EPIDEMIOLOGY OF EQUINE HERPESVIRUS INFECTIONS

ZHEN FANG FU

A thesis presented in partial fulfilment (75%)

of the requirement for the degree of

Master of Philosophy in Veterinary

Science at Massey University

January 1985

ABSTRACT

The epidemiology of infections with equine herpesvirus (EHV) types 1 and 2 in foals on a Thoroughbred stud in New Zealand was investigated. As part of this study an ELISA test was developed to measure antibody titres to EHV-2 in equine sera. All the sera collected from the foals before the ingestion of colostrum were negative for antibodies to both EHV-1 and EHV-2. Soon after sucking, these foals had serum antibody levels against these two viruses similar to those of their dams. The maternally derived antibody to EHV-1 lasted for 3-4 months and antibody titres rose again at around weaning time. In contrast, passively acquired antibody to EHV-2 was rapidly supplemented by actively produced antibody.

Serological evidence suggested that most of the foals (85%) became infected with EHV-1, and 25% were reinfected in their first ten months of life; however EHV-1 was not recovered either from these mares or their foals during the investigation period despite the large increase in antibody titres. Serological evidence of EHV-1 infection in foals indicated that this occurred around the time of weaning when the maternally derived antibody had declined to a level which was presumably unprotective. The clinical signs which developed after EHV-1 infection were very mild, the main symptom observed being a profuse nasal discharge usually lasting two or three days, occasionally with an elevation of body temperature. The source of EHV-1 infection in foals could not be determined and there was no evidence to suggest that their dams were infected with EHV-1 around the time when the foals became infected. However, a relationship between preinfection antibody titres ($\log 10^3$) against EHV-1 and the viral infection was observed.

In contrast, EHV-2 was isolated from all of the foals by 2 to 4 months of age. The virus infection persisted in these animals for 2 to 6 months and stimulated continuous production of antibody. As soon as the antibody level against EHV-2 reached a peak, the

isolation of the virus decreased, and eventually EHV-2 was no longer isolated from these foals by 9 months of age. The foals possibly contracted EHV-2 infection from their dams since some of them excreted the virus around the time when EHV-2 was isolated from their foals. Clinical reactions at around the time of EHV-2 infection varied from foal to foal, ranging from subclinical to fever, mucopurulent nasal discharge and swollen submandibular lymph nodes. Two severely affected foals from which EHV-2 was isolated died of complications resulting from secondary bacteraemia. From these findings, an association between EHV-2 and the respiratory disease observed in these foals was postulated. However, the possible role of EHV-2 as a pathogen for young foals needs confirmation by further studies including experimental infection of gnotobiotic foals.

A trail for evaluation of Pneumabort-K (an EHV-1 subtype 1 vaccine) was conducted in these foals. Animals inoculated with the vaccine at the age of 30 and 60 days failed to respond serologically to the immunization, and it was assumed that this was due to the intereference of the high levels of passively acquired antibody. Based on this observation, another EHV-1 vaccination procedure for foals commencing at 80-90 days was recommended.

ACKNOWLEDGEMENT

The work described in this thesis was jointly supported by a China-NZ exchange scholarship, the Department of Veterinary Patholgy and Public Health, Massey University, and Mr. D.Benjamin of Field House, Matamata.

Thanks are due to Professor B.W.Manketelow who kindly made available the facilities of the Department of Veterinary Pathology and Public Health, Massey University for this study.

I would particularly like to thank Dr. R.B.Marshall, my supervisor, for his continuous help and encouragement throughout this project; Dr. D.J.Hampson for his commentary on some of the experiments, and particularly in the preparation of this thesis; Dr. M.R.Alley for his help whenever needed; and Mrs. Linley Denby for her excellent technical assistence.

The friendship and effort of the staff of the Matamata Veterinary Services, involving collection of all the materials, useful discussions and generous hospitality, are greatly appreciated, and special thanks are due to Merss L.G.Dickinson, J.B.Grimmett, and P.D.Jolly.

I am also indeted to Dr. A.J.Robinson, director of MRC Virus Research Unit, for his enthusiasm and useful suggestions; Mr. G.W.Horner, Veterinary Investigation Officer of Ruakura Animal Health Laboratory, Hamilton for advice and kindly suppling EHV-1 and EHV-2 viruses.

Finally, I would also like to thank all the staff in the Department of Veterinary Pathology and Public Health, especially Professor D.K.Blackmore for guidance in Epidemiology, Dr. K.M.Moriaty for advice in Immunology, Dr. G.V.Peterson for help with statistical analysis, Mr. P.N.Wildbore for administrative assistance, and Mr. T.Law for the preparation of the photographs.

TABLE OF CONTENTS

INDEX OF TABLES

INDEX OF FIGURES AND PLATES

Chapter 1

INTRODUCTION

Respiratory disease among young horses in the first year of life appears to be a common problem on thoroughbred studs in New Zealand (Jolly et al., 1985). Preliminary investigations by Ruakura Animal Health Laboratory (RAHL), Hamilton (G.W.Horner, personal communication) and by Massey University (A.J. Robinson, personal communication) of the horse population of New Zealand, and of one stud in particular, have indicated that equine respiratory viruses are frequently involved and may be the initiators of the diseases observed. In these studies equine herpesvirus types 1 (EHV-1) and 2 (EHV-2) in particular have been implicated. Recently, a serological survey was conducted on a series of 71 sera from young horses from throughout New Zealand and with a history of respiratory disease. Seventy percent (46/66) of them were found to be positive to EHV-1, and 99% (66/67) were positive to EHV-2 (Jolly, 1983). Influenza viruses, which are one of the important causes of respiratory disease worldwide, have still not been detected in New Zealand.

EHV-1 infection of horses was first found to be present in New Zealand in 1964 when 31 sera from horses from this country were tested for antibodies against EHV-1 (Matumoto et al., 1965). All but one of these sera tested were positive to EHV-1 by serum neutralisation (SN) and/or complement fixation (CF) tests. Later, Pearce and Alley (1976) conducted a survey of aborted foetuses and foals (from 1972 to 1975) and found neither histological nor virological evidence of EHV-1 infection. Subsequently, Hutton and Durham (1977) reported the first outbreak of EHV-1 induced abortion in New Zealand, which occurred on a South Island stud, and EHV-1 was isolated from the aborted foetuses. In the following season, two more outbreaks occurred, one on an Auckland property and the other on the same South Island property (Jolly, 1983). However, EHV-1 abortion has not been reported since then, while respiratory disease associated with EHV-1 in young horses is still frequently reported

(Horner et al., 1976; Jolly, 1983). In 1976, Horner et al. reported an outbreak of respiratory disease in suckling foals and yearlings on a Thoroughbred stud, and EHV-1 was isolated from nasal swabs. These viruses recovered from aborted feotuses and from respiratory tracts were confirmed to be EHV-1 subtypes 1 and 2 respectively by cross neutralization tests (Horner, 1981) and by restriction endonuclease analysis (Studdert et al., 1984). This is in agreement with the general pattern observed overseas where viruses recovered from aborted foetuses show the pattern of EHV-1 subtype 1 and those from respiratory tracts show the subtype 2 pattern.

The first isolation of EHV-2 in New Zealand was made from the same outbreak of respiratory disease of foals and at the same stud from which EHV-1 was recovered (Horner et al., 1976, Horner, 1981). Subsequently, a number of EHV-2 isolates have been made from both, apparently healthy animals as well as those with respiratory disease. These EHV-2 isolates were identified on the basis of characteristic CPE, slow growth in tissue culture and formation of striking Cowdry type A inclusions (Horner et al., 1976; Horner, 1981), and also by the restriction endonuclease analysis technique (G.P. Allen and G.W. Horner, personal communication). The latter method also revealed that all the isolates were not genetically identical.

The property from which both EHV-1 and EHV-2 had previously been isolated (Horner et al., 1976) was made this subject of the current investigation. When it was first established in the Waikato area, there were only 10-12 mares and no stallions. In the breeding season these mares had to travel considerable distances to be served. In subsequent years after the reported outbreak of respiratory disease, more foals become infected,and typically 2-3 foals died of complications. In the 1980-1981 foaling season, 5 foals died of respiratory disease. Investigations at that time suggested that aspects of management might be contributing, paticularly the travelling which might stress young foals. Infection with EHV-1 was also suspected. In the 1981-1982 foaling season, the first stallion was purchased, and a killed EHV-1 subtype

1 vaccine (Peumabort-K) was recommended for use on this property. The situation in that season was apparently better than in previous years. In the 1982-1983 season, however, three foals died of respiratory disease. In general, foals became ill at around 50 days of age, developing a serous or purulent nasal discharge and swollen lymph nodes. There was an increase in body temperature (39-41 °C) and the foals were depressed. Most of them recovered uneventfully after one or two weeks, but each year 2-3 foals died either soon after primary infection or after a long period of clinical episodes. Post-mortem examination revealed that most had pneumonia and lymphoadenopathy, and a few had purpura haemorrhagica. EHV-2 was isolated from nearly all of these foals before death and/or at post mortem. EHV-1 and equine adenovirus were also recovered from one animal (Horner et al., 1985).

The present study was established to investigate the incidence of EHV-1 and EHV-2 infections and their causative role as agents of respiratory diseases of foals on this stud farm. The main objectives of this project were firstly, to investigate the epidemiolgy of EHV1 and EHV-2 infections of foals in their first ten months of life. Secondly, to evaluate the effect of immunization with the killed EHV-1 vaccine (Pneumabort-K) in young foals. Finally, to attempt to establish an association between infection with equine herpesviruses and respiratory diseases in foals observed previously.

Chapter 2

LITERATURE REVIEW

2.1 Equine Herpesviruses

2.1.1 Introduction

The family Herpesviridae is large and diverse; individual members cause a variety of diseases in many species, but each virus is usually host-specific. Those which cause diseases in the family Equidae have been designated equine herpesvirus type 1 (EHV-1), type 2 (EHV-2) and type 3 (EHV-3) (Plummer and Watson, 1963; Roizman, 1973). EHV-1 causes abortion in mares and respiratory disease, particularly in young horses (Doll and Bryans, 1963a; Campbell and Studdert, 1983), while EHV-3, also known as coital exanthema virus, induces an acute vesicular venereal infection of stallions and mares (Girard et al, 1968; Studdert, 1974). However, there has been no published data indicating a definite association between EHV-2 and any clinical problems (Studdert, 1974; G.P. Allen and G.W. Horner, personal communication). These three viruses are readily distinguished from one another by cross neutralization tests (Plummer et al., 1973; Studdert, 1974) and also by certain unique physicochemical and biological properties (O'Callaghan et al., 1978). This review concerns only literature relating to EHV-1 and EHV-2.

2.1.2 Virion Morphology and Structure

As determined by electron ultramicroscopy of negatively stained virions and by thin sections, equine herpesviruses (EHVs), like herpesviruses in other animals, contain a core of DNA between 30-75 nm in diameter surrounded by a protein coat (nucleocapsid) that exhibits icosahedral symmetry. The nucleocapsid in turn is enclosed

in an envelope which is derived from modified nuclear membrane and appears trilayered and non-rigid. Between the capsid and the envelope is the tegument area containing layers of amorphous material and lacking the morphology of a unit-membrane structure (Plummer and Waterson, 1963; Darlington and James, 1966; O'Callaghan et al., 1978).

The intact virions are approximately 150-198 nm in diameter. The capsid, about 100 nm in diameter, is icosahedral and contains 162 hollow sub-units (capsomers) which are 8 x 12.5 nm in size and project outwards, and are radially displaced and linked to one another by 2 nm long intercapsomeric fibres (Bagust, 1971; O'Callahan et al., 1978).

Viral DNA: The genome of EHV-1 is well characterised. It comprises a linear, double-stranded DNA with a molecular weight (MW) of 9.3 x 10^6 daltons and a sedimentation coeficient of 49-52S (Soehner et al., 1965; O'Callaghan et al., 1978). The density of the DNA in CsCl is 1.716 g/ml and its G + C content is 57 moles% (Soehner et al, 1965; Plummer et al., 1969). O'Callaghan et al. (1978) reported the use of restriction endonuclease analysis to investigate the genomes of strains of EHV-1 grown in vivo (hamster, EHV-1ha) and in vitro (tissue culture, EHV-1te). Eleven to thirteen fragments varying in size from 1 x 10^6 to 25-30 x 10^6 daltons were resolved in gel electrophoretic analysis of EcoR1 digests of the two strains of EHV-1.

