2, initial dilution 1:50. Doubling dilutions → to column 11.

Row A - *D. arnfieldi* antigen in PBS	All dilutions in 2.5% Marvel
Row B - EDTA treated *D. arnfieldi* antigen	
Row C - Periodate treated *D. arnfieldi* antigen	
Row D - Marvel only	
Row E - *D. arnfieldi* antigen in PBS	All dilutions in diluting buffer
Row F - EDTA treated *D. arnfieldi* antigen	
Row G - Periodate treated *D. arnfieldi* antigen	
Row H - Diluting buffer alone	

   Incubate 37°C, 30 min.

   Wash.

3. Add biotinylated antibody (R_2B) 1:100. All wells.

   Incubate 37°C, 30 min.

   Wash.

4. Add streptavadin peroxidase 1:1000. All wells.

   Incubate 37°C, 30 min.

   Wash.

Appendix 2.24 (Cont'd)

5. Add substrate O.P.D. All wells.
   Incubate 37^{o}C in dark, 30 min.

Appendix 2.24 (Cont'd)

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0.891	0.499	0.382	0.278	0.202	0.195	0.184	0.178	0.166	0.174	0.018
B	0	0.362	0.222	0.215	0.192	0.160	0.162	0.149	0.154	0.159	0.160	0.064
C	0	0.333	0.205	0.189	0.180	0.185	0.186	0.176	0.184	0.182	0.180	0.037
D	0.002	0.160	0.177	0.182	0.167	0.220	0.170	0.166	0.164	0.171	0.174	0.011
E	0	0.900	0.690	0.550	0.428	0.309	0.312	0.296	0.279	0.304	0.269	0.085
F	9.999	0.401	0.377	0.354	0.323	0.253	0.300	0.284	0.290	0.265	0.343	0.074
G	0	0.317	0.293	0.334	0.299	0.263	0.302	0.303	0.310	0.274	0.266	0.096
H	0	0.298	0.322	0.334	0.319	0.292	0.296	0.315	0.337	0.307	0.297	0.104

Appendix 2.25

ELISA - Titration of R_2 with *D. arnfieldi* Antigen in PBS or Horse Serum Diluted in Marvel or Diluting Buffer with R_2B Using Streptavadin Peroxidase at 1:1000

All volumes 50 µl/well.

1. Coat plate antibody (R_2) 1:100. All wells.

   Incubate 37°C. 2 hr.

   Wash.

2. Starting column 2. Initial dilution 1:10. Doubling dilutions → to column 11.

   Row A - *D. arnfieldi* antigen in PBS)
) Dilutions in
   Row B & C - *D. arnfieldi* antigen in horse serum) 2.5% Marvel
   Row D - Horse serum only)

   Row E - *D. arnfieldi* antigen in PBS)
) Dilution in
   Row F & G - *D. arnfieldi* antigen in horse serum) diluting buffer
   Row H - Horse serum only)

   Incubate 37°C, 30 min.

   Wash.

3. Add biotinylated antibody (R_2B) 1:100. All wells.

   Incubate 37°C, 30 min.

   Wash.

4. Add streptavadin peroxidase 1:1000. All wells.

   Incubate 37°C, 30 min.

   Wash.

5. Add substrate O.P.D. All wells.

   Incubate 37°C in dark, 30 min.

Appendix 2.25 (Cont'd)

6. Stop with acid.

   Read 492 nm.

Appendix 2.25

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.001	1.010	0.747	0.555	0.415	0.328	0.243	0.205	0.152	0.153	0.149	9.981
B	0.001	0.853	0.658	0.511	0.386	0.325	0.246	0.220	0.179	0.197	0.195	0.019
C	0.003	0.840	0.638	0.483	0.371	0.300	0.241	0.215	0.172	0.187	0.197	0.028
D	0.001	0.285	0.235	0.230	0.202	0.220	0.213	0.210	0.179	0.209	0.203	0.032
E	0.001	1.053	0.953	0.748	0.640	0.535	0.444	0.416	0.322	0.375	0.327	0.132
F	0	0.853	0.790	0.672	0.629	0.479	0.373	0.347	0.278	0.295	0.309	0.122
G	0.001	0.895	0.815	0.727	0.630	0.504	0.398	0.450	0.293	0.315	0.322	0.139
H	0.001	0.259	0.335	0.407	0.448	0.412	0.355	0.335	0.260	0.288	0.320	0.133

Appendix 2.26

ELISA - Titration of R_2 with D. arnfieldi Antigen in Horse Serum and Serum Samples from Animals with R_2B Using Streptavadin at 1:1000

All volumes 50 µl/well.

1. Coat plates antibody (R_2) 1:100. All wells.
   Incubate 37°C, 2 hr.
   Wash.
2. All plates. Starting column 2, initial dilution 1:10. Doubling dilutions → to column 11.
   Row A - Horse serum with D. arnfieldi antigen.
   Row B - Horse serum only.
   Row C - Bovine serum only.
   Rows D-H - Samples from animals - one animal per row.
   Incubate 37°C, 30 min.
   Wash.
3. Add biotinylated antibody (R_2B) 1:100. All wells.
   Incubate 37°C, 30 min.
   Wash.
4. Add streptavadin peroxidase 1:1000. All wells.
   Incubate 37°C, 30 min.
   Wash.
5. Add substrate O.P.D. All wells.
   Incubate 37°C in dark, 30 min.
6. Stop with acid.
   Read 492 nm.

Appendix 2.26 (Cont'd)

Plate 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0.574	0.458	0.304	0.233	0.191	0.164	0.133	0.128	0.131	0.143	0.117
B	0	0.176	0.138	0.129	0.152	0.112	0.114	0.107	0.114	0.104	0.138	0.152
C	0	0.118	0.087	0.091	0.101	0.089	0.079	0.073	0.084	0.073	0.089	0.093
D	0	0.217	0.121	0.121	0.117	0.102	0.107	0.087	0.104	0.089	0.122	0.107
E	0	0.292	0.209	0.159	0.137	0.119	0.115	0.101	0.115	0.104	0.118	0.118
F	9.999	0.159	0.142	0.138	0.127	0.118	0.114	0.105	0.120	0.102	0.128	0.123
G	0	0.192	0.144	0.133	0.155	0.124	0.115	0.271	0.121	0.120	0.126	0.125
H	0.001	0.263	0.177	0.228	0.199	0.118	0.118	0.109	0.113	0.102	0.134	0.123

Plate 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0.528	0.378	0.285	0.211	0.153	0.143	0.120	0.103	0.122	0.108	0.109
B	9.999	0.157	0.098	0.098	0.091	0.086	0.088	0.089	0.079	0.102	0.108	0.095
C	0	0.212	0.133	0.119	0.105	0.101	0.104	0.110	0.099	0.113	0.116	0.144
D	0	0.077	0.098	0.093	0.090	0.091	0.098	0.104	0.097	0.110	0.111	0.120
E	0	0.081	0.095	0.090	0.088	0.088	0.093	0.105	0.142	0.112	0.121	0.112
F	9.999	0.384	0.226	0.156	0.132	0.118	0.106	0.113	0.145	0.169	0.137	0.101
G	0	0.113	0.124	0.123	0.126	0.125	0.123	0.148	0.126	0.131	0.144	0.130
H	0	0.120	0.145	0.146	0.138	0.120	0.133	0.157	0.163	0.163	0.180	0.167

Appendix 2.26 (Cont'd)

Plate 3

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0.568	0.450	0.317	0.210	0.167	0.144	0.120	0.130	0.105	0.107	0.102
B	0	0.171	0.124	0.114	0.089	0.083	0.084	0.082	0.080	0.070	0.072	0.080
C	0	0.248	0.152	0.108	0.092	0.085	0.082	0.079	0.090	0.091	0.154	0.091
D	9.999	0.249	0.172	0.136	0.117	0.098	0.099	0.107	0.103	0.087	0.154	0.098
E	0	0.147	0.131	0.117	0.113	0.101	0.113	0.126	0.174	0.124	0.117	0.119
F	0	0.087	0.089	0.077	0.064	0.056	0.056	0.059	0.067	0.104	0.153	0.074
G	0	0.165	0.159	0.138	0.133	0.115	0.110	0.139	0.140	0.121	0.138	0.145
H	0	0.304	0.187	0.184	0.140	0.113	0.118	0.119	0.134	0.132	0.138	0.142

Appendix 2.27

ELISA - Titration of R_2 and Serum Samples, Untreated and EDTA-Treated, from Animals with R_2B Using Streptavadin Peroxidase at 1:1000

All volumes 50 µl/well.

1. Coat plates antibody (R_2) 1:100. All wells.

   Incubate 37°C, 2 hr.

   Wash.

2. Starting column 2. Initial dilution 1:100. Doubling dilutions → to column 11. All dilutions in 2.5% Marvel.

   Row A - D. arnfieldi antigen in horse serum.

   Row B - Horse serum only.

   Row C - Marvel only.

   Rows D-H - Samples from sanimals - one animal per row.

   Plate 1 - untreated samples.

   Plate 2 - all samples treated with EDTA before use.

   Incubate 37°C, 30 min.

   Wash.

3. Add biotinylated antibody (R_2B) 1:100. All wells.

   Incubate 37°C, 30 min.

   Wash.

4. Add streptavadin peroxidase 1:100. All wells.

   Incubate 37°C, 30 min.

   Wash.

5. Add substrate O.P.D. All wells.

   Incubate 37°C in dark, 30 min.

6. Stop with acid.

   Read 492 nm.

Appendix 2.27 (Cont'd)

Plate 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	1.493	1.428	1.181	0.971	0.758	0.536	0.365	0.247	0.187	0.138	0.098
B	0.001	0.185	0.118	0.089	0.093	0.088	0.079	0.081	0.108	0.091	0.086	0.092
C	0.001	0.089	0.078	0.097	0.104	0.092	0.099	0.100	0.099	0.104	0.100	0.102
D	0	0.175	0.127	0.095	0.107	0.097	0.099	0.093	0.099	0.108	0.108	0.104
E	0	0.110	0.095	0.091	0.108	0.105	0.100	0.102	0.114	0.123	0.122	0.112
F	0	0.129	0.096	0.094	0.115	0.110	0.108	0.114	0.124	0.125	0.128	0.123
G	0.001	0.544	0.320	0.146	0.141	0.129	0.129	0.188	0.128	0.120	0.122	0.126
H	0.001	0.312	0.160	0.098	0.127	0.107	0.106	0.106	0.105	0.124	0.135	0.120

Plate 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	1.454	1.325	1.145	1.019	0.827	0.561	0.427	0.278	0.224	0.184	0.102
B	0.001	0.214	0.149	0.105	0.099	0.098	0.091	0.106	0.098	0.117	0.131	0.092
C	0.001	0.096	0.098	0.108	0.094	0.113	0.083	0.107	0.089	0.121	0.092	0.086
D	0	0.178	0.139	0.106	0.097	0.099	0.094	0.106	0.092	0.104	0.098	0.075
E	0	0.145	0.116	0.131	0.100	0.103	0.095	0.099	0.090	0.123	0.094	0.072
F	0	0.135	0.079	0.113	0.102	0.104	0.118	0.108	0.100	0.118	0.113	0.091
G	0.001	0.162	0.112	0.121	0.112	0.114	0.100	0.112	0.108	0.124	0.047	0.088
H	0.002	0.122	0.091	0.100	0.117	0.086	0.080	0.082	0.077	0.095	0.071	0.073

Appendix 2.28

ELISA - Titration of R_2 and Serum Samples from Donkeys with R_2B Using Streptavadin Peroxidase at 1:1000

All volumes 50 µl/well.

1. Coat plate antibody (R_2) 1:100. All wells.

   Incubate 37°C, 2 hr.

   Wash.

2. Starting column 2. Doubling dilutions → in 5% Marvel.

   Row A - D. arnfieldi antigen in horse serum - initial dilution 1:10.

   Row B - Horse serum only - initial dilution 1:10.

   Row C - Marvel 5%.

   Row D-H - Samples from donkeys - neat.

   Incubate 37°C, 40 min.

   Wash.

3. Add biotinylated antibody (R_2B) 1:100. All wells.

   Incubate 37°C, 40 min.

   Wash.

4. Add streptavadin peroxidase 1:1000. All wells.

   Incubate 37°C, 40 min.

   Wash.