The nucleic acid of EHV-2 is also double-stranded DNA, which appears to be non-cross linked, but contains a number of single-stranded nicks as determined by sedimentation in alkaline sucrose (Hsiung et al., 1969; Wharton et al., 1981). It has an average density in CsCl of 1. 716 g/ml, which corresonds to a G + C ratio of 57.7 moles% (Plummer et al., 1969; Wharton et al., 1981). By sedimentation and restriction endonuclease analysis, the latter authors demonstrated that the viral DNA has a MW of about 121-126 x 10^6 daltons and a sedimentation coefficient of approximately 61S, which is in contrast to a MW of 84 x 10^6 daltons and a sedimentation coeffient of 49S reported by Russell and Crawford (1964).

Structural Proteins of Virions: EHV-1 virions are composed of at least 33 polypeptides ranging in MW from 276,000 to 16,000 daltons with 27 of these being located in the envelope or tegument structures (Kemp et al., 1974; Perdue at el., 1974; O'Callaghan and Randall, 1976), of which there are at least 4 glycoproteins, 4 lipoproteins and 9 glycolipoproteins (Perdue et al, 1974). Recently, Turtinen and Allen (1982) demonstrated 11 glycoproteins (MW 260k, 150k, 138k, 90k, 87k, 65k, 62k, 50k, 46k, and 24k) which probably correspond to virion proteins (VP) 1, 2, 9b, 10, 13, 14, 16, 17, 18, 21, 22a, and 25 respectively.

Three distinct species of EHV-1 nucleocapsids have been observed and designated as L (light, P=1.237 g/cm^3), I (intermediate, P=1.244 g/cm^3), and H (heavy, P=1.258 g/cm^3). These nucleocapsids comprise five major structural proteins: nucleocapsid proteins (NC) (NC1=148K, NC2=59K, NC3=46K, NC4=37K, NC5=18K) which correspond to VP 9a, 19, 22, 23, and 26, respectively (Kemp et al., 1974; Perdue et al., 1974; O'Callaghan et al., 1978). Three types of intranuclear nucleocapsids (empty capsids, capsids with an electron lucent core, and mature capsids with electron dense core) have been reported to be present in EHV-2 infected cells (Wharton et al., 1981).

2.1.3 Resistence of EHVs to Inactivation

EHV-1 infectivity is characteristically heat-labile, being readily inactivated at 56°C for 10 minutes. It is unstable at -20°C, but is relatively stable at 4°C and -40°C or below (Doll et al., 1959a; Fouad, 1965; Rasmussen, 1966) and keeps well when lyophilised (Bagust, 1971). Doll et al. (1959a) showed that the addition of 10-20% serum to saline diluent reduced the thermal inactivation rate of EHV-1. The infectivity of this virus suspended in distilled water decreases ten-fold during one hour at 22°C (Fouad, 1965). However, it remains infective on iron, glass and straw surfaces for several days and on wood, paper and manilla rope for 7 to 14 days under field conditions of temperature and humidity (Doll et al., 1959a). Furthermore, these authors reported that contaminated horse hair or oily burlap remains infective for 35 to

42 days.

The virus is rapidly inactivated at <pH 4 or >pH 10 (Doll et al., 1959a; Fouad, 1965), while optimum survival occurs at pH 6.0-6.7. EHV-1 is sensitive to ether, chloroform, sodium deoxycholate and trypsin (Kawakami et al., 1962) as well as 10% formalin, 0.1% methanol, ethanol and heparin (Fouad, 1965).

EHV-2 is sensitive to ether and chloroform (Karpas, 1966; Hsiung et al., 1969; Erasmus, 1970; Harden et al., 1974), and susceptible to growth inhibition by halogenated nucleotides (Karpas, 1966). However, Erasmus (1970) reported that the resistence to trypsin is variable, some isolates being susceptible while others are resistent. The virus also survives trypsinization of kidney cells during tissue culture preparation (Hsiung et al., 1969; Erasmus, 1970) and is inactivated by heating at 56° C for 30 minutes (Studdert, 1974).

2.1.4 Propagation of EHVs

EHV-1 can infect a variety of cells both in vitro and in vivo. However, EHV-2 has only a narrow host range, which is in common with other cytomegaloviruses.

In horses direct intrauterine and intrafoetal inoculation with EHV-1 regularly results in abortion (Doll, 1953). Syrian hamsters are satisfactory laboratory animals (Doll et al., 1953), and have been used for studies of EHV-1 infection (Wilks and Coggins, 1977a), assessment of the immunogenicity of EHV-1 vaccines (Doll et al., 1959b; Papp-vid and Derbyshire, 1978), production of high titres of virus (Bryans and Doll, 1959) and studies of EHV-1 structures (O'Callaghan et al., 1978). The susceptability of other animals such as mice, rats, rabbits, cats, calves and guinea-pigs has been equivocal (Bagust, 1971).

Since Shimizu et al. (1959) successfully grew EHV-1 in equine kidney cell culture, a variety of primary cells as well as cell lines have been adapted for propagation of the virus (Bagust, 1971;

O'Callaghan et al., 1978). Kidney cultures from equine, rabbit, caprine, porcine and bovine species and bovine testis are extensively used for isolation and propagation of EHV-1 (Kawakami et al., 1962; Plummer and Waterson, 1963; Mayr et al., 1965). Of those cell lines tested, KyED and RK-13 are widely employed for production and serological tests for this virus (Burrows and Goodridge, 1973; Thomson, 1978).

On cell monolayers, EHV-1 is characterised by formation of eosinophilic intranuclear inclusion bodies (Cowdry type A) and of giant-cells (Bartha, 1970; Bagust, 1971; Studdert, 1974). The CPE in EHV-1 infected cells develops rapidly and destroys the cell sheet (Jolly, 1983). Cells in the monolayer become rounded, form grape-like clusters, and detach from the culture flask. EHV-1 forms plaques under agar-overlayers but the size of these plaques and the time required for their formation varies between the two subtypes (Studdert and Blackney, 1979; Buxton and Fraser, 1977; Dutta and Myrup, 1983). In single-step growth curve studies, Studdert (1974) observed that absorption of this virus is followed by an eclipse period of 6 hours. Maximum levels of cell-associated virus are attained by 20 to 24 hours, and the maximum levels of extracellular EHV-1 is reached about 2 hours later.

So far, no experimental animal other than the horse has been successfully used for growth of EHV-2 (Plummer and Waterson, 1963; Erasmus, 1970; Blakeslee et al., 1975). In vitro, this virus grows well only on cell cultures (primary, secondary or cell lines) of equine and rabbit origin (Wharton et al., 1981). It also replicates in lamb and cat kidney cultures (Erasmus, 1970), but its growth on other monolayers such as Vero, foetal bovine endothelial cells, PS, PRK, human and hamster kidney cultures is limited and varies according to the strain of EHV-2 used (Hsiung et al., 1969; Harden et al., 1974; Horner et al., 1976). Viral pathology in permissive equine and rabbit cells is characterised by spreading foci of cytomegalic cells which exhibit enlarged nuclei containing striking Cowdry type A inclusions surrounded by a clear halo (Erasmus, 1970; Kemeny and Pearson, 1970; Wharton et al., 1981). In addition, marked syncytia formation and vacuolization are seen in infected RK

cells (Wharton et al., 1981). In agar-overlayers, EHV-2 also produces plaques of varible size (1-2 mm), usually smaller than those formed by EHV-1 (Kemney and Pearson, 1970; Harden et al., 1974).

In cell culture, EHV-2 has an eclipse period of 18-48 hours, maximum titres being reached at 96-120 hours after infection. Approximately 40-50% of total infectious EHV-2 remains cell associated in cell cultures (Studdert, 1974; Wharton et al., 1981). These features of EHV-2 are reflected in a delayed CPE. Thus, on primary isolation, 10-14 days are needed before initial foci of rounded cells are seen, so that blind passage is often required before such foci are detected (Studdert, 1974). On subsequent passages the incubation period becomes shorter and may vary between 2 and 7 days (Roeder and Scott, 1975).

2.2 Equine Herpesvirus Type 1

2.2.1 Clinical Manifestations and Pathology of EHV-1 Infection

EHV-1 is the most important of the EHVs, and four syndromes associated with this virus infection have been recognized, namely respiratory disease, abortion, perinatal mortality and paralysis.

Respiratory disease

EHV-1 is a major cause of upper respiratory tract disease in horses worldwide and affects young horses under 2 years of age especially severely (Bagust, 1971; Studdert, 1974; Bryans, 1980a). In the USA, foals usually become infected in the autumn and winter months at weaning (Doll, 1961; Doll and Bryans, 1963a) at the time when maternally derived antibody has declined to undetectable levels. Horses become infected with EHV-1 by inhalation and ingestion (Shimizu et al., 1961; Prickett, 1970). The virus replicates in the nasal mucosa and results in rhinitis and other respiratory distress (Shimizu et al., 1961). Characteristic clinical signs are fever (39-41.5 ^{o}C) lasting 2-5 days, transient anorexia, inappetance, general malaise and a profuse, serous, or purulent nasal discharge accompanied by visable congestion of the nasal and conjunctival mucosa and enlargement of the submandibular lymph nodes. Leucopaenia occurs in parallel with fever, and both lymphocyte and neutrophil numbers are reduced during the first two days, although this is followed by a leucocytosis on recovery (Maurer and Jones, 1943; Bagust, 1971; Thomson, 1978). In some cases, there is a short period of coughing and oedematous swellings of the subcutis and tendon sheaths of the legs (Bagust, 1971; Doll, 1972; Powell, 1975). Recovery is usually seen within one to two weeks in uncomplicated cases.

The pathological findings are mainly confined to the respiratory system, there being oedema and bronchopneumonia, especially in the apical lobes (Doll and Bryans, 1963a, Prickett, 1970; Studdert, 1974; Powell, 1975). Purulent or serofibrinous exudates may be present in the bronchi and trachea. A marked

congestion and swelling of the nasal mucosa and atelectasis in small areas of the lung have also been seen (Shimizu et al., 1961). Histologically, neutrophilic infiltrations are frequently seen in the bronchi and peribronchioli, and perivascular infiltrations of round cells are present in the local alveoli. Cowdry type A inclusions have been observed in the affected epithelium (Prickett, 1970). The latter author also described extensive necrosis of epithelial cells in the upper respiratory tract, and necrosis and inclusions in the germinal centers of the bronchial lymph nodes.

EHV-1 infection in old or reinfected horses may be symptomless apart from a transient elevation of body temperature. This is probably due to protection by antibody produced against a previous challenge (Doll et al., 1954; Burrows and Goodridge, 1972). Nevertheless a mild illness and reduced performance in Thoroughbreds in training has been associated with reinfection (Powell et al., 1974; Thomson, 1978) and this was described as the "poor performance" syndrome by Mumford and Rossdale (1980). Doll and Bryans (1963a) have demonstrated that horses can become reinfected at intervals of 3 to 6 months, particularly in their first two years of life (Thomson, 1978).

Respiratory disease initiated by EHV-1 infection can be exacerbated by secondary bacterial invasion, the most damaging organisms being group C Streptococci (Studdert, 1971; 1974). In addition, Escherchia coli, Shigella equuli, Pseudomonas spp and Bordetella bronchiseptica are sometimes involved (Doll, 1972; Thomson, 1978).

The role of bacterial invasion in the pathogenesis of EHV-1 associated respiratory disease is unclear since these organisms might not merely be secondary invaders but play a part in the production of classical rhinopneumonitis (Doll, 1972; Studdert, 1974). In experiments using gnotobiotic foals, Thomson (1978) observed that most clinical disease in the foals occurred when EHV-1, EHV-2 and β-haemolytic Streptoccoci were active together and that the majority of EHV-1 infections alone appeared non-pathogenic.

Abortion

"Epizootic abortion in mares" was first described in the 1930's by Dimock and Edwards (1933) and EHV-1 was identified as the cause of this syndrome after isolation of a herpes virus from aborted foetuses (Shimizu et al., 1959). Abortion is now generally believed to be a sequela to respiratory infection with the subtype-1 (foetal strains) of EHV-1.

Abortion is generally recorded 1 to 4 months after infection, taking place between the 4th and 11th months of pregnancy, with the majority (95%) of cases seen from the 7th month after conception onwards (Doll, 1953; Doll and Bryans, 1962a; Bryans, 1980a). Experimentally, Doll (1953) was able to induce abortion 3-9 days after in utero inoculation of foetuses with EHV-1. In addition, Turner et al. (1970) reported that abortion was induced 72 to 84 hours after infection with a EHV-1 virus (EH39) by the intrauterine route.

Abortion occurs suddenly without prior indication or premonitory signs. The placenta is usually expelled with the foetus or soon after, and mares have a high conception rate 30 days after abortion (McGee, 1970). EHV-1 can be recovered readily from the organs of the aborted foetus.

Vulvovaginitis associated with EHV-1 has also been reported (Studdert, 1974), but is infrequent and rather milder than that caused by EHV-3, and the perineal sheath is not involved (Studdert, 1974; Campbell and Studdert, 1983).

The pathogenesis of abortion caused by EHV-1 has not been fully elucidated. Whether or not abortion occurs in infected mares depends both on the nasopharyngeal resistence to viral infection and on the virulence of the infecting strain of virus (Bryans, 1969; Burrows and Goodridge, 1972). When local immunity is insufficient (neutrilization titre <1:100), the virus will grow rapidly in the nasopharyngeal mucosa and is picked up by leucocytes. This cell-associated viremia transports the virus from the respiratory

tract to the target organ, the placenta. By being located intracellularly, the virus is protected from neutralizing antibody in the blood stream. The virus is then released from leucocytes, crosses the placenta by some unknown mechanism and infects the foetus to cause abortion (Bryans, 1969; Bryans and Prickett, 1970; Gleeson and Coggins, 1980).

The pathological changes in aborted foetuses have been extensively recorded (Dimock and Edwards, 1936; Doll, 1953; Doll and Kintner, 1954; Jeleff, 1957) and were summarized by both Prickett (1970) and Studdert (1974). The lesions in aborted foetuses vary with their age; foetuses aborted before 6 months of gestation are usually autolyzed with widespread cell necrosis and inclusions in many tissues, but most commonly in the liver and lung. In foetuses aborted after 6 months of pregnancy, the following classical lesions are observed: jaundice, petechiation of the visible mucosa, subcutaneous oedema, excessive pleural fluid, pulmonary oedema, splenic enlargement with prominent lymph follicles and white, cream-coloured foci of necrosis in the liver. The differences in the pathological changes between these two age groups are believed to be due to the ability of the foetuses to produce inflammatory and immune responses (Studdert, 1974).

Microscopically, the lesions are of bronchiolitis, pneumonitis, severe necrosis of the splenic white pulp without cellular infiltration, and focal areas of necrosis in the liver. Typical herpetic inclusions are invariably seen, especially in and around the necrotic areas.

Lesions in aborted mares are only observed in the reproductive tract, including an intense perivascular infiltration of lymphocytes and plasma cells in the sub-endometric blood vessels, a fine intravillar necrosis with complete separation of the allantochorion and uterine endometrium, and marked distension of the regional lymph vessels (Prickett, 1970; Bryans and Prickett, 1970).

Perinatal Mortality

This syndrome has received little attention, and is generaly included with the description of EHV-1 abortion (McGee, 1970;). Recently perinatal death associated with EHV-1 infection has been reported in the USA (Bryans et al. ,1977) and Australia (Dixon et al., 1978; Hartley and Dixon, 1979). In the report of Bryans et al. eight of nine foals died between 9 and 14 days after birth, EHV-1 being isolated from four of them, although bacterial infection was also involved. Foals developed respiratory distress, watery, non-fetid diarrhoea and weakness prior to death. The authors proposed that this condition was due to infection with EHV-1 during the terminal phase of gestation.

The clinical signs observed in the outbreak of perinatal death associated with EHV-1 infection in Australia (Hartley and Dixon, 1979) were somewhat different. Almost all the losses occurred in the foals at full term, but without any abortions at the time. Some of these foals were born dead, some alive but weak and dying within 24 hours, and others apparently healthy when born but becoming ill and dying within 3 days. Clinical signs of respiratory distress and systemic illness were observed in all the foals prior to death, but were more severe in the foals that survived 1-3 days. Severe dyspnoea with marked cyanosis of the mucosal membranes were seen in the latter foals (Dixon et al., 1978).

In the foals which died within 3 days of birth, there were no gross lesions other than oedematous and atelectic lungs which became plum purple in colour. Histologically acute focal necrotising bronchiolitis, pneumonia, oedema and congestion of the lung were seen with the presence of intranuclear inclusions in bronchiolar epithelium. In many of the foals that survived longer than 6 hours there was hyaline membrane formation in the lungs and a tendency for greater lymphoid depletion and degeneration in the thymus and spleen (Hartley and Dixon, 1979).

In contrast, Bryans et al. (1977) reported that neonatal EHV-1 infection causes interstitial pneumonia with extensive depletion and/or degeneration of lymphocytes in spleen and thymus, this paving the way for lethal secondary bacterial infection.

Paresis and Paralysis

Saxegaard (1966) firstly reported the isolation of EHV-1 from the spinal cord and brain of a stallion with severe lumbar paralysis in Norway. Subsequently, neurological disease associated with this virus resulting in paresis was described in naturally occurring infections (Roberts, 1965; Platt et al., 1980; Thein, 1981;), was induced experimentally (Jackson and Kendrick, 1971; Patel et al., 1982), and was observed in vaccinated horses (Thomson, G. W. et al., 1979) with or without the recovery of EHV-1. Paresis and paralysis occurs mainly in mares but has also been recorded in stallions, and in foals (Saxegaard, 1966; Greenwood and Simson, 1980)

The incubation period is approximately 7 days (Jackson and Kenderic,1971; Greenwood and Simson, 1980) and clinical signs range from severe paralysis and recumbancy through to a mild ataxia, especially with posterior incoordination, urinary incontinence and diarrhoea (Jackson et al., 1977; Platt et al., 1980). Greenwood and Simson (1980) observed that the clinical picture in foals was less severe than in adults. Affected horses may die in a few days, require euthanasia, or recover in a few weeks.

The pathogenesis of myeloencephalitis caused by EHV-1 is more complicated than that of the other EHV-1 associated sydromes. Whether it is due to direct EHV-1 infection of neurons or by immune reaction is still controversial. The main pathological findings are a severe and widespread necrotising vasculitis in the brain, spinal cord, and in the sheaths of nerves, capsules of ganglia, meninges and occasionally elsewhere in the body. Focal malacia in both grey and white matter of the brain and spinal cord occurs with minimal involvement of neurons, and neither neuronophagia nor inclusion bodies are seen (Jackson and Kenderic, 1971; Little and Thorsen, 1976; Jackson et al., 1977; Thomson G, W. et al., 1979). Jackson et al. (1977) proposed that during a cell-associated viraemia, in association with high levels of circulating antibody, virus spreads directly from the circulating infected cells to endothelial cells,

then to contiguous endothelial cells without an extracellular phase. Once the endothelial infection is initiated virus spreads centrifugally to infect adjacent parenchymal cells. These authors also assumed that malacic foci develop secondary to thrombosis of local vessels, and paralysis results from ischaemia and metabolic changes in the nervous tissue. In contrast, Platt et al. (1980) suggested that the vascular lesions were due to an EHV-1 specific immunological reaction in the vascular endothelium, since high antibody titres to EHV-1 in horses with myeloencephalitis have been reported in the natural and experimental condition (Saxegaard, 1966; Jackson and Kenderic, 1971; Thomson G. W. et al., 1979). This is in agre ement with the view of Dinter and Klingeborn (1976) that the nervous disease occurs because of reinfection or possible recurrent infection of antibody positive horses.

The virus has been recovered infrequently from the CNS of myeloencephalitic horses (Saxegaard, 1966; Little and Thorsen, 1976; Thein et al., 1981). Very recently, Patel et al. (1982) recovered the virus readily from the CNS of foals experimentally inoculated with an isolate originally recovered from paralytic horses. Furthermore, Patel and Edington (1983) detected the virus in neurons throughout the brain of mice inoculated intracerebrally with EHV-1 subtype 1 or isolates from equine paresis. These findings indicate that certain strains of EHV-1 can infect the CNS and causes paresis or the paralysis syndrome, which is supported by the observation that herpesviruses in other species, such as pseudorabies virus in pigs, bovine herpesvirus type 1 in cattle and herpes simplex virus in man are neurotropic and are capable of multiplying in neurons causing neuronophagia (Platt et al., 1980).

2.2..2 Epidemiology of EHV-1 Infection

Although EHV-1 infection occurs all over the world (Matumoto et al., 1965), the epidemiology may vary somawhat between localities. Outbreaks of abortion due to EHV-1 are relatively common in Northern America and some European countries, but are reported rarely in Australia (Sabine et al., 1983), England (Burrows and Goodridge, 1979), and New Zealand (Hutton and Durham, 1977), while respiratory

disease occurs frequently in young horses in all these countries.

EHV-1 is transmitted horizontally as well as vertically, foetuses being infected through the placenta, and foals and horses being infected by direct or indirect contact (Prickett, 1970). Thomson (1978) pointed out that aerosal transmission is probably important in explosive outbreaks of this disease.

Recently, the restriction endonuclease technique has been applied to diffentiate EHV-1 isolates and the results showed two distict patterns of fingerprints, although there were some minor differences by a loss or gain of restriction enzyme cleavage sites within each subtype (Sabine et al., 1981; Studdert et al., 1981; Turtinen et al., 1981; Allen et al., 1983). These findings are in agreement with the early serological results that there are two subtypes of EHV-1 (Bartha, 1970; Burrows and Goodridge,1973; Thomson, 1978). Subtype 1 viruses (prototype KyD) are usually isolated from aborted foetuses, while subtype 2 viruses (prototype H-45) are recovered from respiratory disease. EHV-1 recovered from neonatal deaths and paralyses also showed the patterns of subtype 1 virus (Studdert, 1983). Based on these findings and also on the results reported by Allen and Turtinen (1982) that only 20% homology was demonstrated between the two EHV-1 subtype genomes by DNA-DNA reassociation, Studdert et al.(1981, 1984) suggested that the two subtypes of EHV-1 should be designated as EHV-1 and EHV-4, respectively.

Although the data accumulated in Central Kentucky over 50 years indicated that the annual incidence of abortion due to EHV-1 was only 1% on average (Bryans, 1980a), this was drawn from the entire population, and the abortion rate in unprotected herds can reach 20% (Doll and Bryans, 1963a). These authors (1963a,c)also suggested that the source of virus infecting pregnant mares was from young horses suffering from respiratory disease, since the aborting mares had been in contact with foals showing respiratory signs attributed to EHV-1. Abortion also induces a more durable immune response to EHV-1 infection than does respiratory disease (Doll, 1961; Doll and Bryans, 1963a). Outbreaks of abortion tend to occur at intervals of

about 3 years (Doll and Bryans, 1963b), and this might be in part due to the waning of immunity in mares to levels where virus entering the respiratory tract can become viraemic and cross the placenta.

Campbell and Studdert (1983) suggested that the aborted foetuses, membranes and fluids may be an important source of infection for other mares. This is supported by the fact that epizootic abortion may occur in a group of mares following an EHV-1 associated abortion of a recently purchased pregnant mare (Kawakami et al., 1970; Burrows and Goodridge, 1979; Sabine et al., 1983).

In contrast to abortions, rhinopneumonitis often occur s every autumn and winter among groups of "immunologically naive" weaners brought together in confined quarters (Doll and Bryans, 1963a, Thomson, 1978). At this time maternally derived antibody declines to an undetectable level (Thomson, 1978). Bryans (1980a) suggested that stress associated with transportation, crowded situations and poor management may play an important part in the occurrence of this disease. In areas where EHV-1 is endemic, virtually all the foals become infected during their first year of life or soon after (Burrows and Goodridge, 1979; Bryans, 1980a). In such situations respiratory disease occurs less frequently in very young foals, although Thomson (1978) has reported that one foal which had no detectable antibody contracted infection as early as 2 weeks of age.

Kaji (1963) demonstrated that both the number with positive titres and the level of antibody titres against EHV-1 in a population increased with age. The prevelence of infection rises rapidly during the first year of life, increases slowly for 3-4 years and finally reaches a state of equilibrium at about 8 years of age. Thomson (1978) also observed this tendency and showed that there was a progressive increase with age in neutralizing antibody against several atrains of EHV-1 in young horses, although the virus was only recovered in a few horses which showed clinical signs. This might have been due to reinfection since immunity following respiratory infection with EHV-1 is short-lived, lasting only 3-4 months (Doll, 1961; Doll and Bryans, 1963b). Hence, subsequent

respiratory infections usually cause only mild disease or are subclinical (Doll, 1972; Burrows and Goodridge, 1973; Thomson, 1978).