5. Add substrate O.P.D. All wells.

   Incubate 37°C in dark, 40 min.

6. Stop with acid.

   Read 492 nm.

Appendix 2.28 (Cont'd)

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.001	1.593	1.535	1.312	1.076	0.858	0.566	0.401	0.305	0.251	0.222	0.176
B	0	0.241	0.203	0.162	0.168	0.152	0.164	0.153	0.159	0.213	0.180	0.161
C	0	0.189	0.188	0.185	0.191	0.196	0.192	0.186	0.191	0.189	0.192	0.207
D	0	0.603	0.414	0.222	0.209	0.192	0.200	0.180	0.187	0.192	0.189	0.176
E	0.001	0.234	0.195	0.181	0.180	0.190	0.198	0.182	0.196	0.205	0.183	0.175
F	0	0.210	0.198	0.173	0.187	0.183	0.181	0.180	0.190	0.196	0.193	0.170
G	0.001	0.237	0.231	0.197	0.197	0.187	0.198	0.183	0.186	0.208	0.223	0.187
H	0.001	0.543	0.344	0.219	0.232	0.194	0.222	0.210	0.196	0.218	0.195	0.199

Appendix 2.29

ELISA - Titration of R_2 and Serum Samples from Animals with R_2 Using Streptavadin Peroxidase at 1:1000

All volumes 50 µl/well.

1. Coat plates antibody (R_2) 1:100. All wells.
   Incubate 37°C, 2 hr.
   Wash.
2. Starting column 2. Doubling dilutions → Dilutions in 2.5% Marvel.
   Row A - D. arnfieldi antigen in horse serum. Initial dilution 1:10.
   Row B - Horse serum only. Initial dilution - neat.
   Row C - Marvel only.
   Row D-H - Samples from animals - one animal per row.
   Incubate 37°C, 30 min.
   Wash.
3. Add biotinylated antibody (R_2B) 1:100. All wells.
   Incubate 37°C, 30 min.
   Wash.
4. Add streptavadin peroxidase 1:1000. All wells.
   Incubate 37°C, 30 min.
   Wash.
5. Add substrate O.P.D. All wells.
   Incubate 37°C in dark, 30 min.
6. Stop with acid.
   Read 492 nm.

Appendix 2.29 (Cont'd)

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0.478	0.469	0.427	0.384	0.312	0.235	0.156	0.113	0.081	0.069	0.037
B	0.001	0.050	0.061	0.050	0.056	0.042	0.040	0.041	0.042	0.041	0.046	0.044
C	0.002	0.066	0.068	0.061	0.057	0.044	0.054	0.049	0.056	0.047	0.039	0.046
D	0.001	0.053	0.083	0.083	0.072	0.066	0.057	0.047	0.055	0.045	0.038	0.045
E	0	0.034	0.056	0.067	0.060	0.066	0.055	0.048	0.056	0.057	0.050	0.051
F	0	0.037	0.064	0.062	0.063	0.070	0.071	0.051	0.062	0.064	0.047	0.062
G	0	0.035	0.070	0.073	0.070	0.072	0.067	0.057	0.063	0.067	0.066	0.057
H	0.001	0.044	0.116	0.101	0.077	0.075	0.056	0.051	0.053	0.053	0.065	0.040

Appendix 2.30

ELISA - Titration of R_2 and Serum Samples from Infected and Non-Infected Donkeys with R_2B Using Streptavadin Peroxidase at 1:1000

All volumes 50 µl/well.

1. Coat plates antibody (R_2) 1:100. All wells.
   Incubate 37°C, 2 hr.
   Wash.
2. Starting column 2. Doubling dilutions → Dilutions in 2.5% Marvel.
   Row A - D. arnfieldi antigen in horse serum. Initial dilution 1:10.
   Row B - Horse serum only. Initial dilution - neat.
   Rows C-H Plate 1. Samples from donkey with patent infection.
   Rows C-G Plate 2. Samples from donkey without patent infection.
   Row H Plate 2. Sample from donkey with patent infection.
   Samples start column 2, initial dilution - neat, one sample per row.
   Incubate 37°C, 30 min.
   Wash.
3. Add biotinylated antibody (R_2B) 1:100. All wells.
   Incubate 37°C, 30 min.
   Wash.
4. Add streptavadin peroxidase 1:1000. All wells.
   Incubate 37°C, 30 min.
   Wash.
5. Add substrate O.P.D. All wells.
   Incubate 37°C in dark, 30 min.
6. Stop with acid.
   Read 492 nm.

Appendix 2.30 (Cont'd)

Plate 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	1.369	1.258	0.856	0.638	0.448	0.288	0.182	0.112	0.088	0.073	0.047
B	0.001	0.142	0.098	0.067	0.063	0.115	0.065	0.064	0.058	0.059	0.067	0.064
C	0.001	0.101	0.094	0.075	0.059	0.071	0.067	0.075	0.064	0.064	0.068	0.062
D	0	0.275	0.102	0.074	0.066	0.076	0.064	0.069	0.065	0.061	0.070	0.061
E	0	0.428	0.148	0.096	0.081	0.090	0.063	0.082	0.077	0.098	0.097	0.077
F	0	0.333	0.126	0.090	0.082	0.091	0.074	0.078	0.082	0.080	0.074	0.082
G	0.001	0.280	0.124	0.103	0.105	0.117	0.102	0.106	0.085	0.083	0.097	0.091
H	0.001	0.213	0.130	0.098	0.092	0.093	0.090	0.092	0.096	0.074	0.089	0.088

Plate 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	9.999	1.516	1.284	0.818	0.583	0.384	0.278	0.164	0.123	0.103	0.097	0.100
B	0.001	0.130	0.071	0.052	0.091	0.075	0.062	0.054	0.055	0.060	0.091	0.090
C	0.001	0.099	0.063	0.054	0.057	0.063	0.085	0.055	0.062	0.102	0.075	0.073
D	0	0.182	0.089	0.049	0.052	0.052	0.062	0.059	0.087	0.067	0.066	0.069
E	0	0.206	0.102	0.064	0.062	0.065	0.082	0.071	0.091	0.082	0.088	0.100
F	0.001	0.172	0.084	0.058	0.062	0.064	0.083	0.068	0.085	0.081	0.110	0.085
G	0	0.270	0.127	0.063	0.073	0.069	0.080	0.074	0.090	0.089	0.104	0.102
H	0.001	0.276	0.121	0.040	0.037	0.035	0.055	0.048	9.993	0.054	0.072	0.034

Appendix 2.31

ELISA - Titration of R_2 and Serum Samples from Animals with R_2B Using Streptavadin Peroxidase at 1:1000

All volumes 50 µl/well.

1. Coat plates antibody (R_2) 1:100. All wells.

   Incubate 37°C, 2 hr.

   Wash.

2. Row A - D. arnfieldi antigen in horse serum. Initial dilution 1:10.

   Row B - Horse serum only. Initial dilution - neat.

   Starting column 2. Doubling dilutions → column 11.

   Dilutions in 2.5% Marvel.

   Rows C-E) Samples added, 3 of each columns 2-12
)
   Rows F-G) excluding Plate 2 columns 10 & 11.

   Incubate 37°C, 30 min.

   Wash.

3. Add biotinylated antibody (R_2B) 1:100. All wells.

   Incubate 37°C, 30 min.

   Wash.

4. Add streptavadin peroxidase 1:1000. All wells.

   Incubate 37°C, 30 min.

   Wash.

5. Add substrate O.P.D. All wells.

   Incubate 37°C in dark, 30 min.

6. Stop with acid.

   Read 492 nm.

Appendix 2.31 (Cont'd)

Plate 1.

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	1.179	1.128	0.886	0.648	0.519	0.354	0.256	0.177	0.128	0.113	0.026
B	0	0.086	0.080	0.051	0.055	0.051	0.045	0.058	0.048	0.047	0.064	0.034
C	0	0.140	0.210	0.103	0.074	0.110	0.049	0.061	0.071	0.053	0.107	0.020
D	0	0.121	0.176	0.122	0.113	0.115	0.071	0.065	0.077	0.087	0.122	0.040
E	0	0.064	0.083	0.069	0.060	0.098	0.071	0.088	0.088	0.088	0.106	0.041
F	0	0.068	0.048	0.074	0.097	0.113	0.220	0.268	0.144	0.120	0.356	0.044
G	0.001	0.079	0.064	0.078	0.087	0.108	0.175	0.210	0.130	0.141	0.272	0.042
H	0.002	0.025	0.028	0.031	0.033	0.049	0.073	0.086	0.070	0.052	0.112	0.046

Plate 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	1.246	1.075	0.793	0.633	0.413	0.255	0.162	0.102	0.067	0.063	9.999
B	0.001	0.106	0.061	0.033	0.025	0.022	0.005	0.012	0.013	0.004	0.027	9.999
C	0	0.178	0.206	0.060	0.066	0.066	0.018	0.022	0.082	0.104	0.167	0.006
D	0.001	0.088	0.140	0.042	0.047	0.051	0.008	0.004	0.060	0.051	0.100	9.999
E	0	0.060	0.106	0.160	0.123	0.067	0.052	0.048	0.078	0.071	0.095	0.004
F	9.999	0.150	0.347	0.083	0.075	0.074	0.057	0.087	0.127	0.109	0.116	0.038
G	9.999	0.124	0.263	0.110	0.106	0.089	0.072	0.089	0.121	0.125	0.154	0.069
H	9.999	0.058	0.149	0.078	0.078	0.070	0.067	0.074	0.097	0.128	0.171	0.007

Appendix 2.32

ELISA - Titration of R_2 and Serum Samples from Animals with R_2B Using Streptavadin Peroxidase at 1:1000

All volumes 50 µl/well.

1. Coat plates antibody (R_2) 1:100. All wells.

   Incubate 37°C, 2 hr.

   Wash.

2. Starting column 2.

   Row A - D. arnfieldi antigen in horse serum. Initial dilution 1:10.

   Row B - Horse serum only. Initial dilution - neat.

   Doubling dilutions → in 2.5% Marvel.

   Rows C & D) Samples in duplicate - one animal per column
)
   Rows E & F) excluding columns 11 & 12, rows G & H, plate 5.
)
   Rows G & H)

   Incubate 37°C, 30 min.

   Wash.

3. Add biotinylated antibody (R_2B) 1:100. All wells.

   Incubate 37°C, 30 min.

   Wash.

4. Add streptavadin peroxidase 1:1000. All wells.

   Incubate 37°C, 30 min.

   Wash.

5. Add substrate O.P.D. All wells.

   Incubate 37°C in dark, 30 min.

6. Stop with acid.

   Read 492 nm.