Doll and Bryans (1963a) suggested that the source of virus infecting young horses is either from a natural reservoir or from adults with persistent infection (carriers). It was also suggested that young horses are most probably infected by reactivated virus shed by older horses, especially their dams. Young foals with acute respiratory disease may shed virus for a period of time, which presumbly increases the dose of infective virus among a group, and thus the rate and incidence of infection. This in turn provides an opportunity for the infection of mares at the appropriate stage of pregnancy, and allows abortion to occur (Doll and Bryans, 1963a; Campbell and Studdert, 1983).

It has also been suggested that there may be a latent state in EHV-1 infection since most abortions occur 1-4 months after infection of the mares (Doll, 1952 ; Doll and Bryans, 1963a). EHV-1 infection has been reactivated by stress factors associated with the administration of Africa Horse Sickness vaccine (Erasmus, 1968). Additional evidence for the reactivation of latent EHV-1 infection has also been obtained from a pony herd kept in isolation in Britain (Burrows and Goodridge, 1979, 1984). Virus has been recovered from ponies shortly after being separated from a foal, being housed, castrated and during the terminal stages of Grass Sickness. However, attempts to demonstrate virus reactivation following corticosteroid treatment or to recove latent virus from trigeminal ganglia were unsuccessful.

Syndromes other than respiratory infection and abortion complicated the epidemiolgy of EHV-1 (Campbell and Studdert, 1983). As already mentioned, Hartley and Dixon (1979) reported an outbreak of neonatal death associated with EHV-1 in the absence of abortion. EHV-1 was recovered from 22 out of 27 foals. Futhermore, a number of neurological infections have been reported with or without recovery of the virus from the CNS (Saxegaard, 1966; Jackson and Kenderic, 1971; Little and Thorson, 1976; Dinter and Klingeborn,

1976; Platt et al., 1980, Greenwood and Simson, 1980; Thein et al., 1981). Initially it appeared that pregnancy and abortion was a pre-requisite for neurological disease, but recently Greenwood and Simson (1980) demonstrated that the disease also occurred in barren mares, stallions, geldings and foals.

2.2.3 Immunity to EHV-1 Infection

A variety of in vitro and in vivo tests have been developed for the detection of humoral and cell mediated immunity (CMI) induced by EHV-1 infections. Those serological tests used for the determination of humoral immunity include complement fixation (CF) (Randall et al., 1950), serum neutralization (SN) (McCollum et al., 1962; Burrows, 1968), haemagglutination (HA) (Semerdjiew, 1962), immunofluorescence antibody (IF) (Ishizaki et al., 1962; Thomson et al., 1976) and ELISA (Jolly, 1983; Dutta et al., 1983); while delayed hypersensitivity (DH), lymphocyte stimulation (LS) (Wilks and Coggins, 1976; Thomson and Mumford, 1977), cytotoxicity (Wilks and Coggins, 1977b) and leucocyte migration inhibition (LMI) tests (Frymus, 1980) have been adopted for the measurement of CMI.

Humoral immunity: Maternal antibody will not cross the equine placenta so that in foals, passsive immunity is derived only from colostrum. Antibody titres in the foals after ingestion of colostrum are similar to those in their dams, and persist for 3-4 months before declining to an undetectable level around weaning time (Thomson, 1978; Kendrick and Stevenson, 1979)

After experimental infection of horses with EHV-1, CF antibodies have been shown to appear 2 weeks later, reach a peak after another week and then diminish markedly from 5 weeks to disappear after 10 weeks (Shimizu et al., 1961; Thomson et al., 1976). Doll and Bryans (1962) noted that CF antibody appeared earlier and persisted longer (3-6 months) in mares than in foals (1-4 months), and suggested this might have resulted from an anamnestic response. The development of SN and IF antibody against EHV-1 is similar to that of CF antibody, but SN and IF reach a peak one week earlier than CF antibody, and persist at a moderate level

up to one year or longer (Shimizu et al., 1961; Doll and Bryans, 1962b; Thomson et al., 1976). Following reinfection, these antibodies increase in the first week and the SN and IF titres become at least 4-fold greater than after the primary response, although CF titres are almost the same as the primary response and then declined quickly to become undetectable 40 weeks after the first inoculation. At this time SN and IF antibody titres are still similar to those occurring in response to the primary infection (Thomson et al., 1976). In contrast to this, Doll and Bryans (1962) reported that horses showed a typical anamnestic reaction in CF antibody following reinfection.

The development, persistence and decline of CF antibody after natural infection have been reported to be similar to those observed after experimental inoculation (Bagust and Pascoe, 1970).

Cell Mediated Immunity (CMI): Dutta et al. (1980) showed that lymphocyte transformation in the presence of EHV-1 appeared as early as 2 days after foals were inoculated, and reached a peak in 7-10 days before subsequent declining. Thomson and Mumford (1977) reported that virus inactivated by heat and UV light induced a considerable response in sensitized lymphocytes, while live virus did so only poorly. Furthermore, in vivo the degree of lymphocyte transformation stimulated by inactivated EHV-1 with Freund's complete adjuvant (FAC) were higher than those following infection with a virulent strain (Thomson, 1978).

Protection: Humoral immunity and CMI both play a role in resistance to and recovery from EHV-1 infection. Wilks and Coggins (1976) demonstrated that a combination of immune spleen cells and antiserum from EHV-1-infected animals conferred the highest protection in hamsters against EHV-1 infection. Furthermore, both serum and peripheral blood from experimentally infected horses are cytotoxic to EHV-1 infected cells (Wilks and Coggins, 1977b).

Humoral immunity is certainly capable of protecting horses against EHV-1 infection. Newborn foals that have ingested maternal antibody are protected from EHV-1 infection up to weaning (Doll and

Bryans, 1963b; Thomson, 1978). Bryans (1969), Burrows and Goodridge (1973) and Neely and Hawkins (1978) were all able to correlate resistance to EHV-1 infection (abortion) in experimentally infected horses with the threshold of antibody levels at 10^{2}, $10^{1.4}$ and $10^{1.9}$, respectively. Moreover, horses that experienced repeated infection, stimulating greater persistence of CF antibody, have been shown to be more resistant to infection (Doll and Bryans, 1963b; Studdert, 1974).

In general, the antibody response to EHV-1 is poor in the first year of life (Dutta and Shipley, 1975; Gerber et al., 1977), but the incidence of seroconversion is greater than the incidence of clinical disease (Studdert, 1974, Thomson, 1978). This suggests that other protective machanisms besides circulating antibody operate in horses against EHV-1 infection. Gerber et al. (1977) reported that foals less than one year of age showed a greater increase in CMI response to EHV-1 than did horses 1-2 years old. Frymus (1980) also demonstrated a large increase in the CMI response following administration of vaccine to foals at 3 weeks and 3 months of age. It is possible that those foals that succumb to infection in the first year of life may be individuals which do not mount a sufficient CMI response (Campbell and Studdert, 1983). Further evidence for the protective role of CMI comes from Pachciarz and Bryans (1978) who reported that vaccination induced a great lymphocyte transformation response in pregnant mares, and this was associated with protection against abortion; those mares that aborted EHV-1 infected foals all had a lower lymphocyte stimulation index. This may explain why some mares with high levels of circulating antibodies abort EHV-1 infected foetuses (Doll and Bryans, 1963a; Bryans, 1969).

The interaction of humoral and CMI responses to EHV-1 is not fully understood. No correlation has been found between the status of CMI and antibody levels (Gerber et al., 1977; Frymus, 1980; Thein et al., 1981). Frymus (1980), using the leucocyte migration inhibition test, demonstrated that foals at the age of both 3 weeks and 3 months developed a CMI response after first and second vaccinations, while the SN response was poor. Gerber et al. (1977)

also reported that 6-8 month old foals showed no or only a slight increase in SN antibody following vaccination and revaccination with a modified live EHV-1 vaccine, but demonstrated a marked increase in CMI response to EHV-1 as measured by lymphocyte transformation. In contrast, horses aged 12-18 months showed a great increase in SN antibody while their CMI was low or absent, although it did increase after revaccination. Thomson and Mumford (1977) noted that animals vaccinated previously showed an increase in SN antibody but not in secondary lymphocyte response after challenge.

It has not been determined whether or not the lower CMI reponse in old horses is due to high circulating antibody levels. However, after repeated infection, horses that show a low CMI response to EHV-1 do have high SN antibody titres (Gerber et al., 1977; Frymus, 1980). Moreover, Wilks and Coggins (1977b) noted that in vitro the decrease in lymphocyte response following the peak reaction was coincidental with a rise in SN antibody, and washed lymphocytes from infected ponies showed a higher response than those in autologous serum. However, Pachciarz and Bryans (1978) reported that the decrease in cellular response to EHV-1 did not correlate with increases in either SN antibody or CF titres.

2.2.4 Immunization against EHV-1 Infection

Both inactivated and attenuated live vaccines have been developed for immunization against EHV-1 infection. These have been summarised and listed by Campbell and Studdert (1983). Recently, a sub-unit vaccine was prepared by Papp-vid and Derbyshire (1978).

In the USA, a formalin inactivated vaccine developed from infected equine foetuses (Doll et al., 1952) and from infected hamster liver (Doll et al., 1959b) was either of poor immunogenicity (Doll et al., 1959b; Doll and Bryans, 1963b, c) or caused side effects including anaphylactic reactions and isoimmune erythrolysis (Doll et al., 1952; Doll and Bryans, 1963b, c). Such vaccines were replaced by a live hamster-adapted EHV-1 vaccine instilled intranasally (Doll, 1961). The live virus was used in a planned

infection program (Doll, 1961, Doll and Bryans, 1963c) and only applied to horses bred seasonally and to those in endemic areas. This program was initiated in July after foaling was completed and the mares were revaccinated in October as a "booster" dose prior to the last trimester of gestation. All horses on a stud were inoculated twice a year regardless of age, sex and breeding status. Once this program was started, it was recommended that it be continued without interruption. The premises were recommended to be quarantined for three weeks following vaccination, since the virus can be transmitted by contact and cause abortion or respiratory disease in susceptible horses. Doll and Bryans (1963c) reported that, after a 4 year period of field trials, the abortion rate was only 0.88% among 6,680 mares on 65 studs compared to 13-14% on affected non-participating farms during the same time, and to 15% abortions on the affected farms before the live vaccine was used. Six years after this somewhat virulent virus vaccine became commercially available, Peacock (1969) considered that the limited use of this .ccine in a planned infection program resulted in reasonabally effective control of horse abortions due to EHV-1.

Nevertheless, it was felt that this live vaccine might regain virulence and cause outbreaks of either abortion or respiratory disease. Bryans (1978) developed a chemically inactivated EHV-1 vaccine with adjuvant "F-3" which produced a SN antibody response in horses and appeared to induce serviceable immunity against challenge with an EHV-1 foetal strain in pregnant mares. The vaccine, administered at intervals of approximately 60 days, induced a level of antibody which lasted for more than 21 weeks in pregnant mares. After challenge, none of the vaccinated mares aborted compared to six of ten control mares which aborted EHV-1 infected foals. Later, the vaccine preparation became commercially available (Pneumobort-K) (Moore and Koonse, 1979) and its safety and efficacy was tested in subsequent field trials in Central Kentucky, France and Ireland (Moore and Koonse, 1979; Bryans, 1980b). These trials established that after vaccination 90% of pregnant mares maintained antibody titres at levels presumbly protective against abortifacient infection (>1:80) over a 5 1/2 to 6 month period of pregnancy. In one of these trials, Moore and Koonse (1979) reported that two of

the eleven vaccinated mares aborted, compared to 3 abortions and 2 cases of paresis in six unvaccinated controls after challenge. Bryans (1980b) noted that on average the abortion rate was 1.6/1000 in vaccinated compared to 12.5/1000 in unvaccinated mares during 1977-1980. It was recommended that pregnant mares should receive doses of vaccine in the 5th, 7th and 9th months of gestation; young horses should be given two injections 3 or 4 weeks apart starting at the age of 3-4 months and a third injection six months later. Race horses should have further injections at intervals of approximately 6 months throughout their first three years.

This vaccine was also licensed in the United Kingdom and trails to evaluate this vaccine were conducted by challenging vaccinated ponies either with subtype 1 virus (Burrows et al., 1984) or with subtype 2 virus (Mumford and Bates, 1984). All these vaccinated animals responded to the vaccine by production of SN and CF antibodies. Those ponies which received three doses of vaccine resisted challenge with subtype 2 virus, while some of the others to which only two doses of vaccine were given were susceptible, although the amount of virus excreted was significantly reduced (Mumford and Bates, 1984). However, all the animals became infected when challenged with subtype 1 virus, one or two months after two or three vaccinations. No obvious differences in the febrile responses, clinical signs and subsequent abortions were observed between vaccinated and control mares. However, the febrile respiratory disease suffered by the vaccinated yearlings and two-year-old ponies was less severe than in the controls and the amount and duration of virus shedding was reduced (Burrows et al., 1984).

Other vaccines either killed or attenuated have been used for immunizing both mares and young horses, such as "Pre-vaccinol" (Mayr et al., 1965), "Rhinomune" (Mayr, 1970; Bass, 1978), and "Rhinoquin" (Purdy et al., 1977; 1978). However, either the efficacy and safety of these vaccines have not been fully demonstrated, or some of them had to be withdrawn because of sequela such as abortion and paralysis (Campbell and Studdert, 1983).

Almost all these vaccines were derived from the abortifacient strain of EHV-1. Shimizu et al.(1974), however, used a combination of both respiratory and abortifacient strains of this virus. A vaccination program was advocated using a live respiratory isolate of H-45 initially, followed by the inactivated foetal strain (KyD hamster adapted) (Kawakami et al., 1974; Shimizu et al., 1974; Kawakami and Shimizu, 1978). It was claimed that vaccination protected horses from respiratory disease as well as abortion. Since the respiratory strain of virus used in the program was insufficiently attenuated and potentially able to give adverse effects in pregnant mares its extensive use was not recommended (Kawakami and Shimizu, 1978).

Using gradient ultracentrifugation and gel electrophoresis, Papp-Vid and Derbyshire (1978) purified various EHV-1 nucleocapsid and envelope components. These fractions were used as subunit vaccines and administrated to hamsters, which were challenged subsequently with hamster-adapted EHV-1. Protection was demonstrated only in those animals vaccinated with envelope-containing materials, especially glycoprotein components of the EHV-1 envelope. Later the authors (1979) showed that, in vitro, only antibody directed against the viral envelope neutralized EHV-1 in a plaque reduction test. They concluded that the critical, protective and immunogenic antigens of EHV-1 were located in the viral envelope. The use of this material as a subunit vaccine may thus be an alternative to conventional vaccines (Campbell and Studdert, 1983).

2.3 Equine Herpesvirus Type 2

2.3.1 Introduction

Plummer and Watson (1963) first isolated equine herpesvirus type 2 (EHV-2) from a nasal swab taken from a horse with catarrh. This isolate was serologically distinct from equine herpesvirus type 1 (EHV-1), previously shown to cause respiratory disease and abortion in Equidae (Doll et al., 1957). A number of EHV-2 isolates have since been recovered at various sites from both ill and apparently healthy horses and from spontaneously degenerated equine cell cultures (Hsiung et asl., 1969; Flammini and Allegri, 1972; Studdert, 1974; Dutta and Campbell, 1978). The virus was also found concurrently with Streptococcous equi infection in cases of strangles (Studdert, 1971), and with equine adenovirus in outbreaks of respiratory disease (Roberts et al., 1974). EHV-2 has also been well known as equine cytomeglovirus and "slow growing" equine herpesvirus because of its slow growth, its tendency to remain cell associated and its restricted host range in tissue culture (Hsiung et al., 1969; Studdert, 1974).

2.3.2 Clinical signs and pathology

The clinical manifestations of EHV-2 infection in horses are unclear. Possible associations between EHV-2 infection and chronic pharyngitis (Blakeslee et al., 1975; McAllister and Blakeslee, 1977), conjunctivitis (Studdert, 1971; Thein, 1978) and respiratory disease in foals (Palfi et al., 1978; Horner, 1985) have been reported.

Chronic pharyngitis: Chronic pharyngitis is a common disease of horses, the essential signs being serous nasal discharge and a persistent cough while other clinical parameters appear normal. Under pharyngeal endoscopy, nasopharyngeal lymphoid hyperplasia and oedema with hyperaemia of the dorsopharyngeal recess can be seen. Most foals develop the condition around weaning or as yearlings (Blakeslee et al., 1975). In 1976 the American Association of

Equine Practioners conducted a survey on the syndrome termed "equine chronic pharyngitis", and in this survey EHV-2 was considered one of the possible causes (Kester 1976). Previously Blakeslee et al. (1975) reported that two young foals developed chronic pharyngitis after experimental exposure to EHV-2 strain LK, with virus being recovered from infected animals and rising titres against EHV-2 being demonstrated. McAllister and Blakeslee (1977) further attempted to draw attention to the association between EHV-2 infection and chronic pharyngitis, pointing to the chronicity of the condition and the ubiquitousness and persistence of EHV-2 in the horse population. Nevertheless, a true cause and effect relationship has still to be established.

Conjunctivitis: Twenty five cases of conjunctivitis were reported in horses between 1973 and 1976 in which the clinical picture consisted in each case of a partial, very painful keratoconjunctivitis superficialis with photophobia, lacrymation and blepharospasm followed by a conjunctival germination of blood vessels (Thein, 1978). Infected horses showed a concentric, irregularly limited darkening of the cornea from 0.5 to 1 cm in diameter with a cloudy ring and responded to treatment with eye ointment containing desoxyuridin. Two EHV-2 isolates were recovered from corneal biopsy samples and eye secretions. The author also found higher levels of circulating antibody to EHV-2 in the sera from horses with conjunctivitis than from normal animals. In an outbreak of respiratory disease, Studdert (1971) also reported that two of six infected horses developed conjunctivitis, and EHV-2 was isolated from ocular swabs from both animals.

Respiratory disease in foals: As mentioned above, only two young foals developed clinical signs of respiratory disease after experimental infection with EHV-2 (Blakeslee et al., 1975). In Hungary, Pálfi et al. (1978) reported a number of outbreaks of respiratory disease in young foals aged 6 to 10 weeks; these occurred in many large studs and in each case EHV-2 was the only viral isolate. Nearly all the foals on a property were infected, and the clinical signs observed were a slight rise in temperature (38.5-39.5 ^{o}C), dullness, and serous nasal discharge lasting for

several days. Infected foals did not respond to antibiotic administration. Two stages could be distinguished during the course of the disease. Most foals appeared to have recovered after one week, but two to three weeks later 30-40% of them become ill again and showed severe respiratory symptoms with a high elevation of body temperature, dyspnoea and prostration. In this secondary phase 30% to 40% of affected animals died. Corynebacterium equii was isolated from the lungs of all the foals that succumbed. Three to four-fold increases in antibody titres against EHV-2 were demonstrated in paired sera, but no increase in titre could be detected against EHV-1, Influenza equi A1 and A2, and PI-3, all of which are common causes of respiratory disease in horses. During outbreaks in subsequent years the course of the disease became milder and losses decreased to around 5%.

A similar situation was recently recognised in New Zealand (Horner, 1985). Over four successive years 12 foals ranging from 8 weeks to 8 months in age died with respiratory disease on one stud, other infected foals showed either mild or severe reactions but recovered.The clinical signs were similar to those described in Hungary (Pálfi et al., 1978) including pyrexia, nasal discharge, anorexia, enlarged lymph nodes, purpura haemorrhagica, and pharyngitis. EHV-2 was isolated from most of these cases, and a number of bacteria were also recovered (eg. Streptococcus zooepidemics, Actinobacillus equuli, Corynebacterium equii, Salmonella typhimurium, Bordetella bronchiseptica and Escherchia coli). In one foal both EHV-1 and equine adenovirus were also isolated.

During an outbreak of respiratory disease in Australia, EHV-2 was recovered from two foals which died of pneumonia (Studdert,1974). Very recently, an outbreak of EHV-2 associated respiratory disease of young foals was described in Japan (Sugiura et al., 1983), with a rise in antibody titres against EHV-2 demonstrated in two animals.

In Hungary the pathological findings were of catarrhal pneumonia with numerous abscesses in the lungs (Pálfi et al., 1978).

In those cases reported by Horner (1985) in New Zealand, however, many different sites were involved, and fatal bacterial infections occurred with various lesions of pneumonia, arthritis, colitis, peritonitis, cellulitis, hepatitis, nephritis and meningitis. Notable changes were also seen in the lymphatic tissue. Lymph nodes were usually enlarged with lymphadenitis characterised by lymphoid and reticuloendothelial hyperplasia, proliferation of immunoblastic cells, oedema, capillary proliferation and fibroplasia, erythrophagocytosis, presence of atypical lymphocytes, formation of syncytial cells and proliferation of macrophages lining the medullary sinuses. Interestingly chronic pharyngitis with follicular hyperplasia of the tonsillar lymphoid tissue was also a constant finding in the foals. Of the 12 foals, four also showed thrombocytopaenic purpura characterised by low platelet counts and normal fibrinogen levels.

Horses may become infected with EHV-2 by contact as young foals (Studdert, 1974). The virus may then persist multiplying in pharyngeal lymphoid follicles (Harden et al., 1974), causing chronic pharyngitis. A viraemia is then likely to follow with the virus being isolated from many sites such as kidney, bone marrow, spleen, testicle and genital tract (Kono and Kobayashi, 1964; Hsiung et al., 1969; Studdert 1974). In uncomplicated cases, EHV-2 infection might appear in a subclinical form or as a very mild disease with signs such as fever, nasal discharge and enlarged lymph nodes (Studdert 1974).

Recently, Blakeslee et al. (1980) demonstrated in vitro that EHV-2 can cause a dose-related suppression of antigen- or mitogen-induced lymphocyte blastogenesis. This finding was confirmed in vivo using the rabbit as a model (Aller et al., 1980).

Based on these findings, Horner (1985) suggested that EHV-2 may replicate in the lymphoid tissue and cause immunosuppression which in turn predisposes foals to bacterial or viral infections. Under conditions of stress, such as malnutrition, transportation and inadequate management, this may lead to overwhelming bacterial infections resulting in death. In most cases reported by Pálfi et

al. (1978) and Horner (1985) potentially pathogenic bacteria were isolated from the foals in the terminal stage of the disease or at post mortem.

2.3.3 Epidemiology and Immunology of EHV-2 Infection

Complement fixation, viral neutralization and immunofluorescence antibody tests have been used to detect EHV-2 infection (Blakeslee et al., 1975, Mumford and Thomson, 1978). The complement fixation test detects both a common antigen of EHV-2 and a herpesvirus group-specific antigen (Plummer, 1964), whereas the viral neutralization test detects type-specific antigens (Karpas, 1966; Plummer et al., 1969, 1973). Mumford and Thomson (1978) compared the above three tests using antiserum produced in rabbits, and found that the immunofluorescence antibody test was preferred due to its broad reactivity amongst EHV-2 isolates, and its lack of cross reaction with EHV-1 antiserum produced in a gnotobiotic foal and with sera against other herpesviruses. The high level of anti-complementory activities in rabbit serum and the widely different activities of different batchs of complement fixation antigen, rendered the complement fixation test inferior to the IF test.

Epidemiology

EHV-2 infection is ubiquitous among horses (Studdert 1974, Pálfi et al., 1978; Blakeslee et al., 1980). Serological evidence suggests that the prevalence in the horse population may appoach 100% (Pálfi et al., 1978; Jolly et al., 1984), so that all horses will probably eventually become infected. Attempts at virus isolation have, however, given varying results. Thus Blakeslee et al. (1980) reported that EHV-2 was recovered from nasal swabs of 30% of 67 horses with upper respiratory illness (URI), while Shernman et al. (1977) demonstrated a 4-6% isolation rate from racetrack horses during outbreaks of URI. In outbreaks of URI in foals in Hungary EHV-2 was recovered from all animals swabbed (Pálfi et al., 1978). In another study, Kemeny and Pearson (1970) recovered EHV-2 from the leucocytes of 71 out of 80 (88.7%)

apparently normal horses. During a period of 6 months, EHV-2 was isolated one or more times from 5 of 7 young foals (Wilks and Studdert, 1974). The differences in frequency of isolation in these various studies may relate to the susceptibility of cell lines used for isolation, or the methods used for recovery of virus (Blakeslee et al., 1980). Another explanation would be that different sites were sampled by different authors.

There is evidence that EHV-2 infection persists (Studdert, 1974, Blakeslee et al., 1980) since the virus has been isolated from leucocytes and nasal swabs from normal horses (Kemeny and Pearson 1970; Harden et al., 1974; Thomson, 1978) and it also can be repeatedly recovered from the same horses for long periods of time (Wilks and Studdert 1974, Blakeslee et al., 1975). Foals apparently shed virus for longer than do older horses (Blakeslee et al., 1975) and this may indicate that aged horses have a solid immunity which affords better protection.

Clinical signs associated with EHV-2 infection are mainly confined to young foals (Blakeslee et al., 1975, Pálfi et al., 1978, Horner, 1985), and the mortality in complicated situations has been thought to reach 30-40% (Pálfi et al., 1978). Old horses shed virus without overt clinical signs although an association with chronic pharyngitis has been made (Blakeslee et al., 1975; McAllister and Blakeslee, 1977).