Appendix 2.32

Plate 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	1.345	1.229	1.014	0.846	0.586	0.462	0.338	0.253	0.198	0.186	0.183
B	0	0.155	0.114	0.113	0.126	0.107	0.120	0.136	0.128	0.125	0.135	0.145
C	0	0.106	0.151	0.143	0.172	0.384	0.153	1.078	0.278	0.142	0.326	0.167
D	9.999	0.093	0.145	0.127	0.151	0.363	0.139	1.017	0.243	0.139	0.324	0.159
E	9.999	0.103	0.145	0.172	0.140	0.133	1.455	0.252	0.137	0.318	0.187	0.719
F	0	0.113	0.152	0.189	0.153	0.135	1.463	0.249	0.143	0.328	0.196	0.714
G	0	0.111	0.222	0.198	0.185	0.213	0.203	0.193	0.170	0.211	0.239	0.195
H	0.001	0.111	0.238	0.200	0.189	0.195	0.197	0.174	0.176	0.195	0.228	0.195

Plate 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.001	1.360	1.224	1.017	0.795	0.610	0.446	0.307	0.234	0.194	0.162	0.164
B	0	0.139	0.113	0.105	0.103	0.105	0.108	0.105	0.111	0.118	0.119	0.122
C	9.999	0.109	0.170	0.163	0.252	0.127	0.978	0.140	0.105	0.105	0.219	0.284
D	0	0.104	0.162	0.160	0.240	0.124	0.930	0.128	0.098	0.102	0.217	0.276
E	0	0.347	0.139	1.657	0.569	0.480	1.114	1.252	0.920	0.102	0.155	0.169
F	0	0.341	0.136	1.622	0.534	0.504	1.094	1.229	0.194	0.099	0.145	0.169
G	0	0.137	0.105	0.191	0.121	0.128	0.359	0.184	0.191	0.479	0.211	0.203
H	0.002	0.133	0.109	0.189	0.123	0.127	0.366	0.207	0.198	0.468	0.181	0.211

Appendix 2.32 (Cont'd)

Plate 3

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	1.293	1.213	1.033	0.810	0.639	0.470	0.313	0.241	0.215	0.182	0.169
B	0	0.151	0.130	0.129	0.117	0.123	0.130	0.138	0.122	0.151	0.139	0.143
C	9.999	0.510	0.123	0.207	0.174	0.892	0.235	0.208	0.154	0.490	0.358	0.199
D	0	0.485	0.126	0.203	0.170	0.871	0.233	0.199	0.157	0.485	0.371	0.190
E	9.999	0.344	0.176	0.365	0.124	0.131	0.564	0.143	0.146	0.189	0.183	0.410
F	0.003	0.353	0.188	0.386	0.132	0.141	0.565	0.140	0.140	0.188	0.176	0.376
G	0	0.788	0.140	0.127	0.193	0.234	0.300	0.135	0.231	0.172	0.168	0.175
H	0	0.787	0.138	0.128	0.181	0.195	0.302	0.120	0.240	0.154	0.163	0.170

Plate 4

	1	2	3	4	5	6	7	8	9	10	11	12
A	9.999	1.162	1.082	0.907	0.699	0.548	0.409	0.293	0.227	0.191	0.152	0.146
B	0	0.125	0.117	0.106	0.098	0.109	0.106	0.110	0.109	0.119	0.116	0.118
C	9.999	0.247	0.234	0.173	0.166	0.126	0.151	0.133	0.157	0.298	0.448	0.338
D	9.999	0.244	0.230	0.157	0.159	0.116	0.153	0.130	0.148	0.302	0.417	0.322
E	0	0.201	0.283	0.172	0.112	0.478	0.123	0.154	0.265	0.131	0.190	0.114
F	0	0.199	0.276	0.168	0.109	0.501	0.121	0.152	0.268	0.136	0.198	0.112
G	0	0.106	0.116	0.452	0.160	0.124	0.877	0.147	0.190	0.410	0.134	0.106
H	0.001	0.111	0.119	0.384	0.166	0.118	0.866	0.151	0.183	0.421	0.144	0.124

Appendix 2.32 (Cont'd)

Plate 5

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	1.172	1.095	0.849	0.680	0.523	0.381	0.262	0.214	0.169	0.155	0.137
B	9.999	0.150	0.109	0.093	0.107	0.098	0.109	0.104	0.110	0.103	0.127	0.113
C	0	0.150	0.358	0.119	0.454	0.153	0.154	0.082	0.122	0.133	0.234	0.107
D	0	0.136	0.352	0.108	0.439	0.145	0.121	0.064	0.103	0.129	0.225	0.091
E	9.999	0.173	0.096	0.106	0.143	0.383	0.094	0.094	0.082	0.885	0.162	0.072
F	0	0.164	0.095	0.106	0.140	0.371	0.091	0.085	0.082	0.867	0.143	0.063
G	9.999	0.216	0.351	0.124	0.208	0.684	0.093	0.114	0.114	0.177	0.133	0.111
H	9.999	0.266	0.322	0.119	0.196	0.624	0.088	0.103	0.105	0.182	0.156	0.113

Appendix 2.33

ELISA - Titration of R_2 and Serum Samples from Animals with R_2B Using Streptavadin Peroxidase at 1:1000

All volumes 50 µl/well.

1. Coat plates antibody (R_2) 1:100. All wells.

   Incubate 37°C, 2 hr.

   Wash.

2. Row A - D. arnfieldi antigen in horse serum. Initial dilution 1:10.

   Row B - Horse serum only. Initial dilution - neat.

   Starting column 2. Doubling dilutions → in 2.5% Marvel.

   Row C & D)
) Samples in duplicate, one animal per column.
   Row E & F)
)
   Row G & H)

   Incubate 37°C, 30 min.

   Wash.

3. Add biotinylated antibody (R_2B) 1:100. All wells.

   Incubate 37°C, 30 min.

   Wash.

4. Add streptavadin peroxidase 1:1000. All wells.

   Incubate 37°C, 30 min.

   Wash.

5. Add substrate O.P.D. All wells.

   Incubate 37°C in dark, 30 min.

6. Stop with acid.

   Read 492 nm.

Appendix 2.33 (Cont'd)

Plate 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.001	1.484	1.417	1.158	0.958	0.650	0.484	0.371	0.306	0.233	0.244	0.223
B	0.001	0.201	0.194	0.163	0.165	0.162	0.173	0.176	0.221	0.166	0.190	0.184
C	0	0.181	0.396	0.282	0.778	0.163	0.148	0.162	0.195	0.123	0.125	0.133
D	0.001	0.198	0.392	0.236	0.776	0.166	0.146	0.138	0.196	0.129	0.130	0.129
E	0.001	0.718	0.622	0.150	0.261	0.505	0.266	0.253	0.236	0.235	0.449	0.195
F	0	0.719	0.620	0.145	0.259	0.525	0.272	0.228	0.250	0.266	0.413	0.193
G	0.001	0.307	0.134	0.226	0.972	0.296	0.238	0.135	0.472	0.161	0.303	0.206
H	0	0.290	0.116	0.213	1.000	0.259	0.246	0.122	0.487	0.150	0.277	0.189

Plate 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	1.520	1.423	1.121	0.917	0.696	0.493	0.375	0.326	0.258	0.281	0.248
B	0	0.163	0.172	0.159	0.169	0.172	0.162	0.178	0.186	0.182	0.225	0.194
C	0	0.291	0.199	0.235	0.205	0.226	0.160	0.216	0.208	0.200	0.347	0.271
D	0.001	0.237	0.191	0.248	0.190	0.211	0.161	0.229	0.207	0.207	0.340	0.237
E	0	0.490	0.238	0.156	0.284	0.632	0.353	0.176	0.482	0.158	0.166	1.174
F	0	0.475	0.230	0.161	0.266	0.684	0.415	0.180	0.466	0.151	0.171	1.063
G	9.999	0.378	0.157	0.178	0.230	0.480	0.193	0.587	0.358	0.283	0.320	0.638
H	0	0.384	0.141	0.161	0.219	0.460	0.192	0.568	0.317	0.240	0.271	0.574

Appendix 2.33 (Cont'd)

Plate 3

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.001	1.531	1.372	1.127	0.912	0.670	0.496	0.365	0.297	0.253	0.235	0.263
B	0	0.213	0.194	0.179	0.186	0.181	0.174	0.187	0.199	0.190	0.199	0.199
C	0.003	0.116	0.168	0.097	0.177	0.227	0.127	0.095	0.245	0.195	0.158	0.179
D	0	0.106	0.162	0.092	0.167	0.229	0.118	0.086	0.259	0.175	0.143	0.182
E	0.001	0.269	0.126	0.123	0.125	0.140	0.159	0.162	0.209	0.260	0.260	0.424
F	0	0.254	0.120	0.117	0.119	0.138	0.219	0.152	0.193	0.263	0.263	0.438
G	0	0.305	0.316	0.391	0.236	0.183	0.121	0.163	0.176	0.149	0.149	0.176
H	0	0.287	0.353	0.364	0.229	0.174	0.114	0.133	0.164	0.123	0.123	0.165

Appendix 2.34

ELISA - Titration of R_2 and Serum Samples from Animals with R_2B Using Streptavadin Peroxidase at 1:1000

All volumes 50 µl/well.

1. Coat plates antibody (R_2) 1:100. All wells.

   Incubate 37°C, 2 hr.

   Wash.

2. Row A - D. arnfieldi antigen in horse serum - Initial dilution 1:10.

   Row B - Horse serum only - Initial dilution - neat.

   Starting column 2 - Doubling dilutions → in 2.5% Marvel.

   Row C & D)
) Samples in duplicate, one animal per column
   Row E & F)
) excluding rows G & H, columns 5-12
   Row G & H)

   Incubate 37°C, 30 min.

   Wash.

3. Add biotinylated antibody (R_1B) 1:100. All wells.

   Incubate 37°C, 30 min.

   Wash.

4. Add streptavadin peroxidase 1:1000. All wells.

   Incubate 37°C, 30 min.

   Wash.

5. Add substrate O.P.D. All wells.

   Incubate 37°C in dark, 30 min.

6. Stop with acid.

   Read 492 nm.

Appendix 2.34 (Cont'd)

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0.342	0.233	0.214	0.166	0.169	0.173	0.163	0.133	0.165	0.167	0.004
B	0.001	0.157	0.147	0.154	0.142	0.158	0.161	0.165	0.126	0.178	0.175	0.009
C	0	0.228	0.963	0.755	0.552	0.260	0.170	0.624	0.139	0.225	0.157	0.014
D	0	0.272	0.962	0.749	0.521	0.289	0.176	0.716	0.141	0.256	0.148	0.010
E	0	0.316	0.231	0.350	0.180	0.293	0.452	0.215	0.159	0.227	0.636	0.026
F	0	0.300	0.236	0.355	0.184	0.284	0.496	0.225	0.201	0.247	0.692	0.034
G	0	0.167	0.168	0.158	0.012	0.024	0.040	0.053	0.013	0.030	0.048	0.043
H	9.999	0.155	0.151	0.143	0.002	0.013	0.032	0.027	0.011	0.025	0.045	0.044

Appendix 2.35

ELISA - Titration of R_2 with Serum Samples, Untreated and EDTA-Treated, from Animals with R_2B Using Streptavadin Peroxidase at 1:1000

All volumes 50 µl/well.

1. Coat plates antibody (R_2) 1:100. All wells.

   Incubate 37°C, 2 hr.

   Wash.

2. Row A - *D. arnfieldi* antigen in horse serum - Initial dilution 1:10.

   Row B - horse serum only. Initial dilution - neat.

   Starting column 2 - Doubling dilutions → in 2.5% Marvel.

   Rows C, E & G - Untreated serum samples

   Rows D, F & H - Serum samples as above but EDTA treated.

   Samples start column 2.

   - indicates no sample.

   Incubate 37°C, 30 min.

   Wash.

3. Add biotinylated antibody (R_2B) 1:100. All wells.

   Incubate 37°C, 30 min.

   Wash.

4. Add streptavadin peroxidase 1:1000. All wells.

   Incubate 37°C, 30 min.

   Wash.

5. Add substrate O.P.D. All wells.

   Incubate 37°C in dark, 30 min.

6. Stop with acid.

   Read 492 nm.

Appendix 2.35 (Cont'd)

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0.930	0.924	0.848	0.786	0.736	0.597	0.473	0.369	0.264	0.233	0.005
B	0	0.148	0.109	0.104	0.087	0.099	0.075	0.078	0.089	0.079	0.095	0.021
C	9.999	0.127	0.126	0.131	0.127	0.127	0.109	0.119	0.147	0.117	0.133	0.019
D	0	-	-	-	-	0.129	-	-	0.143	-	-	0.022
E	0	0.123	0.117	0.120	0.108	0.121	0.110	0.118	0.132	0.122	0.142	0.031
F	0	-	-	-	-	-	-	-	-	-	-	0.017
G	0	0.135	0.176	-	0.145	0.121	0.124	0.131	0.126	0.139	0.145	0.029
H	0	-	-	-	-	-	-	-	0.129	-	-	0.028

Appendix 2.36

ELISA - Titration of R_2 and Serum Samples, Untreated and EDTA-Treated, from Animals with R_2B Using Streptavadin Peroxidase at 1:1000

All volumes 50 µl/well.

1. Coat plates antibody (R_2) 1:100. All wells.

   Incubate 37°C, 2 hr.

   Wash.

2. Row A - D. arnfieldi antigen in horse serum. Initial dilution 1:10.

   Row B - Horse serum only. Initial dilution - neat.

   Starting column 2. Doubling dilutions → in 2.5% Marvel.

   Rows C, E & G - Untreated serum samples.

   Rows D, F & H - EDTA treated serum samples above.

   Samples start column 2.

   Incubate 37°C, 30 min.

   Wash.

3. Add biotinylated antibody (R_2B) 1:100. All wells.

   Incubate 37°C, 30 min.

   Wash.