Foals are believed to be infected with the virus in the first few weeks (6-10 weeks) of life (Wilks and Studdert, 1974; Pálfi et al., 1978), possibly by inhalation or by contact with their dams ; the virus is then probably transmitted among the group (Kemeny and Pearson, 1970; Studdert, 1974). Once infected, a foal possibly maintains its infection and becomes a life-long carrier and a constant shedder of the virus (Studdert, 1974). The later author also postulated that the cells which support the persistent infection of EHV-2 might be lymphocytes since the virus was recovered from the buffy coat in a high proportion of cases (Kemeny and Pearson, 1970); it was also recovered to a lesser degree from other sites, in particular the epithelial cells of kidney, spleen

and testicle (Karpas, 1966; Studdert, 1974).

Harden et al. (1974) reported that some EHV-2 isolates were completely resistent to the action of antisera raised against other isolates. This suggests that EHV-2 comprises a number of antigenically distinct heterologous viruses.

The antigenic differences between EHV-2 isolates detected by the virus neutralization test are also reflected in their genetic differences. Thus, nine isolates of EHV-2 isolated from a stud in New Zealand, including three isolated on the same day, all showed different patterns of DNA fingerprints when examined by the restriction endonuclease technique (Allen and Horner, Personal communication).

Since EHV-2 isolates are antigenically and genetically different, horses could be infected with more than one strain during their life. Thus Mumford and Thomson (1978) reported that sera from conventional horses which yeilded isolates of EHV-2 had a much broader reactivity than did rabbit antiserum raised against one isolate only. This may also explain the multiple episodes of respiratory disease experienced by horses (Studdert 1974).

Unlike human cytomegalovirus, EHV-2 has not yet been shown to cause intrauterine infection, and thus all of a series of foal sera sampled before colostrum consumption were negative to EHV-2 (Studdert, 1974; Wilks and Studdert, 1974). Milk and urine are common modes of transmission for human cytomegalovirus, but these have not been investigated as a source of EHV-2 in foals.

Immunology

Colostrum confers on foals passive immunity against EHV-2, and titres against EHV-2 found in the foals' sera are comparable to those of their mares (Wilks and Studdert, 1974). These authors (1974) also showed that the maternally derived antibody declined to its lowest level at around 90-120 days. Subsequently rising titres can be readily detected in paired sera collected from diseased

animals, especially in young foals (Blakeslee et al., 1975, Pálfi et al., 1978). The report of respiratory disease occurring in young foals aged 6-10 weeks (Pálfi et al., 1978, Horner, 1985) are consistent with these findings. Thus at this stage, a lowered passive antibody titre would not be protective. These findings are supported by the work of Belák et al. (1980). Thus foals passively immunized with hyperimmune serum against EHV-2 immediately after birth did not develop clinical signs, and shedding of EHV-2 did not occur. In contrast, foals not receiving γ-globulin developed respiratory symptoms and started to shed EHV-2 in the nasal discharge at about 3 weeks of age. Repeated injections of hyperimmune serum at monthly interval apparently prevented respiratory disease in the foals on one stud where the disease had been severe in the previous years. These findings indirectly support the hypothesis that EHV-2 or certain strains of EHV-2 play a role in respiratory disease in young foals, even though secondary bacterial infection should be held responsible for the deaths reported by Pálfi et al. (1978) and by Horner (1985). Furthermore these findings indicate that passive immunity might be useful in preventing this complication in young foals infected with EHV-2.

Chapter 3

MATERIALS and METHODS

Most of the materials and methods applied in this study are described below. Detailed procedures for specific techniques are given in the relevant chapters.

3.1 Animals

This study was undertaken on a Thoroughbred stud farm in which respiratory disease was a problem in young foals aged 2-4 months. Sixteen mares and their foals were selected randomly and blood samples and nasal swabs were taken from these mares before parturition and thereafter at monthly intervals. Their foals were sampled at the same time commencing when they were around 30 days of age. Since the foaling season in the southern hemisphere starts in August and September, the survey was initiated in September 1983 and ceased in the following July. Serum and nasal swabs from mares were not taken beyond the time of weaning but the collection of samples from foals continued throughout the entire period.

3.2 Sampling Procedures for Horses

Sera were separated from whole blood and stored at -20 ^{o}C. Nasal swabs immersed in transport medium (see below) were either stored at -18 ^{o}C temporarily before transportation or packed in ice and sent to the laboratory.

3.2.1 Nasal Swabs:

Nasal swabs were 15 cm long and cotton-tipped. A sterile swab was fully inserted into each horse's nostril (right or left as convenient) and left for between 30-60 seconds. The swab was then withdrawn and the end broken off and immediately immersed in 2 ml of transport medium containing T199 (Wellcome, and Flow lab. Inc.) with 0.2% bovine serum albumen and antibiotics. The latter

consisted of 200 IU/ml of penicillin, 2 mg/ml of streptomycin, 2mg kanamycin and 0.5 mg/ml of amphotericin B.

Before their inoculation onto cell cultures, the swabs were squeezed out into transport medium with sterile forceps and this medium was then centrifuged at 1000g for 10 minutes. The supernatant was stored at -70 ^{o}C until virus isolation was attempted.

3.2.2 Sera

Blood was collected from the jugular vein into plain sterile vacuum tubes. The tubes were kept at 4 ^{o}C overnight and then centrifuged at 500g for 8 minutes before the serum was poured off and stored at -20 ^{o}C.

3.3 Cell Culture

Throughout the project, RK-13, a commercial rabbit kidney cell line was passaged serially by conventional methods and used for virus isolation, titration and identification of EHVs. Growth medium consisted of 5-10 v/v foetal calf serum (FCS) in T199 medium (Appendix I) with the addition of penicillin (100 u/ml), streptomycin (10 mg/ml), kanamycin (10 mg/ml) mixture (PSK) to 1% v/v. The maintenance medium was the same as growth medium except that FCS was reduced to 1-2% v/v. All the materials used for cell culture, including media, flasks, microtitre plates and glassware were sterilized by ultrafiltration, γ-radiation, or by autoclaving as appropriate.

3.3.1 Storing and Reconstitution of Cells

Cell stocks were kept in sealed glass ampoules and stored in liquid nitrogen. Each ampoule contained approximately 1×10^{6} cells in 1.0 ml of T199 medium to which had been added 20% FCS and 10% dimethyl sulphoxide (DMSO).

In order to reconstitute cells, ampoules stored in liquid nitrogen were removed and thawed rapidly in a water bath at 37 ^{o}C.

Each ampoule of cell suspension (1.0 ml) was diluted with 9.0 ml of growth medium and the cells were pelleted by centrifugation at 1000g for 10 minutes and then resuspended in 10 ml growth medium for cell counting.

3.3.2 Growth and Trypsinization of Confluent Monolayers

Approximately $1x10^5$ RK-13 cells/ml in 20 ml growth medium were dispensed into plastic tissue culture flasks (Falcon 75 2 cm, Cat. No. 3024) and then incubated at 37°C. Confluent monolayers were usually formed after 3-5 days, and then the medium was changed to maintainance medium (T199 + 2% FCS + 1% PSK) prior to use.

Before trypsinization, the medium was removed and cell monolayers were washed twice with 10 ml of sterile phosphate buffered saline (PBS. pH 7.2) (Appendix I) and 3 ml of a mixture containing Antibiotics-Trypsin-Versine (ATV) (Appendix I) was then added. The flask was incubated at 37°C for 10 minutes, shaken to separate aggregates of cells and then 7.0 ml of growth medium was added before cell counting.

Cell counting was performed by the Trypan Blue exclusion method. Thus 0.2 ml of cell suspension was mixed with 1.8 ml Trypan Blue solution (Appendix I) and the number of unstained viable RK-13 cells per ml of the original solution estimated using an Improved Neubauer Haemocytometer (Spencer, Gemany).

After cell numbers were determined, suspended cells were distributed into tissue culture flasks and tissue culture plates (Linbro trays) (Flow Laboratories Inc. McLean, Virginia, U.S.A.) at approximately $1x10^5$ cells per ml, or into microtitre plates at approximately $1.75x10^5$ cells per ml according to requirement.

3.4 Virus Isolation

Virus isolation was attempted using the above RK-13 cells in tissue culture plates (Linbro trays). Prior to monolayer formation, duplicate wells were each inoculated with 0.2 ml of the

fluids obtained from the nasal swabs. Infected cultures were maintained at 37°C with 2-5% FCS in T199. The cells were examined daily under an inverted microscope for the appearence of cytopathic effects (CPE). After 14 days the tissue culture plates were frozen at -70°C and later thawed for further passages. Samples were considered negative after the third passage if no CPE was observed.

3.5 Production of Virus Stocks

3.5.1 EHV-1

The EHV-1 virus used in this work was isolated from an aborted foetus at Wallaceville Animal Health Laboratory, Upper Hutt, Wellington (Hutton and Durham, 1977). RK-13 cell cultures were used for the propagation of EHV-1. Confluent monolayers in tissue culture flasks were washed twice with PBS, the medium was changed to T199 without any serum added, and the flasks were then inoculated with stock virus at approximately 0.1 tissue culture infective dose (TCID) per cell. Cultures were harvested when 90-100% of cells showed CPE. This usually occurred within 48 hours after infection and the virus were then stored at -70°C.

The same procedures were used for the preparation of viral antigen for ELISA except that an equal numbers of control cell cultures were included and treated identically but without virus inoculation.

3.5.2 EHV-2

The stock EHV-2 virus was isolated from a foal on the stud being investigated (A.J. Robinson, personal communication) and later identified as EHV-2 (G.W. Horner, personal communication). Methods of production of EHV-2 were the same as described for EHV-1 except that the cell cultures were inoculated with EHV-2 before the cell monolayers were completed and maintanance medium was used after virus inoculation. The time for EHV-2 to produce a CPE varied from one isolate to another but usually required 3-10 days.

3.6 Titration of Virus

Confluent monolayers of RK-13 cells were prepared by inoculation of 100 µl of material containing 1.75 x 10^5 cells per ml into each well of a 96-well microtitre plate (Falcon, Cat. No. 3040 and 3041,USA.) which was incubated at 37°C for 3-5 days. After washing with PBS, each well of the microtitre plate was inoculated with 50 µl of a serial 10-fold dilution of virus preparation in maintanance medium. Ten wells were inoculated with each dilution and an appropriate number of controls were included. Each plate was incubated at 37°C until CPE was fully developed. The virus titres were calculated by the method of Reed and Muench (1938) and expressed as a tissue culture infective dose (TCID).

3.7 Virus Identification

All virus isolates made in this study were identified by the procedures described below.

3.7.1 Observation of Virus Behaviour in Cell Culture

After inoculation with extract from the nasal swabs, the cell cultures in the Linbro tray were observed daily for the development of a characteristic CPE and the time required for its initial appearance.

3.7.2 Electron Microscopy (EM)

Negative staining was used to examine these isolates. Infected cells were firstly centrifuged at 1000g for 10 minutes and then the supernatant was pelleted through a 45% (W/V) sucrose cushion at 21000 g for two and half hours. The viral material in the pellet was then lysed by addition of 0.2 ml distilled water.

One drop (0.25 ml) of BSA was added to a carbon-formvar coated grid, followed by a drop of the virus lysate. After washing with distilled water, phosphotungstic acid (pH 7.0) was then added. Excess fluid was absorbed with filter paper at each step and the

grids finally examined under a Philips EM 200 (Philips, Eindhoven, the Netherland).

3.7.3 Chloroform Sensitivity

The methods used were those of Feldman and Wang (1961). Duplicate 1 ml samples of virus solution were dispensed in bijoux bottles and to one was added 0.05 ml of chloroform. After shaking intermittently for 10 minutes at room temperature, both chloroform treated and virus control samples were centrifuged at 1000 g for 5 minutes. The supernatant was then removed and added to confluent monolayers of RK-13 cells in tissue culture plates (Linbro trays) which were observed daily to compare the appearence of CPEs.

3. 7. 4 Staining of Inclusion Bodies

RK-13 cells grown on coverslips in a Linbro tray were inoculated with 0.2 ml of virus sample into each well and stained using Haemotoxylin and Eosin (H & E) after CPE had developed. Coverslips removed from the Linbro tray were rinsed in PBS for 10 seconds before being fixed in Bouin's fixative for 5 seconds. After washing in three changes of 70% ethanol, the coverslips were stained in Erhlicks Haemotoxylin for 5 minutes, washed in tap water and Scott's tapwater. The coverslips were then immersed in 1% Eosin solution for 10 minutes and dehydrated in 95% and 100% ethanol for 10 seconds each. Finally, the coverslips were washed in xylol and mounted onto slides in DPX for examination under the light microscope.

Chapter 4

THE DEVELOPMENT OF AN ELISA TEST FOR DETECTING ANTIBODIES AGAINST EHV-2 IN EQUINE SERA

4.1 Introduction

Serum neutralization, complement fixation and immunofluorescence antibody tests have been adapted for the detection of antibodies to EHV-2 (Mumford and Thomson, 1978). However, a test that is more sensitive, specific and easier to perform would be useful for investigations into the seroepidemiolgy of, and humoral immune response to EHV-2 infections.

The enzyme linked immunosorbent assay (ELISA) originally described by Engvall and Perlmann (1971) has the potential to fulfill the above requirements and was therefore developed for the measurement of antibody against EHV-2 in equine sera.

4.2 Materials and Methods

4.2.1 Preparation of Antigen

The virus used as the ELISA antigen in this study was isolated in 1982 from the Thoroughbred stud under investigation (A.J. Robinson, personal communication) and identified as EHV-2 by the RAHL (G.W. Horner, personal communication). The virus was propagated in RK-13 cell monolayers in tissue culture flasks (Falcon 75x2 cm, cat. no. 3024). Before the monolayers were formed, the cell cultures were washed twice with PBS, then T199 medium with 2-5% FBS and antibiotics were added. Each flask (20 ml) was inoculated with 1.0 ml of stock virus. The same number of uninfected control cells were also included. The concentration of the stock virus was approximately 1×10^6 TCID. When complete CPE was observed, usually 8 days after inoculation, the cell cultures were harvested and

stored at -70°C.

Prior to their use, the cultures were frozen and thawed three times in order to release all the viruses remaining in the cells. Equal volumes of infected and uninfected cells were then treated by the polyethylene glycol (PEG) precipitation method as described by Jolly (1983). Briefly, 360 mls (from 18 tissue culture flasks) of both infected and uninfected cell culture materials were adjusted to 1.0 M NaCl and 8% W/V PEG (of average molecular weight 6000-7500). The preparations were stirred for 2 hours at 4°C, then left to stand overnight. The precipitate that formed was recovered by centrifugation in a Sorvall GSA rotor at 8000 rpm (10,000g) for 30 minutes, and resuspended in 10 ml of NaCl-Tris-EDTA (NTE) buffer (Appendix I). The same procedure was repeated for a second PEG precipitation, only this time the preparation was centrifuged after stirring for two hours without standing overnight. After centrifugation, the final pellet was resuspended in 5,0 ml of NTE buffer, sonicated 4 x 1 minute on ice and stored at 4°C. Samples were taken for assay of total protein concentration by the method of Lowry et al. (1951).

4.2.2 ELISA Procedures

The procedure for this indirect ELISA was based on that of Engvall and Perlmann (1971) as modified by Voller et al. (1979). Tests were performed in 96 well plastic microplates (Nunc-Immuno Plate I, Cat. No. 23454, Intermed, Denmark) using the antigen prepared as above. Horseradish peroxidase (HRPO) was chosen as the enzyme label and ortho-phenylenediamine (OPD) as the substrate. Colourless OPD changed to an orange/brown reaction product in the presence of this enzyme and the colour change was measured as absorbance at 490 nm using a Micro-ELISA Reader II (Dynatech, Virginia, USA).

The detailed procedures were as follows: 200 µl of antigen or control antigen diluted in bicarbonate buffer (pH 9.6) (Appendix I) were added to appropriate wells in Nunc-Immuno Plates. These plates were incubated overnight in a humid chamber at 4°C. After overnight

incubation, plates were emptied by rapid inversion and vigorous shaking. Plates were then rinsed once with phosphate buffered saline plus 0.05% Tween-20 (PBS-Tween) (Appendix I) and emptied as above. Three sequential washes were made by using a semi-automatic MINIWASH Washer Aspirator (Dynatech, Virginia, U.S.A.). Each well was filled twice with approximately 300 µl PBS-Tween and emptied by suction, then filled a third time and left to stand for 3 minutes. Plates were then emptied by rapid inversion, shaken dry, and the process repeated twice more. Sera were diluted in PBS-Tween plus 4% W/V bovine serum albumin (Appendix I) and 200 µl aliquots added to microplate wells. Plates were incubated at 37 °C for 2 hours before the wash procedure was repeated. The enzyme labeled antiglobulin conjugate diluted in PBS-Tween-4%BSA was added to all wells and then the plates were again incubated at 37 °C for another 2 hours before the plates were emptied and washed as above. Substrate solution (Appendix I) (200 µl per well) was added and the plates were incubated in the dark for 30 minutes at room temperature. The substrate used was 0.04% W/V OPD and 0.012% hydrogen peroxide in phosphate-citrate buffer (pH 5.0) (Appendix I) prepared fresh immediately before use. The absorbance value of each well was then measured at a wavelength of 490 nm using the micro-ELISA minireader II. A well that had been incubated without antigen, serum and conjugate but containing substrate was included in each plate as a blank.

4.2.3 Standardization of ELISA

Chequer-board titrations were performed to determine the optimum concentrations of serum, conjugate and antigen for routine use.

A. Titration of Serum and Conjugate

Rabbit anti-horse IgG (whole molecule) labelled with horseradish peroxidase was used as conjugate in this study (Miles-Yeda Ltd, Krryat Weizmann, Rehovot. Israel). This conjugate was diluted with PBS-Tween-4% BSA. Preliminary titration of this conjugate against equine gamma-globulin (EGG) indicated that

dilutions of 1/4000 - 1/40,000 were required in subsequent chequerboard titration of serum vs. conjugate (Jolly, 1983).

EHV-2 antigen at 1/50 dilution was used for the serum vs. conjugate titration. Four dilutions of conjugate (1/400, 1/4000, 1/12,000 and 1/40,000) and seven doubling dilutons of serum from 1/50 to 1/3200 were titrated against viral and control antigens (each at a 1/50 dilution) and "no antigen" (200 µl coating buffer alone in each well) on the same microplate. Both serum and conjugate were diluted in PBS-Tween-4%BSA. "No serum" control wells incubated with PBS-Tween-4% BSA only during the serum incubation step were included for each conjugate dilution against viral antigen, control antigen and "no antigen" containing wells.

B. Determination of Optimum Antigen Concentration

Five dilutions (1/50, 1/75, 1/100, 1/150 and 1/200) of both viral and control antigens were titrated against 4 dilutions (1/100, 1/400, 1/1600 and 1/6400) of a positive reference serum and against three dilutions (1/100, 1/400 and 1/1600) of a negative serum. "No antigen" and "no serum" controls were included on each plate.

C. Colour Development

Optimum substrate concentration and the time required for the substrate reaction were adopted from the ELISA test for EHV-1 (Jolly, 1983). The mixture of 0.04% W/V OPD and 0.012% hydrogen peroxide in phosphate-citrate buffer (pH 5.0) was prepared immediately before use. Thirty minutes was chosen as the standard reaction time.

To stablize the colour development for reading, the reaction between the substrate and enzyme used in ELISA may be terminated after a predetermined substrate incubation period by the addition of strong acid or alkali. Sulphuric acid (2.5 molar) is used for the peroxidase/OPD reaction in the ELISA test (Miranda et al., 1977; Voller et al., 1979). In this experiment, OD values were measured in two plates that had the same reagents.

3.2.4 Titration of Test Sera

Forty-two selected sera used in these titrations were collected from foals aged 5-11 months in different districts throughout N.Z. as part of a national survey in 1981. These sera were assayed for EHV-2 virus neutralization antibodies at the Diagnostic Laboratory, New York State College of Veterinary Medicine, Cornell, U.S.A. and had serum neutralisation (SN) titres ranging from negative to 1:128 (A.J. Robinson and D. Lein personal communication). Each of these sera in eight tripling dilutions from 1/50 to 1/109,350 was titrated against the optimum dilution of viral antigen preparation and against a control.

4.2.5 Expression of the ELISA Result

The endpoint titres determined by the method of Jolly (1983) were used to express the results of the ELISA test in this study. The titre of an individual serum was calculated as follows: An ELISA specific absorbence (ESA) value was calculated by subtracting the absorbence of the control antigen well from that of test antigen well. Logit transformations of each titration curve (ESA vesus log reciprocal serum dilution) were then carried out, with logit values calculated as

$$\text{logit } S = (S/M-S) \log_e$$

where M = maximum ESA for an individual titration curve and

S = the ESA value for each subsequent serum dilution on the same curve such that (M>S>0.00)

Linear regressions were then compiled by the method of least squares for each set of logit data against the reciprocal serum dilution. These regression lines were used to determine the end point titres of individual sera. An ESA value of 0.20 at a 1/50 dilution was selected as the postive and negative cut off point. Sera with an ESA value less than 0.2 at a dilution of 1/50 were considered negative, and the lowest ELISA titre possible became 1/50.

4.2.6 Comparison of ELISA Results with SN Results

and with Virus Isolation

The results obtained from this ELISA test were compared with the SN results reported from the American Diagnostic Laboratory and also with the virus isolation conducted in this survey (for details see chapter 6).

4. 3 Results

4. 3. 1 Antigen

The PEG method increased the protein content of viral antigen by 36.6% and that of the control antigen by only 12.3% (table 4-1).

4.3.2 Standardization of Serum and Conjugate:

The results of the titration of serum versus conjugate are shown in table 4-2 and figure 4-1. Non-specific absorbence occurred with increasing conjugate and serum concentrations (column No.4, 8 and 12). These reactions were mainly due to non-specific binding of serum antibody to the plastic plate rather than to non-specific bindings of the conjugate since all wells in row A (no serum) had minimal OD values. The concentration of the conjugate amplified the effect of the non-specific binding of the serum antibody. The 'no antigen' wells had relatively higher absorbence values compared to the control antigen wells and this might be attributed to the inhibition of non-specific binding of serum protein by the protein in the control antigen. There was no reaction between the viral antigen and the conjugate since the wells in row A showed almost the same absorbence values at the same dilution of the conjugate. In addition, the progressive decrease in absorbences with increasing serum dilution indicated their dependence on serum concentration.

Dilutions of conjugate at both 1/400 and 1/4000 produced the highest absorbence values against the EHV-2 antigen, but also

resulted in high control values. Since OD values over 1.9 Å are not reliably detected by this Micro-ELISA Reader II, a 1/12,000 dilution of conjugate and eight tripling dilutions of serum from 1/50 to 1/109,350 were chosen for the titration of test sera. This achieved the most accurate quantitation of specific activity in sera with a wide range of antibody concentrations.

4.3.3 Determination of Optimum Antigen Concentration

Serial dilutions of both EHV-2 and control antigens at 1/50, 1/75, 1/100, 1/150 and 1/200 were incubated in a Nunc-Plate and titrated against three sera at dilutions of 1/100, 1/400, 1/1600 and 1/6400. Of these three sera, two were known to be positive by SN and the third was a presuckling foal's serum (ie. presumably negative). The results are shown in figures 4-2 and 4-3. The absorbence values for the antigen at dilutions of 1/75, 1/100 and 1/150 were similar, but a pro-zone phenomenen was observed for the strong positive serum at the 1/50 dilution of viral antigen. The control antigen produced no reaction since the OD values for the strong positive, medium positive and negative sera were the same at the same serum dilution. The 1/100 dilution of both antigens was, therefore, chosen for use in the test for reasons of convenience and economy.

4.3.4 Colour Development

After 30 minutes substrate reaction time, 50 µl of 2.5 M sulphuric acid was added to each well on one of the two plate in which the same reagents were incorporated. The addition of this stopping solution resulted in an average 4 fold increase in OD values. This OD value was well above the maximum of 1.9 Å which could be read on the ELISA reader, therefore this addition of acid as a termination step was not used in this test. Since the reader deals with a whole plate within 2 minutes it was not considered necessary to stop the reaction.

4.3.5 Titration of Test Sera:

Each of the 42 test sera at tripling dilutions from 1/50 to 1/109,350 was titrated against the viral and control antigens at a dilution of 1/100. The titration curves of all sera were sigmoidal in shape. Examples of a strongly positive serum (No.36) and weakly positive serum (No.4) are shown in figure 4-4. Control antigen absorbences were relatively higher at the lower serum dilutions and depended largely on the titres of individual sera.