4. Add streptavadin peroxidase 1:1000. All wells.

   Incubate 37°C, 30 min.

   Wash.

5. Add substrate O.P.D. All wells.

   Incubate 37°C in dark, 30 min.

6. Stop with acid.

   Read 492 nm.

Appendix 2.36 (Cont'd)

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.001	1.104	1.098	1.066	1.068	1.036	0.985	0.788	0.776	0.608	0.467	0.141
B	0.001	0.134	0.113	0.101	0.099	0.104	0.102	0.101	0.101	0.109	0.107	0.139
C	0.001	0.101	0.094	0.113	0.111	0.138	0.142	0.128	0.119	0.123	0.127	0.152
D	0	0.101	0.099	0.106	0.103	0.131	0.111	0.112	0.109	0.115	0.119	0.159
E	0	0.182	0.109	0.115	0.127	0.119	0.120	0.112	0.115	0.135	0.129	0.167
F	0	0.178	0.106	0.114	0.127	0.123	0.130	0.136	0.109	0.136	0.117	0.179
G	0	0.112	0.111	0.130	0.150	0.135	0.122	0.172	0.119	0.137	0.147	0.201
H	0	0.099	0.136	0.120	0.121	0.108	0.114	0.156	0.096	0.108	0.121	0.155

Appendix 2.37

ELISA - Titration of R_2 and Serum Samples, Untreated and EDTA-Treated, from Animals with R_2B Using Streptavadin Peroxidase at 1:1000

All volumes 50 µl/well.

1. Coat plates antibody (R_2) 1:100. All wells.

   Incubate 37°C, 2 hr.

   Wash.

2. Row A - D. arnfieldi antigen in horse serum. Initial dilution 1:10.

   Row B - Horse serum only. Initial dilution - neat.

   Starting column 2. Doubling dilutions → in 2.5% Marvel.

   Rows C, E & G. Untreated serum samples.

   Rows D, F & H. EDTA treated serum samples as above.

   Samples start column 2.

   Incubate 37°C, 30 min.

   Wash.

3. Add biotinylated antibody (R_2B) 1:100. All wells.

   Incubate 37°C, 30 min.

   Wash.

4. Add streptavadin peroxidase 1:1000. All wells.

   Incubate 37°C, 30 min.

   Wash.

5. Add substrate O.P.D. All wells.

   Incubate 37°C in dark, 30 min.

6. Stop with acid.

   Read 492 nm.

Appendix 2.37 (Cont'd)

	1	2	3	4	5	6	7	8	9	10	11	12
A	9.999	-	-	-	-	1.882	1.713	1.549	1.240	0.989	0.786	0.019
B	0	0.313	0.262	0.236	0.242	0.235	0.217	0.244	0.230	0.223	0.252	0.038
C	0	1.680	-	1.713	1.716	1.421	1.525	0.983	1.696	1.725	-	0.011
D	9.998	0.377	1.549	0.243	0.288	0.372	0.274	0.241	0.467	0.218	0.261	0.017
E	0	1.824	1.493	0.314	0.405	0.609	0.233	0.578	0.245	1.621	0.310	0.022
F	0	0.344	0.249	0.267	0.291	0.300	0.274	0.261	0.266	0.218	0.269	0.027
G	9.999	1.254	0.320	0.300	0.296	0.272	0.483	1.057	0.326	1.628	1.039	0.028
H	9.999	0.341	0.272	0.273	0.307	0.266	0.289	0.267	0.276	0.270	0.304	0.014

Appendix 2.38

ELISA - Titration of R_2 and Serum Samples, Untreated and EDTA-Treated, from Animals with R_2B Using Streptavadin Peroxidase at 1:1000

All volumes 50 µl/well.

1. Coat plates antibody (R_2) 1:100. All wells.

   Incubate 37°C, 2 hr.

   Wash.

2. Row A - D. arnfieldi antigen in horse serum. Initial dilution 1:10.

   Row B - Horse serum only. Initial dilution - neat.

   Starting column 2. Doubling dilutions → in 2.5% Marvel.

   Rows C, E & G. Untreated serum samples.

   Rows D, F & H. EDTA treated serum samples as above.

   Samples start column 2.

   Incubate 37°C, 30 min.

   Wash.

3. Add biotinylated antibody (R_2B) 1:100. All wells.

   Incubate 37°C, 30 min.

   Wash.

4. Add streptavadin peroxidase 1:1000. All wells.

   Incubate 37°C, 30 min.

   Wash.

5. Add substrate O.P.D. All wells.

   Incubate 37°C in dark, 30 min.

6. Stop with acid.

   Read 492 nm.

Appendix 2.38 (Cont'd)

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.001	1.311	1.213	1.037	0.919	0.767	0.628	0.483	0.382	0.281	0.194	9.994
B	0	0.146	0.077	0.057	0.063	0.053	0.067	0.082	0.081	0.075	0.086	9.999
C	0	0.110	0.195	0.702	0.255	0.283	0.086	0.419	0.249	0.278	0.201	0.006
D	9.999	0.107	0.120	0.143	0.124	0.104	0.074	0.134	0.146	0.093	0.092	0.008
E	0.001	0.106	0.095	0.174	0.081	0.145	0.101	0.094	0.132	0.138	0.281	0.007
F	0	0.078	0.095	0.090	0.060	0.096	0.111	0.078	0.104	0.096	0.111	0.008
G	0	0.302	0.143	0.057	0.092	0.621	0.455	0.083	0.499	0.154	0.142	9.999
H	0	0.136	0.144	0.098	0.096	0.250	0.105	0.095	0.117	0.094	0.100	9.999

Appendix 2.39

ELISA - Titration of R_2 and Serum Samples, Untreated and EDTA-Treated, from Animals with R_2B Using Streptavadin Peroxidase at 1:1000

All volumes 50 µl/well.

1. Coat plates antibody (R_2) 1:100. All wells.

   Incubate 37°C, 2 hr.

   Wash.

2. Row A - D. arnfieldi antigen in horse serum. Initial dilution 1:10.

   Row B - Horse serum only. Initial dilution - neat.

   Starting column 2. Doubling dilutions → in 2.5% Marvel.

   Rows C, E & G. Untreated serum samples

   Rows D, F & H. EDTA treated serum samples as above.

   Samples start column 2. Excluding Plate 2, rows G & H, columns 10 & 11.

   Incubate 37°C, 30 min.

   Wash.

3. Add biotinylated antibody (R_2B) 1:100. All wells.

   Incubate 37°C, 30 min.

   Wash.

4. Add streptavadin peroxidase 1:1000. All wells.

   Incubate 37°C, 30 min.

   Wash.

5. Add substrate O.P.D. All wells.

   Incubate 37°C in dark, 30 min.

6. Stop with acid.

   Read 492 nm.

Appendix 2.39 (Cont'd)

Plate 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	1.080	1.104	1.051	0.899	0.810	0.605	0.491	0.393	0.326	0.224	0.004
B	0	0.145	0.177	0.144	0.150	0.133	0.126	0.121	0.136	0.149	0.135	0.004
C	0	0.124	0.229	0.153	0.289	0.250	1.103	0.132	0.375	0.275	0.059	0.009
D	9.999	0.172	0.228	0.150	0.288	0.256	1.117	0.152	0.349	0.228	0.917	0.018
E	9.999	0.343	0.244	0.009	0.341	0.157	0.254	0.030	0.205	0.905	0.176	0.024
F	9.999	0.261	0.247	0.602	0.319	0.190	0.271	0.912	0.250	0.474	0.205	0.027
G	0	0.162	0.904	0.537	0.245	0.550	0.368	0.261	0.148	0.232	0.965	0.036
H	9.999	0.172	0.621	0.396	0.299	0.530	0.422	0.216	0.173	0.210	0.981	0.032

Plate 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0.969	0.858	0.991	0.895	0.753	0.657	0.520	0.392	0.290	0.201	9.987
B	0	0.197	0.170	0.139	0.150	0.134	0.136	0.147	0.147	0.139	0.113	0.007
C	0	0.421	0.118	0.657	0.223	0.173	0.972	1.521	1.047	0.815	0.165	0.004
D	9.999	0.362	0.195	0.433	0.176	0.208	0.949	0.289	0.843	0.595	0.152	0
E	0	0.194	0.412	0.107	0.219	0.134	0.934	0.172	0.763	0.160	0.142	9.996
F	9.999	0.223	0.289	0.160	0.222	0.162	0.472	0.203	0.457	0.174	0.168	0.012
G	0	0.123	0.132	0.251	0.679	0.175	0.971	0.611	0.165	x	x	0.008
H	9.999	0.168	0.179	0.248	0.241	0.186	0.833	0.641	0.180	x	x	0.022

Appendix 2.40

ELISA - Titration of R_2 and Serum Samples, Untreated and EDTA-Treated, from Animals with R_2B Using Streptavadin Peroxidase at 1:1000

All volumes 50 µl/well.

1. Coat plates antibody (R_2) 1:100. All wells.

   Incubate 37°C, 2 hr.

   Wash.

2. Row A - D. arnfieldi antigen in horse serum. Initial dilution 1:10.

   Row B - Horse serum only. Initial dilution - neat.

   Starting column 2. Doubling dilutions → in 2.5% Marvel.

   Rows C, E & G. Untreated serum samples.

   Rows D, F & H. EDTA treated serum samples as above.

   Samples start column 2.

   Incubate 37°C, 30 min.

   Wash.

3. Add biotinylated antibody (R_2B) 1:100. All wells.

   Incubate 37°C, 30 min.

   Wash.

4. Add streptavadin peroxidase 1:1000. All wells.

   Incubate 37°C, 30 min.

   Wash.

5. Add substrate O.P.D. All wells.

   Incubate 37°C in dark, 30 min.

6. Stop with acid.

   Read 392 nm.

Appendix 2.40 (Cont'd)

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0.793	0.697	0.571	0.430	0.344	0.232	0.151	0.097	0.067	0.097	0.057
B	0	9.984	9.987	9.985	9.984	9.991	9.990	9.992	9.997	0.003	0.033	0.054
C	0	0.021	0.054	0.052	0.087	0.034	0.978	0.019	0.172	0.078	0.428	0.128
D	9.999	0.020	0.050	0.258	0.084	0.023	0.976	0.020	0.069	0.077	0.241	0.076
E	9.999	0.028	0.091	0.060	0.019	0.074	0.014	0.062	0.033	0.030	0.036	0.128
F	9.999	0.031	0.053	0.068	0.025	0.068	0.033	0.055	0.065	0.052	0.067	0.151
G	0.001	0.247	0.146	0.101	0.033	0.045	0.707	0.152	0.050	0.307	0.090	0.087
H	9.999	0.206	0.119	0.105	0.042	0.049	0.681	0.114	0.061	0.262	0.083	0.096

Appendix 2.41

ELISA - Titration of R_2 and Serum Samples, Untreated and EDTA-Treated, from Animals with R_2B Using Streptavadin Peroxidase at 1:1000

All volumes 50 µl/well.

1. Coat plates antibody (R_2) 1:100. All wells.

   Incubate 37°C, 2 hr.

   Wash.

2. Row A - D. arnfieldi in horse serum. Initial dilution 1:10.

   Row B - Horse serum only. Initial dilution - neat.

   Starting column 2. Doubling dilutions → in 2.5% Marvel.

   Rows C, E & G. Untreated serum samples.

   Rows D, F & H. EDTA treated serum samples as above.

   Samples start column 2. Excluding plate 2, rows E & F.

   Incubate 37°C, 30 min.

   Wash.

3. Add biotinylated antibody (R_2B) 1:100. All wells.

   Incubate 37°C, 30 min.

   Wash.

4. Add streptavadin peroxidase 1:1000. All wells.

   Incubate 37°C, 30 min.

   Wash.

5. Add substrate O.P.D. All wells.

   Incubate 37°C in dark, 30 min.

6. Stop with acid.

   Read 492 nm.

Appendix 2.41 (Cont'd)

Plate 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.001	1.067	1.013	0.879	0.803	0.678	0.520	0.384	0.322	0.013	0.205	0.005
B	0	0.206	0.114	0.107	0.113	0.114	0.127	0.130	0.133	0.011	0.143	0.004
C	0.001	0.865	0.112	0.295	0.305	0.229	0.159	0.269	0.346	0.016	0.155	0.006
D	0.001	0.887	0.108	0.289	0.294	0.238	0.167	0.267	0.367	0.024	0.150	0.008
E	0.001	0.155	0.301	0.215	0.781	0.211	0.133	0.112	0.216	0.017	0.412	0.007
F	0	0.173	0.284	0.214	0.775	0.214	0.127	0.113	0.226	0.023	0.454	0.008
G	0	0.261	0.113	0.256	0.192	0.132	0.121	0.107	0.173	0.027	0.173	0.017
H	0	0.286	0.119	0.266	0.203	0.129	0.119	0.108	0.190	0.021	0.161	0.014

Plate 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0.984	1.025	0.892	0.842	0.690	0.556	0.444	0.332	0.271	0.231	0.013
B	0.001	0.136	0.112	0.113	0.106	0.109	0.122	0.125	0.135	0.138	0.154	0.013
C	0	0.158	0.117	0.146	0.411	0.215	0.407	0.165	0.162	0.189	0.387	0.014
D	0	0.159	0.127	0.144	0.346	0.220	0.407	0.151	0.166	0.188	0.424	0.021
E	-	-	-	-	-	-	-	-	-	-	-	-
F	-	-	-	-	-	-	-	-	-	-	-	-
G	0	0.340	0.138	0.171	0.173	0.220	0.242	0.882	0.166	0.195	0.223	0.030
H	9.999	0.321	0.124	0.151	0.144	0.225	0.220	0.866	0.128	0.156	0.187	9.997

Appendix 2.42

ELISA - Titration of R_2 and Serum Samples, Untreated and EDTA-Treated, from Animals with R_2B Using Streptavadin Peroxidase at 1:1000

All volumes 50 µl/well.

1. Coat plates antibody (R_2) 1:100. All wells.

   Incubate 37°C, 2 hr.

   Wash.

2. Row A - Columns 2 - 7 - D. arnfieldi antigen. Initial dilution 1:10.
   Doubling dilutions → to column 11.

   Columns 8 - 11- Horse serum only. Initial dilution - neat.
   Doubling dilutions →

   Rows B, D & F - Untreated samples.

   Rows C, E & G - EDTA treated samples.

   Row H - Columns 2, 4, 6, 8, 10 & 12 - Untreated samples.

   Columns 3, 5, 7, 9, 11 - EDTA treated samples.

   Row A - Column 12 - EDTA treated sample.

   Incubate 37°C, 30 min.

   Wash.

3. Add biotinylated antibody (R_2B) 1:100. All wells.

   Incubate 37°C, 30 min.

   Wash.

4. Add streptavadin peroxidase 1:1000. All wells.

   Incubate 37°C, 30 min.

   Wash.

5. Add substrate O.P.D. All wells.

   Incubate 37°C in dark, 30 min.

Appendix 2.42 (Cont'd)

6. Stop with acid.

   Read 492 nm.

Appendix 2.42 (Cont'd)

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.007	0.963	0.850	0.750	0.563	0.417	0.042	0.116	0.024	0.017	0.022	0.130
B	0.009	0.167	0.247	0.150	0.726	0.290	0.193	0.398	0.199	0.141	0.397	0.074
C	0.011	0.111	0.121	0.113	0.106	0.114	0.273	0.127	0.112	0.118	0.399	0.201
D	0.008	0.218	0.403	0.112	0.532	0.224	0.221	0.128	0.131	0.303	0.232	0.135
E	0.011	0.069	0.199	0.066	0.047	0.041	0.169	0.074	0.059	0.069	0.080	0.081
F	0.009	0.100	0.133	0.266	0.103	0.144	0.145	0.312	0.208	0.123	0.158	0.153
G	0.011	0.094	0.076	0.084	0.049	0.045	0.074	0.081	0.054	0.052	0.070	0.088
H	0.011	0.268	0.127	0.215	0.128	0.116	0.143	0.159	0.128	0.127	0.159	0.159

Appendix 2.43

LISA - Titration of R and Serum Samples, Untreated and EDTA-Treated, from Animals with R_2B Using Streptavadin Peroxidase at 1:1000

All volumes 50 µl/well.

1. Coats plates antibody (R_2) 1:100. All wells.
   Incubate 37°C, 2 hr.
   Wash.
2. Row A - Columns 2-11 - D. arnfieldi antigen in horse serum.
   Initial dilution 1:10. Doubling dilutions →
   Row B - Columns 2-11 - Horse serum only. Initial dilution - neat.
   Doubling dilutions →
   Rows C, E & G - Untreated samples excluding rows E & G, columns 10-12.
   Rows D, F & H - EDTA treated samples excluding rows F & H, columns 10-12
   Incubate 37°C, 30 min.
   Wash.
3. Add biotinylated antibody (R_2B) 1:100. All wells.
   Incubate 37°C, 30 min.
   Wash.
4. Add streptavadin peroxidase 1:1000. All wells.
   Incubate 37°C, 30 min.
   Wash.
5. Add substrate O.P.D. All wells.
   Incubate 37°C in dark, 30 min.
6. Stop with acid.
   Read 492 nm.

Appendix 2.43 (Cont'd)

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.001	1.005	0.863	0.631	0.556	0.403	0.321	0.261	0.221	0.199	0.181	0.028
B	0	0.105	0.104	0.102	0.107	0.107	0.104	0.110	0.094	0.127	0.128	0.023
C	9.999	0.063	0.157	0.294	0.107	0.211	0.106	0.114	0.113	0.122	0.124	0.027
D	0.002	0.114	0.121	0.117	0.104	0.124	0.124	0.121	0.143	0.142	0.142	0.036
E	0.001	0.220	0.168	0.125	0.140	0.175	0.158	0.185	0.237	-	-	-
F	0	0.191	0.192	0.131	0.139	0.135	0.129	0.164	0.154	-	-	-
G	9.999	0.240	0.236	0.359	0.402	0.536	0.485	0.745	0.738	-	-	-
H	0	0.108	0.105	0.109	0.129	0.112	0.123	0.157	0.138	-	-	-

Appendix 2.44

ELISA - Titration of R_2 and Serum Samples, Untreated and EDTA-Treated, from Animals with R_2B Using Streptavadin Peroxidase at 1:1000

All volumes 50 µl/well.

1. Coat plates antibody (R_2) 1:100. All wells.

   Incubate 37°C, 2 hr.

   Wash.

2. Row A - Columns 2-9 D. arnfieldi antigen in horse serum.

   Initial dilution 1:10. Doubling dilutions →

   Rows B, D & F - Untreated samples. Starting column 2.

   Rows C, E & G - EDTA treated samples. Starting column 2.

   Row H - Columns 2, 4, 6, 8, 10 & 12 - Untreated samples.

   Columns 3, 5, 7, 9, 11 - EDTA treated samples.

   Row A - Column 11 - Untreated sample.

   Columns 10 & 12 - EDTA treated samples.

   Incubate 37°C, 30 min.

   Wash.

3. Add biotinylated antibody (R_2B) 1:100. All wells.

   Incubate 37°C, 30 min.

   Wash.

4. Add streptavadin peroxidase 1:1000. All wells.

   Incubate 37°C, 30 min.

   Wash.

5. Add substrate O.P.D. All wells.

   Incubate 37°C in dark, 30 min.

6. Stop with acid.

   Read 492 nm.

Appendix 2.44 (Cont'd)

Plate 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	9.998	0.979	0.792	0.685	0.115	0.107	0.101	0.107	0.094	0.249	0.178
B	0.001	0.164	0.181	0.151	0.105	0.172	0.099	0.303	0.291	0.307	0.199	0.324
C	0.001	0.074	0.066	0.077	0.074	0.093	0.091	0.097	0.091	0.091	0.085	0.091
D	0	0.189	0.110	0.182	0.183	0.103	0.389	0.124	0.255	0.153	0.137	0.100
E	0.001	0.072	0.063	0.072	0.075	0.083	0.086	0.087	0.108	0.081	0.102	0.103
F	0.001	0.097	0.076	0.085	0.159	0.211	0.120	0.163	0.096	0.084	0.244	0.157
G	0	0.082	0.069	0.088	0.096	0.109	0.091	0.112	0.100	0.092	0.116	0.127
H	0	0.107	0.084	0.122	0.090	0.113	0.100	0.094	0.111	0.219	0.114	0.170

Plate 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.001	1.014	0.865	0.857	0.716	0.123	0.120	0.084	0.084	0.097	0.123	0.103
B	9.999	0.510	0.208	0.638	0.204	0.258	0.116	0.139	0.381	1.040	0.113	0.300
C	0.001	0.099	0.088	0.094	0.109	0.097	0.086	0.081	0.083	0.104	0.082	0.103
D	0.001	0.267	0.319	0.148	0.121	0.114	0.116	0.163	0.118	0.761	0.387	0.217
E	0	0.127	0.102	0.121	0.121	0.117	0.085	0.103	0.111	0.107	0.101	0.098
F	0.001	0.094	0.250	0.254	0.359	0.223	0.517	0.121	0.141	0.135	0.100	0.176
G	0	0.106	0.102	0.128	0.127	0.118	0.124	0.100	0.117	0.126	0.097	0.110
H	9.999	0.232	0.127	0.286	0.132	0.513	0.118	0.218	0.103	0.475	0.090	0.148

Appendix 2.44 (Cont'd)

Plate 3

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	1.241	1.201	1.059	0.835	0.128	0.134	0.119	0.107	0.126	0.229	0.155
B	0.001	0.147	0.105	0.099	0.242	0.300	0.434	0.284	0.440	0.141	0.170	0.165
C	0.001	0.089	0.087	0.083	0.095	0.101	0.098	0.094	0.094	0.094	0.101	0.110
D	0	0.189	0.155	0.316	0.373	0.577	0.297	0.502	0.592	0.233	0.225	0.838
E	0	0.109	0.109	0.108	0.136	0.123	0.123	0.121	0.122	0.124	0.130	0.146
F	9.999	0.282	0.211	0.112	0.133	0.114	0.198	0.284	0.315	0.275	0.632	0.130
G	0	0.116	0.115	0.107	0.150	0.130	0.120	0.128	0.103	0.108	0.132	0.132
H	0.001	0.133	0.131	0.192	0.197	0.144	0.137	0.137	0.124	0.284	0.142	0.169

Appendix 2.45

ELISA - Titration of R_2 and Serum Samples, Untreated and EDTA-Treated, from Animals with R_2B Using Streptavadin Peroxidase at 1:1000

All volumes 50 µl/well.

1. Coat plates antibody (R_2) 1:100. All wells.

   Incubate 37°C, 2 hr.

2. Row A - Columns 2-6 - D. arnfieldi in horse serum. Initial dilution 1:10. Doubling dilutions →

   Columns 7-11 - Horse serum only. Initial dilution - neat. Doubling dilutions →

   Rows B, D & F - Untreated samples.

   Rows C, E & G - EDTA treated samples.

   Row H - No samples.

   Incubate 37°C, 30 min.

   Wash.

3. Add biotintylated antibody (R_2B) 1:100. All wells.

   Incubate 37°C, 30 min.

   Wash.

4. Add streptavadin peroxidase 1:1000. All wells.

   Incubate 37°C, 30 min.

   Wash.

5. Add substrate O.P.D. All wells.

   Incubate 37°C in dark, 30 min.

6. Stop with acid.

   Read 492 nm.

Appendix 2.45 (Cont'd)

Plate 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.001	1.300	1.279	1.202	1.113	0.959	0.136	0.128	0.119	0.142	0.122	0.157
B	0.001	0.339	0.096	0.296	0.322	0.105	0.087	0.236	0.379	0.128	0.079	0.212
C	0.001	0.121	0.124	0.222	0.138	0.129	0.125	0.115	0.137	0.129	0.121	0.134
D	0	0.103	0.460	0.092	0.230	0.182	0.654	0.085	0.089	0.195	0.147	0.446
E	0.001	0.129	0.116	0.121	0.128	0.142	0.132	0.126	0.137	0.139	0.127	0.137
F	0.001	0.237	0.158	0.536	0.128	0.112	0.473	0.319	0.150	0.445	0.128	0.110
G	0	0.111	0.145	0.154	0.163	0.153	0.155	0.156	0.162	0.181	0.154	0.156
H	0	0.005	0.007	0.013	0.020	0.017	0.024	0.026	0.028	0.032	0.027	0.029

Plate 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	1.053	1.131	0.961	0.818	0.671	0.075	0.074	0.072	0.057	0.052	0.100
B	0	0.332	0.359	0.069	0.070	0.342	0.069	0.075	0.298	0.398	0.066	0.069
C	0	0.064	0.062	0.053	0.057	0.059	0.044	0.092	0.061	0.063	0.069	0.066
D	0.001	0.244	0.445	0.074	0.062	0.115	0.059	0.074	0.191	0.669	0.199	0.153
E	0.001	0.103	0.096	0.098	0.088	0.101	0.076	0.078	0.103	0.085	0.088	0.106
F	9.999	0.167	0.204	0.150	0.171	0.144	0.163	0.075	0.145	0.074	0.095	0.232
G	0	0.123	0.097	0.101	0.101	0.119	0.089	0.100	0.098	0.090	0.091	0.107
H	0	0.005	9.988	9.995	9.992	0.001	9.991	9.990	9.992	9.983	9.995	0.003

Appendix 2.46

ELISA - Titration of R_2 and Serum Samples, Untreated and EDTA-Treated, from Animals with R_2B Using Streptavadin Peroxidase at 1:1000

All volumes 50 µl/well.

1. Coat plates antibody (R_2) 1:100. All wells.

   Incubate 37°C, 2 hr.

   Wash.

2. Row A - Columns 2-6 - D. arnfieldi antigen in horse serum. Initial dilution 1:10. Doubling dilutions →

   Columns 7-11 - Horse serum only. Initial dilution - neat. Doubling dilutions →

   Rows B, D & F - Untreated samples.

   Rows C, E & G - EDTA treated samples.

   Row H - Columns 2, 4, 6, 8 & 10 - Untreated samples.

   Columns 3, 5, 7, 9 & 11 - EDTA treated samples.

   Incubate 37°C, 30 min.

   Wash.

3. Add biotinylated antibody (R_2B) 1:100. All wells.

   Incubate 37°C, 30 min.

   Wash.

4. Add streptavadin peroxidase 1:1000. All wells.

   Incubate 37°C, 30 min.

   Wash.

5. Add substrate O.P.D. All wells.

   Incubate 37°C in dark, 30 min.

6. Stop with acid.

   Read 492 nm.

Appendix 2.46 (Cont'd)

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	1.262	1.187	1.085	1.014	0.847	0.106	0.080	0.064	0.066	0.077	0.108
B	0	0.146	0.146	0.151	0.173	0.478	0.423	0.452	0.442	0.126	0.222	0.221
C	0	0.108	0.111	0.110	0.129	0.088	0.100	0.111	0.093	0.102	0.109	0.104
D	0	0.225	0.089	0.075	0.165	0.211	0.175	0.166	0.443	0.096	0.613	0.134
E	0	0.111	0.104	0.108	0.106	0.100	0.098	0.096	0.097	0.102	0.115	0.114
F	0	0.094	0.107	0.174	0.327	0.082	0.078	0.155	0.116	0.188	0.348	0.136
G	0.001	0.134	0.106	0.117	0.104	0.093	0.124	0.120	0.106	0.118	0.118	0.124
H	0.001	0.413	0.106	0.104	0.133	0.091	0.129	0.090	0.210	0.170	0.134	0.146

Appendix 2.47

ELISA - Titration of R_2 and Serum Samples, Untreated and EDTA-Treated, from Animals with R_2B Using Streptavadin Peroxidase at 1:1000

All volumes 50 µl/well.

1. Coat plates antibody (R_2) 1:100. All wells.

   Incubate 37°C, 2 hr.

   Wash.

2. Row A - Columns 2-6 - D. arnfieldi antigen in horse serum. Initial dilution 1:10. Doubling dilutions →

   Columns 7-11 - Horse serum only. Initial dilution - neat. Doubling dilutions →

   Rows B, D & F - Untreated samples.

   Rows C, E & G - EDTA treated samples.

   Row H - Columns 2, 4, 6, 8, 10 & 12 - Untreated samples.

   Columns 3, 5, 7, 9, 11 - EDTA treated samples.

   Row A - Column 11 - Untreated sample.

   Columns 10 & 12 - EDTA treated samples.

   Incubate 37°C, 30 min.

   Wash.

3. Add biotinylated antibody (R_2B) 1:100. All wells.

   Incubate 37°C, 30 min.

   Wash.

4. Add streptavadin peroxidase 1:1000. All wells.

   Incubate 37°C, 30 min.

   Wash.

5. Add substrate O.P.D. All wells.

   Incubate 37°C in dark, 30 min.

Appendix 2.47 (Cont'd)

6. Stop with acid.

   Read 492 nm.

Appendix 2.47 (Cont'd)

Plate 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0.496	0.546	0.989	0.379	0.463	1.046	0.150	0.084	0.107	0.081	0.114
B	0.001	1.381	1.421	1.374	1.406	1.350	0.696	1.388	0.185	0.150	0.189	0.398
C	0	0.385	1.308	1.354	1.372	0.977	0.452	0.272	0.178	0.161	0.252	0.149
D	0	0.245	0.394	1.024	1.033	0.879	0.467	0.362	0.325	0.550	0.739	0.557
E	0	0.178	0.485	0.450	0.986	0.828	1.112	0.772	0.551	0.106	0.264	0.221
F	0	0.289	0.142	0.499	1.114	0.798	0.716	0.623	0.269	1.240	0.407	0.255
G	0	0.099	0.164	0.360	0.713	0.665	0.710	1.017	1.091	0.364	0.294	0.415
H	0	0.124	0.093	0.264	0.191	0.368	0.544	0.755	1.179	1.013	0.352	0.420

Plate 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.001	0.425	0.276	0.235	0.170	0.051	0.056	0.047	0.056	0.091	0.082	0.096
B	0	0.055	0.132	0.165	0.104	0.057	0.130	0.062	0.071	0.062	0.085	0.093
C	0.003	0.080	0.077	0.118	0.117	0.095	0.102	0.074	0.101	0.091	0.080	0.082
D	0	0.211	0.115	0.302	0.560	0.353	0.148	0.223	1.382	1.372	0.130	1.282
E	0.001	0.271	0.194	0.218	0.183	0.119	0.112	0.124	0.454	0.362	0.107	0.110
F	0.001	1.314	0.306	0.213	0.135	0.223	0.179	0.158	0.126	0.101	1.407	0.090
G	0.001	0.731	0.237	0.205	0.135	0.217	0.122	0.115	0.119	0.106	0.123	0.141
H	0.002	0.136	0.140	0.150	0.156	0.801	0.136	0.153	0.148	0.078	0.203	0.527

Appendix 2.48

ELISA - Titration of R_2 and Serum Samples, Untreated and EDTA-Treated, from Animals with R_2B Using Streptavadin Peroxidase at 1:1000

All volumes 50 µl/well.

1. Coat plates antibody (R_2) 1:100. All wells.

   Incubate 37°C, 2 hr.

   Wash.

2. Row A - Columns 2-6 - D. arnfieldi antigen in horse serum. Initial dilution 1:10. Doubling dilutions →

   Colums 7-11 - Horse serum only. Initial dilution - neat. Doubling dilutions →

   Rows B, D & F - Untreated samples.

   Rows C, E & G - EDTA treated samples.

   Row H - Columns 2, 4, 6, 8, 10 & 12 - Untreated samples.

   Columns 3, 5, 7, 9, 11 - EDTA treated samples.

   Row A - Column 11 - Untreated sample.

   Columns 10 & 12 - EDTA treated samples.

   Incubate 37°C, 30 min.

   Wash.

3. Add biotinylated antibody (R_2B) 1:100. All wells.

   Incubate 37°C, 30 min.

   Wash.

4. Add streptavadin peroxidase 1:1000. All wells.

   Incubate 37°C, 30 min.

   Wash.

Appendix 2.48 (Cont'd)

5. Add substrate O.P.D. All wells.

   Incubate 37°C in dark, 30 min.

6. Stop with acid.

   Read 492 nm.

Appendix 2.48 (Cont'd)

	1	2	3	4	5	6	7	8	9	10	11	12
A	9.999	0.916	0.738	0.731	0.694	0.201	0.209	0.239	0.209	0.326	0.764	0.224
B	0	1.085	0.776	1.158	1.123	1.216	0.561	1.189	1.146	1.230	1.170	0.314
C	0	0.469	0.506	0.507	0.668	0.607	0.583	0.497	0.461	0.370	1.161	0.283
D	0	1.035	1.066	0.946	1.122	1.208	1.047	1.168	1.108	1.133	0.288	0.317
E	0.001	0.541	0.683	0.924	0.662	0.952	0.847	0.651	1.152	0.339	0.661	0.437
F	0	0.394	0.538	0.595	0.385	0.342	1.085	0.395	0.333	0.425	0.682	0.552
G	0	0.210	0.493	0.507	0.391	0.253	0.507	0.268	0.236	0.237	0.306	0.784
H	0.001	0.239	0.208	0.516	0.195	1.250	0.294	1.182	0.801	0.365	0.483	1.220

Appendix 2.49

ELISA - Titration of R_2 and Serum Samples, Untreated and EDTA-Treated, from Animals with R_2B Using Streptavadin Peroxidase at 1:1000

All volumes 50 µl/well.

1. Coat plates antibody (R_2) 1:100. All wells.

   Incubate 37°C, 2 hr.

   Wash.

2. Row A - Columns 2-6 - *D. arnfieldi* in horse serum. Initial dilution 1:10. Doubling dilutions →

   Columns 7-11 - Horse serum only. Initial dilution - neat. Doubling dilutions →

   Rows B, D & F - Untreated samples.

   Rows C, E & G - EDTA treated samples.

   Row H - Columns 2, 4, 6, 8, 10 & 12 - Untreated samples.

   Columns 3, 5, 7, 9, 11 - EDTA treated samples.

   Row A - Column 11 - Untreated sample.

   Columns 10 & 12 - EDTA treated samples.

   Incubate 37°C, 30 min.

   Wash.

3. Add biotinylated antibody (R_2B) 1:100. All wells.

   Incubate 37°C, 30 min.

   Wash.

4. Add streptavadin peroxidase 1:1000. All wells.

   Incubate 37°C, 30 min.

   Wash.

Appendix 2.49 (Cont'd)

5. Add substrate O.P.D. All wells.
   Incubate 37°C in dark, 30 min.
6. Stop with acid.
   Read 492 nm.

Appendix 2.49 (Cont'd)

Plate 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.004	0.921	0.582	0.405	0.358	0.302	0.160	0.124	0.156	0.152	0.167	0.170
B	0.002	0.965	1.090	0.732	0.640	0.916	0.202	0.201	0.203	0.152	0.268	0.191
C	0.002	0.947	1.107	0.430	0.313	0.222	0.212	0.176	0.137	0.159	0.158	0.168
D	0	0.353	0.494	0.365	0.509	0.494	0.182	0.179	0.280	0.199	0.173	0.188
E	0.001	0.154	0.179	0.159	0.196	0.210	0.247	0.232	0.180	0.223	0.229	0.203
F	0.016	0.117	0.225	0.308	0.192	0.198	0.267	0.547	0.323	0.385	0.314	0.198
G	0.003	0.137	0.188	0.146	0.190	0.163	0.170	0.173	1.150	0.271	0.171	0.194
H	0.018	0.110	0.143	0.423	0.151	0.184	0.165	0.161	0.164	0.585	0.177	0.239

Plate 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0.665	0.532	0.428	0.340	0.090	0.100	0.107	0.123	0.183	0.357	0.181
B	0.002	0.264	0.128	0.105	0.182	0.368	0.414	0.150	0.121	0.190	0.489	0.186
C	0	0.110	0.121	0.114	0.112	0.122	0.121	0.134	0.125	0.138	0.143	0.150
D	0	0.106	0.264	0.264	0.367	0.358	0.243	0.145	0.237	0.160	0.146	0.326
E	0	0.126	0.144	0.142	0.138	0.117	0.167	0.126	0.138	0.144	0.169	0.137
F	0	0.370	0.244	0.119	0.235	0.402	0.124	0.114	0.224	0.120	0.356	0.273
G	0.002	0.129	0.171	0.136	0.172	0.124	0.129	0.131	0.142	0.127	0.124	0.139
H	0	0.129	0.143	0.268	0.142	0.188	0.131	0.277	0.136	0.100	0.138	0.101

Appendix 2.50

ELISA - Titration of R_2 and Serum Samples, Untreated and EDTA-Treated, from Animals with R_2B Using Streptavadin Peroxidase at 1:1000

All volumes 50 µl/well.

1. Coat plates antibody (R_2) 1:100. All wells.

   Incubate 37°C, 2 hr.

   Wash.

2. Row A - Columns 2-6 - D. arnfieldi antigen in horse serum. Initial dilution 1:10. Doubling dilutions →

   Columns 7-11 - Horse serum only. Initial dilution - neat. Doubling dilutions →

   Rows B, D & F - Untreated samples.

   Rows C, E & G - EDTA treated samples.

   Row H - Columns 2, 4, 6, 8, 10 - Untreated samples.

   Columns 3, 5, 7, 9, 11 - EDTA treated samples.

   Row A - Column 11 - Untreated sample.

   Column 12 - EDTA treated sample.

   Incubate 37°C, 30 min.

   Wash.

3. Add biotinylated antibody (R_2B) 1:100. All wells.

   Incubate 37°C, 30 min.

   Wash.

4. Add streptavadin peroxidase 1:1000. All wells.

   Incubate 37°C, 30 min.

   Wash.

Appendix 2.50 (Cont'd)

5. Add substrate O.P.D. All wells.
   Incubate 37°C in dark, 30 min.
6. Stop with acid.
   Read 492 nm.

Appendix 2.50 (Cont'd)

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0.732	0.636	0.516	0.414	0.307	0.104	0.094	0.096	0.101	0.269	0.151
B	0	0.090	0.101	0.787	0.139	0.197	0.216	0.228	0.204	0.346	0.730	0.281
C	0	0.086	0.084	0.105	0.095	0.108	0.125	0.130	0.110	0.106	0.113	0.115
D	0	0.171	0.290	0.317	0.127	0.086	0.026	0.287	0.130	0.244	0.280	0.163
E	0	0.083	0.094	0.095	0.092	0.099	0.083	0.081	0.118	0.111	0.099	0.106
F	0.001	0.202	0.177	0.570	0.462	0.148	0.183	0.290	0.791	0.179	0.459	0.105
G	0	0.089	0.117	0.112	0.136	0.112	0.106	0.101	0.129	0.131	0.122	0.112
H	0	0.097	0.093	0.894	0.094	0.137	0.103	0.107	0.129	0.204	0.252	0.028

Appendix 2.51

ELISA - Titration of R_2 and Serum Samples, Untreated and EDTA-Treated, from Animals with R_2B Using Streptavadin Peroxidase at 1:1000

All volumes 50 µl/well.

1. Coat plates antibody (R_2) 1:100. All wells.

   Incubate 37°C, 2 hr.

   Wash.

2. Row A - Columns 2-6 - D. arnfieldi antigen in horse serum. Initial dilution 1:10. Doubling dilutions →

   Columns 7-11 - Horse serum only. Initial dilution - neat. Doubling dilutions →

   Rows B, D & F - Untreated samples.

   Rows C, E & G - EDTA treated samples.

   Row H - Columns 2, 4, 6, 8, 10 & 12 - Untreated samples.

   Columns 3, 5, 7, 9, 11 - EDTA treated samples.

   Row A - Column 11 - Untreated sample.

   Columns 10 & 12 - EDTA treated samples.

   Incubate 37°C, 30 min.

   Wash.

3. Add biotinylated antibody (R_2B) 1:100. All wells.

   Incubate 37°C, 30 min.

   Wash.

4. Add streptavadin peroxidase 1:1000. All wells.

   Incubate 37°C, 30 min.

   Wash.

Appendix 2.51 (Cont'd)

5. Add substrate O.P.D. All wells.
   Incubate 37°C in dark, 30 min.
6. Stop with acid.
   Read 492 nm.

Appendix 2.51 (Cont'd)

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0.003	0.010	0.005	0.007	0.003	0.007	0.011	0.016	0.021	0.029	0.042
B	0	9.997	9.999	0.009	0.007	0	0.036	0.008	0.013	0.025	0.023	0.014
C	9.999	0.011	0.008	0.017	0.017	0.010	0.008	0.016	0.024	0.027	0.027	0.017
D	0	0.006	0.012	0.025	0.032	0.029	0.021	0.022	0.039	0.094	0.026	0.023
E	9.999	0.034	0.038	0.049	0.038	0.028	0.020	0.067	0.090	0.148	0.086	0.044
F	0	0.065	0.130	0.250	0.072	0.182	0.125	0.398	0.493	0.226	0.173	0.316
G	9.999	0.130	0.172	0.153	0.129	0.203	0.128	0.210	0.226	0.169	0.206	0.129
H	0	0.256	0.143	0.225	0.146	0.136	0.046	0.032	0.153	0.376	0.184	0.322

Appendix 2.52

ELISA - Titration of R_2 and D. arnfieldi Antigen in Horse Serum and Marvel with R_2B Using Streptavadin Peroxidase at 1:1000

All volumes 50 µl/well.

1. Coat plates antibody (R_2) 1:100. All wells.

   Incubate 37°C, 2 hr.

   Wash.

2. Row A - Columns 2-6) Horse serum starting columns 2 & 8. Initial
   Columns 8-12) dilution - neat. Doubling dilutions → in 2.5% Marvel.

   Rows B-H - Columns 2-6 - D. arnfieldi antigen starting column 2. Initial dilution 1:2. Doubling dilutions → in 2.5% Marvel.

   Columns 8-12 - D. arnfieldi antigen in horse serum starting column 8. Initial dilution 1:2. Doubling dilutions → in 2.5% Marvel.

   Incubate 37°C, 30 min.

   Wash.

3. Add biotinylated antibody (R_2B) 1:100. All wells.

   Incubate 37°C, 30 min.

   Wash.

4. Add streptavadin peroxidase 1:1000. All wells.

   Incubate 37°C, 30 min.

   Wash.

Appendix 2.52 (Cont'd)

5. Add substrate O.P.D. All wells.
   Incubate 37°C in dark, 30 min.
6. Stop with acid.
   Read 492 nm.

Appendix 2.52 (Cont'd)

Plate 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.001	0.034	9.996	0.002	0.012	0.004	0.005	0.008	0.020	0.017	9.957	0.020
B	0	0.805	0.708	0.633	0.516	0.437	9.953	0.794	0.666	0.552	0.003	9.980
C	0	0.887	0.794	0.701	0.650	0.521	9.957	0.846	0.713	0.626	0.439	0.205
D	9.999	0.865	0.801	0.729	0.655	0.482	9.975	0.871	0.745	0.647	0.435	0.396
E	9.999	0.837	0.820	0.715	0.586	0.444	9.985	0.829	0.685	0.638	0.391	0.384
F	0	0.822	0.734	0.684	0.565	0.424	9.995	0.750	0.521	0.581	0.206	0.440
G	0	0.889	0.694	0.556	0.434	0.287	0.039	0.760	0.493	0.121	0.120	0.479
H	0	0.518	0.379	0.445	0.174	0.059	0.040	0.152	0.450	0.410	0.384	0.499

Appendix 2.53

ELISA - Titration of R_2 and Serum Samples, Untreated and EDTA-Treated, from Donkeys with R_2B Using Streptavadin Peroxidase at 1:1000

All volumes 50 µl/well.

1. Coat plates antibody (R_2) 1:100. All wells.

   Incubate 37°C, 2 hr.

   Wash.

2. Row A - D. arnfieldi antigen. Starting column 2. Initial dilution 1:10

   Doubling dilutions → in 1.25% Marvel.

   Row B - Horse serum. Starting column 2. Initial dilution - neat.

   Doubling dilutions → in 1.25% Marvel.

   Row C - Columns 2-8 - Untreated donkey samples.

   Row D - Columns 2-8 - EDTA treated donkey samples.

   Rows E, F & G - No samples.

   Row H - Horse serum. Starting column 2 - Initial dilution - neat.

   Doubling dilutions → 1.25% Marvel.

   Incubate 37°C, 30 min.

   Wash.

3. Add biotinylated antibody (R_2B) 1:100. All wells.

   Incubate 37°C, 30 min.

   Wash.

4. Add streptavadin peroxidase 1:1000. All wells.

   Incubate 37°C, 30 min.

   Wash.

5. Add substrate O.P.D. All wells.

   Incubate 37°C in dark, 30 min.

Appendix 2.53 (Cont'd)

6. Stop with acid.

   Read 492 nm.

Appendix 2.53 (Cont'd)

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	1.099	1.069	0.925	0.836	0.714	0.545	0.400	0.307	0.224	0.157	0.124
B	0	0.077	0.067	0.065	0.073	0.069	0.072	0.056	0.064	0.079	0.074	0.067
C	0.001	0.050	0.148	0.207	0.097	0.100	0.072	0.080	-	-	-	-
D	0.002	0.080	0.086	0.095	0.123	0.098	0.098	0.088	-	-	-	-
E	-	-	-	-	-	-	-	-	-	-	-	-
F	-	-	-	-	-	-	-	-	-	-	-	-
G	-	-	-	-	-	-	-	-	-	-	-	-
H	0.001	1.208	0.957	0.841	0.643	0.456	0.314	0.238	0.192	0.167	0.079	0.080

Appendix 2.54

ELISA - Titration of R_2 and Serum Samples, Untreated and EDTA-Treated, from Animals with R_2B Using Streptavadin Peroxidase at 1:1000

All volumes 50 µl/well.

1. Coat plates antibody (R_2) 1:100. All wells.

   Incubate 37°C, 2 hr.

   Wash.

2. Row A - Columns 2-5 - D. arnfieldi antigen in horse serum.
   Initial dilution 1:10.
   Doubling dilutions → in 2.5% Marvel.

   Columns 6-9 - Horse serum. Initial dilution - neat.
   Doubling dilutions → in 2.5% Marvel.

   Rows B, D & F - Untreated samples.

   Rows C, E & G - EDTA treated samples.

   Row H - Columns 2, 4, 6, 8, 10 & 12 - Untreated samples.
   Columns 3, 5, 7, 9 & 11 - EDTA treated samples.

   Row A - Column 11 - Untreated sample.
   Columns 10 & 12 - EDTA treated samples.

   Incubate 37°C, 30 min.

   Wash.

3. Add biotinylated antibody (R_2B) 1:100. All wells.

   Incubate 37°C, 30 min.

   Wash.

4. Add streptavadin peroxidase 1:1000. All wells.

   Incubate 37°C, 30 min.

   Wash.

Appendix 2.54 (Cont'd)

5. Add substrate O.P.D. All wells.
   Incubate 37^{o}C in dark, 30 min.

6. Stop with acid.
   Read 492 nm.

Appendix 2.54 (Cont'd)

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.001	0.771	0.729	0.569	0.059	0.067	0.055	0.058	0.060	0.051	0.052	0.075
B	0.002	0.081	0.052	0.063	0.386	0.169	0.082	0.059	0.046	0.518	0.066	0.698
C	0.001	0.044	0.048	0.051	0.046	0.044	0.041	0.043	0.047	0.045	0.040	0.046
D	0.001	0.043	0.056	0.164	0.133	0.044	0.062	0.155	0.102	0.054	0.088	0.071
E	0	0.040	0.049	0.041	0.044	0.043	0.045	0.056	0.054	0.048	0.048	0.042
F	0.001	0.186	0.046	0.093	0.359	0.050	0.117	0.063	0.092	0.048	0.059	0.150
G	0.001	0.055	0.056	0.052	0.057	0.060	0.052	0.058	0.061	0.056	0.061	0.060
H	0.002	0.109	0.062	0.121	0.058	0.054	0.059	0.044	0.055	0.060	0.052	0.055

Appendix 2.55

ELISA - Titration of R_2 and Serum Samples, Untreated and EDTA-Treated, from Animals with R_2B Using Streptavadin Peroxidase at 1:1000

All volumes 50 µl/well.

1. Coat plates antibody (R_2) 1:100. All wells.

   Incubate 37°C, 2 hr.

   Wash.

2. Row A - Columns 2-8 - D. arnfieldi antigen. Initial dilution - neat.
   Doubling dilutions → in 2.5% Marvel.
   Starting column 2.

   Row B - Columns 2-8 - Horse serum. Initial dilution - neat.
   Doubling dilutions → in 2.5% Marvel.
   Starting column 2.

   Rows C, E & G - Columns 2-12)
   Row A - Columns 9-12) Untreated samples

   Rows D, F & H - Columns 2-12)
   Row B - Columns 9-12) EDTA treated samples

   Incubate 37°C, 30 min.

   Wash.

3. Add biotinylated antibody (R_2B) 1:100. All wells.

   Incubate 37°C, 30 min.

   Wash.

4. Add streptavadin peroxidase 1:1000. All wells.

   Incubate 37°C, 30 min.

   Wash.

Appendix 2.55 (Cont'd)

5. Add substrate O.P.D. All wells.
   Incubate 37°C in dark, 30 min.
6. Stop with acid.
   Read 492 nm.

Appendix 2.55 (Cont'd)

	1	2	3	4	5	6	7	8	9	10	11	12
A	9.999	0.751	0.729	0.610	0.523	0.415	0.303	0.257	0.061	0.053	0.057	0.105
B	9.999	0.533	0.442	0.355	0.257	0.187	0.133	0.099	0.018	0.011	0.027	0.082
C	9.999	0.082	0.020	0	0.076	0.053	9.997	9.997	0.011	0.052	0.056	0.024
D	9.999	9.991	9.999	0.005	0.010	0.004	0.016	0.010	0.015	0.013	0.021	0.021
E	0	9.946	0.037	0.012	0.012	9.957	9.966	0.052	0.043	9.968	9.975	9.989
F	0	9.946	9.958	9.968	9.980	9.963	9.963	9.980	9.984	9.972	9.993	9.984
G	9.999	9.951	9.934	9.938	9.945	0.023	9.946	9.951	9.958	9.964	0.002	9.990
H	0	0.002	0.007	0.011	0.014	9.993	0.003	0.014	0.020	0.009	0.031	0.030

Appendix 2.56

ELISA - Titration of *D. arnfieldi* Antigen and R_1 or Serum from an Infected Mare with anti-Rabbit or anti-Horse IgG Peroxidase

All volumes 50 µl/well.

1. Coat plates *D. arnfieldi* antigen - Columns 2-6 and columns 7-11.
   Initial dilution 1:10. Doubling dilutions →
   Incubate 37°C, 2 hr.
   Wash.
2. Columns 2-6 - Add antibody (R_1). Initial dilution 1:10.
   Doubling dilutions → starting row A, in 2.5% Marvel.
   Columsn 7-11 - Add serum from a *D. arnfieldi* infected mare.
   Initial dilution 1:10. Doubling dilutions →
   starting row A, in 2.5% Marvel.
   Incubate 37°C, 30 min.
   Wash.
3. Columns 2-6 - Add anti-rabbit IgG peroxidase 1:500.
   Columns 7-9 - Add anti-horse IgG peroxidase 1:500.
   Columns 10-11 - Add anti-horse IgG peroxidase 1:1000.
   Incubate 37°C, 30 min.
   Wash.
4. Add substrate O.P.D. All wells.
   Incubate 37°C in dark, 30 min.
5. Stop with acid.
   Read 492 nm.

Appendix 2.56 (Cont'd)

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0.956	0.993	0.756	0.608	0.475	0.998	1.002	0.931	0.298	0.222	0.022
B	0	0.939	1.050	0.685	0.545	0.407	0.968	0.995	0.887	0.276	0.206	0.017
C	0	0.936	0.978	0.679	0.505	0.321	0.891	0.948	0.812	0.232	0.225	0.027
D	0	0.863	0.883	0.588	0.349	0.249	0.868	0.862	0.776	0.203	0.168	0.031
E	0.001	0.733	0.750	0.465	0.238	0.155	0.695	0.720	0.617	0.158	0.142	0.044
F	0.001	0.564	0.561	0.332	0.145	0.095	0.525	0.529	0.495	0.113	0.102	0.036
G	0	0.465	0.358	0.201	0.088	0.049	0.342	0.380	0.346	0.090	0.068	0.037
H	0.001	0.293	0.224	0.116	0.051	0.037	0.257	0.261	0.236	0.087	0.061	0.041

Appendix 2.57

ELISA - Titration of *D. arnfieldi* Antigen and Serum Samples, in Triplicate, from Infected Animals with anti-Horse IgG at 1:500

All volumes 50 µl/well.

1. Coat plate *D. arnfieldi* antigen 1:40. All wells.

   Incubate R.T. overnight.

   Wash.

2. Add samples as below. Initial dilution - neat. Doubling dilutions ↓ Starting rows A and E.

   Columns 2-4, Columns 5-7, Columns 8-10, Columns 11-12 — Rows A - D

   One animal per group of three columns

   (except 11 & 12 when only 2 samples)

   Columns 2-4, Columns 5-7, Columns 8-10, Columns 11-12 — Rows E - H

   One animal per group of three columns

   (except 11 & 12 when only 2 samples)

   Incubate 37°C, 30 min.

   Wash.

3. Add anti-horse IgG 1:500. All wells.

   Incubate 37°C, 30 min.

   Wash.

4. Add substrate O.P.D. All wells.

   Incubate 37°C in dark, 30 min.

5. Stop with acid.

   Read 492 nm.

Appendix 2.57 (Cont'd)

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0.423	0.504	0.524	0.413	0.452	0.447	0.503	0.376	0.493	0.249	0.266
B	0	0.558	0.510	0.618	0.480	0.440	0.528	0.526	0.480	0.494	0.207	0.202
C	0.001	0.632	0.608	0.690	0.532	0.545	0.553	0.535	0.553	0.535	0.200	0.195
D	0	0.628	0.637	0.727	0.594	0.564	0.609	0.551	0.514	0.544	0.170	0.167
E	0.001	0.359	0.474	0.434	0.371	0.452	0.437	0.603	0.454	0.564	0.455	0.261
F	0	0.605	0.593	0.570	0.457	0.437	0.440	0.578	0.557	0.578	0.475	0.450
G	0.001	0.648	0.623	0.633	0.448	0.435	0.418	0.581	0.570	0.596	0.435	0.445
H	0.001	0.558	0.586	0.616	0.575	0.353	0.319	0.522	0.500	0.509	0.375	0.356

Appendix 2.58

ELISA - Titration of *D. arnfieldi* Antigen and Serum Samples, in Triplicate, from Infected Animals with anti-Horse IgG at 1:500

All volumes 50 µl/well.

1. Coat plate *D. arnfieldi* antigen 1:40. All wells.

   Incubate R.T. overnight.

   Wash.

2. Add samples as below. Initial dilution 1:10. Doubling dilutions ↓ Starting rows A and E.

   Columns 2-4, Columns 5-7, Columns 8-10, Columns 11-12 — Rows A - D — One animal per group of three columns (except 11 & 12 when only 2 samples)

   Columns 2-4, Columns 5-7, Columns 8-10, Columns 11-12 — Rows E - H — One animal per group of three columns (except 11 & 12 when only 2 samples)

   Incubate 37°C, 30 min.

   Wash.

3. Add anti-horse IgG 1:500. All wells.

   Incubate 37°C, 30 min.

   Wash.

4. Add substrate O.P.D. All wells.

   Incubate 37°C in dark, 30 min.

5. Stop with acid.

   Read 492 nm.

Appendix 2.58 (Cont'd)

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.001	0.854	0.823	0.934	0.757	0.760	0.765	0.688	0.684	0.687	0.317	0.315
B	0	0.877	0.815	0.891	0.738	0.720	0.733	0.699	0.638	0.641	0.257	0.267
C	0	0.823	0.810	0.840	0.644	0.579	0.621	0.643	0.600	0.560	0.187	0.200
D	0.001	0.567	0.415	0.496	0.395	0.413	0.386	0.479	0.302	0.405	0.103	0.135
E	0	0.366	0.354	0.376	0.349	0.390	0.332	0.325	0.245	0.279	0.157	0.202
F	9.999	0.290	0.288	0.310	0.320	0.335	0.282	0.210	0.196	0.217	0.109	0.145
G	0	0.244	0.243	0.255	0.261	0.276	0.234	0.171	0.156	0.175	0.100	0.119
H	0.001	0.261	0.222	0.236	0.225	0.220	0.196	0.126	0.121	0.137	0.084	0.083

Appendix 2.59

ELISA - Titration of *D. arnfieldi* Antigen and Serum Samples, in Triplicate, from Infected Animals with anti-Horse IgG at 1:500

All volumes 50 µl/well.

1. Coat plate *D. arnfieldi* antigen 1:40. All wells.

   Incubate R.T. overnight.

   Wash.

2. Add samples as below. Initial dilution - neat. Doubling dilutions ↓ Starting rows A and E.

   Columns 2-4, Columns 5-7, Columns 8-10, Columns 11-12 — Rows A - D
   One animal per group of three columns
   (except 11 & 12 when only 2 samples)

   Columns 2-4, Columns 5-7, Columns 8-10, Columns 11-12 — Rows E - H
   One animal per group of three columns
   (except 11 & 12 when only 2 samples)

   Incubate 37°C, 30 min.

   Wash.

3. Add anti-horse IgG 1:500. All wells.

   Incubate 37°C, 30 min.

   Wash.

4. Add substrate O.P.D. All wells.

   Incubate 37°C in dark, 30 min.

5. Stop with acid.

   Read 492 nm.

Appendix 2.59 (Cont'd)

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0.421	0.462	0.463	0.346	0.336	0.352	0.391	0.395	0.420	0.154	0.107
B	0	0.406	0.459	0.442	0.356	0.343	0.364	0.373	0.374	0.385	0.167	0.176
C	0	0.367	0.433	0.425	0.361	0.350	0.356	0.365	0.382	0.372	0.170	0.182
D	9.999	0.375	0.399	0.396	0.334	0.337	0.347	0.348	0.347	0.358	0.171	0.183
E	9.999	0.274	0.346	0.336	0.260	0.286	0.325	0.262	0.331	0.388	0.168	0.144
F	0	0.341	0.367	0.323	0.259	0.301	0.312	0.405	0.399	0.443	0.141	0.162
G	0	0.330	0.356	0.329	0.230	0.228	0.276	0.372	0.400	0.439	0.134	0.137
H	0	0.295	0.326	0.317	0.180	0.199	0.227	0.341	0.345	0.449	0.134	0.116

Appendix 2.60

ELISA - Titration of *D. arnfieldi* Antigen and Serum Samples, in Triplicate, from Infected Animals with anti-Horse IgG at 1:500

All volumes 50 µl/well.

1. Coat plate *D. arnfieldi* antigen 1:20. All wells.

   Incubate 37°C, 2 hr.

   Wash.

2. Add samples as below. Initial dilution - neat. Doubling dilutions ↓ Starting rows A and E.

   Columns 2-4, Columns 5-7, Columns 8-10, Columns 11-12 — Rows A - D

   One animal per group of three columns (except 11 & 12 when only 2 samples)

   Columns 2-4, Columns 5-7, Columns 8-10, Columns 11-12 — Rows E - H

   One animal per groups of three columns (except 11 & 12 when only 2 samples)

   Incubate 37°C, 30 min.

   Wash.

3. Add anti-horse IgG 1:500. All wells.

   Incubate 37°C, 30 min.

   Wash.

4. Add substrate O.P.D. All wells.

   Incubate 37°C in dark, 30 min.

5. Stop with acid.

   Read 492 nm.

Appendix 2.60 (Cont'd)

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0.669	0.670	0.645	0.444	0.378	0.388	0.439	0.376	0.419	0.197	0.178
B	0	0.600	0.598	0.556	0.454	0.446	0.433	0.459	0.470	0.439	0.148	0.132
C	0	0.569	0.590	0.511	0.485	0.432	0.438	0.457	0.454	0.465	0.145	0.130
D	0	0.488	0.533	0.609	0.460	0.444	0.422	0.417	0.415	0.412	0.141	0.111
E	0	0.480	0.513	0.499	0.437	0.457	0.416	0.502	0.522	0.550	0.612	0.536
F	0	0.440	0.488	0.439	0.365	0.344	0.355	0.504	0.475	0.505	0.537	0.519
G	0.001	0.368	0.454	0.420	0.361	0.329	0.306	0.437	0.454	0.483	0.562	0.497
H	0.001	0.299	0.405	0.383	0.317	0.273	0.282	0.391	0.408	0.432	0.575	0.509