After calculation of the titres as described above, it was found that the goodness of fit of logit data in linear regression was remarkably high, with individual line correlation coefficients ranging from 0.971-0.999 over all titrations. The ELISA titres from these 42 sera are summarised in table 4-3.

4.3.6 Comparison of ELISA Titres with SN Titres:

The ELISA titres from this study were compared with the SN titres from the American Diagnostic Laboratory (table 4-3). The only negative serum by SN which became positive by ELISA was serum No.42. With all other sera, titres given by the ELISA test were about 300 - 5000 times higher than those from SNT, with most being increased approximately 1500 times. The distribution of log SNT vesus log ELISA titres is shown in Figure 4-5 , and histograms showing the frequency distributions of log titres from both assays are depicted in figure 4-6. An 81.5% correlation was achieved between the ELISA titres recorded in this study and the SNT titres from the American Laboratory.

When this ELISA test was applied for detection of antibodies in sera collected for this investigation, it detected rises in antibody titres in 13/14 cases where isolation of EHV-2 was also achieved at this or at the subsequent sampling. The increase of antibody level in the other foal was demonstrated 2 months after the first isolation of the virus. All the presuckling sera were negative by this ELISA test (for details see chapter 6).

4.4 Discussion

The ELISA test adopted here for the detection of antibodies against EHV-2 in horse sera proved to be highly sensitive and specific compared with the serum neutralization test and could therefore be used as a tool for seroepidemiology and serodiagnosis of EHV-2 infections.

The highly sensitive nature of the test was reflected in the average 1500 times increase in the antibody titres recorded in this ELISA test over those in the SN test, which is in agreement with other ELISA systems. Bolton et al. (1981) found their standard ELISA test for IBR to be 100 - 1000 times more sensitive than the SN test. The titres obtained in the ELISA for EHV-1 were 100 - 100,000 times greater than those in an SN test (Jolly, 1983).

Serum neutralization antibody has traditionally been regarded as the indicator of exposure to virus and resistance to infection (Bolton et al., 1981). The SN test detects antibodies specific to viral components involved in the binding of virus to cells. The increased sensitivity of ELISA over SNT may indicate that the ELISA test detects not only the neutralizing antibody but also antibodies induced by other viral components.

This ELISA system also appeared to be specific for the detection of antibodies against EHV-2. All the positive sera measured by the SN test in the USA were also positive in the ELISA test performed in New Zealand. A correlation of 81.5% in titres between the ELISA test and the SN test was obtained in these 42 test sera even though these two tests were performed in separate laboratories using different EHV-2 isolates as antigens. These sera were also used for standardization of the ELISA system for EHV-1, and in this case the correlation between the ELISA test and the SN results reported from the American Diagnostic Laboratory was 62.5%, and 71.9% between the ELISA and the SN when both were done in this laboratory using the same antigen (Jolly, 1983).

In addition, this test detected an increase in antibody titres against EHV-2 in 13 out of 14 animals following infection with this virus. The other foal did not show the increase until two months after the first virus isolation was made. However, this animal had the highest maternally derived antibody titres among these foals being sampled. It may be that this high level of passively aquired antibody interfered with this foal's ability to mount its own humoral response to EHV-2.

The purity of the virus antigens used in ELISA tests varies (Voller and Bidwell, 1976; Gilman and Docherty, 1977; Denoyel et al., 1980), but it is generally believed that highly purified antigen is preferred (Bolton et al., 1981; Abraham et al.,1984). An unpurified antigen produces higher non-specific reaction due to competition for binding sites from contaminating macromolecules (Herrmann et al., 1979) and a non-purified antigen preparation also needs more antigen and the inclusion of a 'control cell antigen' (Engvall, 1977; Denoyel et al., 1980). However, preparation of a highly purified antigen is time consuming and needs more viral growth. Furthermore, only a small proportion of total infectious virus is recovered in the final purified antigen preparation, with most being lost in the purification procedure (Bolton et al ., 1981; Jolly, 1983). The later author compared a highly purified EHV-1 antigen with a crude infected cell culture extract concentrated by PEG precipitation and found that there was little difference in performence between these two preparations. In this study for EHV-2, the crude culture extract was also found to be a suitable ELISA antigen and had the advantage that a high yield of virus could be produced with relative ease.

The majority of background absorbences in the ELISA system were attributed to the non-specific binding of serum immunoglobulins to the plates. This is in contrast to findings in other ELISA systems in which high concentrations of uninfected control antigen caused high background absorbances (Gilman and Docherty, 1977; Bolton et al., 1981). In fact, the antigen prepared in this study was found to have a slight inhibitory effect on this non-specific reaction, and this is in agreement with a report for EHV-1 (Jolly, 1983).

De Savingny and Voller (1980) reviewed the communication of ELISA results from the laboratory to the clinician and they concluded that no single method has been found to satisfy the ideal requirements for ELISA serological reports. In this study, the titration method was chosen to express relative antibody activities as these antibody activities can be compared between sera from different horses and sera from the same horse taken at different times. In other titration methods for ELISA, the titres were calculated either as signal/noise ratio (Saunders et al., 1977; Bolton et al., 1981) or from ESA upon the highest serum dilution (Gilman and Docherty, 1977) and therefore the results are not on a continuous scale. In the ELISA system for EHV-1, Jolly (1983) developed the method of calculating the ELISA titres from the interpolation on a linear transformation of individual serum titration curves and this gives the most truly quantitative detection of relative antibody activities. This method was therefore adopted here for EHV-2.

The setting of the "cut off" between positive and negative is a crucial element in every assay for specific antibody activity. In the ELISA system, it is usual to set the minimum positive response value at two or three times the mean negative group, or at the mean negative value plus two (or three) standard deviations of the mean if more reference sera are tested. However, a more statistically valid method is to determine the upper limit of normal by setting this at the 97.5th or 100th percentile (de Savigny and Voller, 1980). All these methods require a number of known negative sera. Unfortunately, most horses become infected with EHV-2 early in their life. Of those sera (67) sent to America,the only one negative in SN became positive in the ELISA. Thus in this study, the setting of ESA at 0.2 as the positive/negative "cut off" point was somewhat arbitrary. Nevertheless, this "cut off" point has been used by many authors for calculating ELISA titres (Gilman and Docherty, 1977; Jolly, 1983) so its use here was considered valid.

Table 4-1 Protein content of viral and control antigens before and after PEG processing

Antigen	Before PEG	After PEG	%Increase
EHV-2	8.2 g/l	11.2 g/l	36.6
Control	7.3 g/l	8.2 g/l	12.3

Table 4-2 Serum-conjugate chequerboard titration: microplate well absorbence at 490 nm

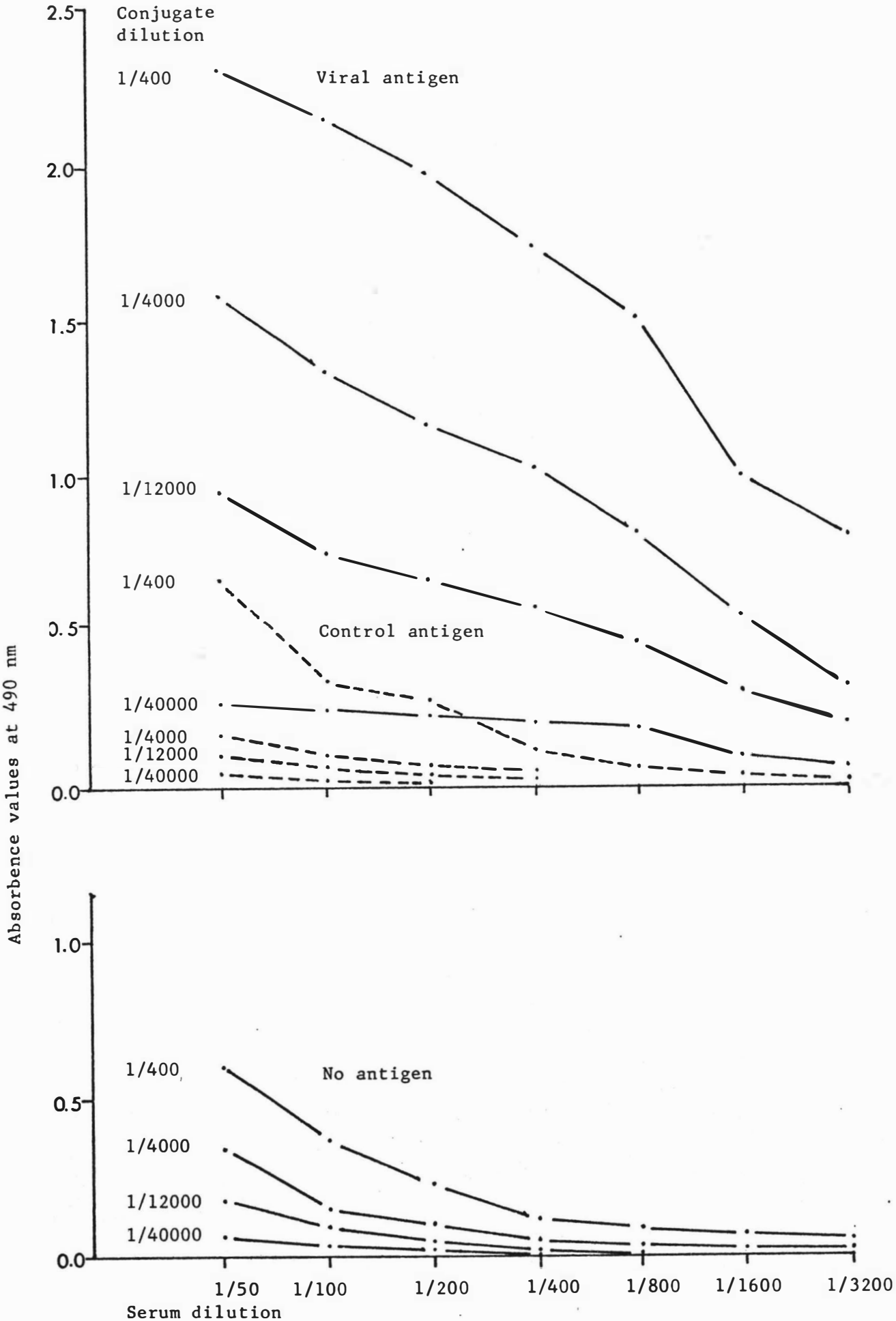
		conjugate dilution												
		No antigen				Viral antigen				Control antigen				Row
		1/40000	1/12000	1/4000	1/400	1/40000	1/12000	1/4000	1/400	1/40000	1/12000	1/4000	1/400	
	No serum	Blank	0.02	0.04	0.08	0.00	0.00	0.03	0.08	0.00	0.01	0.03	0.10	A
	1/50	0.06	0.19	0.32	0.60	0.28	0.95	1.57	2.26	0.05	0.11	0.18	0.58	B
Serum	1/100	0.04	0.10	0.16	0.36	0.26	0.77	1.33	2.14	0.02	0.06	0.11	0.33	C
dilution	1/200	0.02	0.04	0.10	0.23	0.22	0.76	1.20	1.99	0.01	0.04	0.08	0.25	D
	1/400	0.01	0.02	0.04	0.12	0.21	0.58	1.09	1.73	0.01	0.03	0.05	0.10	E
	1/800	0.00	0.01	0.03	0.10	0.20	0.46	0.84	1.51	0.00	0.00	0.03	0.05	F
	1/1600	0.00	0.00	0.02	0.06	0.08	0.32	0.57	0.99	0.00	0.00	0.01	0.03	G
	1/3200	0.00	0.00	0.01	0.04	0.06	0.24	0.34	0.81	0.00	0.00	0.00	0.01	H
	Column	1	2	3	4	5	6	7	8	9	10	11	12	

Table 4-3 Antibody titres to EHV-2 in equine sera measured by a serum neutralization (SN) test and by an ELISA test

	Antibody titres	
Serum No.	SNT (USA)	ELISA (NZ)
1	16	53802
2	32	61660
3	4	6180
4	3	15224
5	32	99449
6	4	6861
7	12	9423
8	16	4128
9	12	16229
10	6	4493
11	3	2724
12	8	8117
13	8	8168
14	12	14118
15	48	40635
16	8	10821
17	8	7640
18	8	9279
19	24	22206
20	8	8892
21	24	23283
22	16	9707
23	8	29792
24	6	2319
25	2	3864
26	8	3069
27	6	4074
28	3	4820
29	6	1703
30	12	6952
31	8	6801
32	6	1709
33	48	137943
34	64	72879
35	32	138995
36	128	230144
37	128	100000
38	4	2239
39	2	8012
40	6	3604
41	3	6357
42	0	6578

Facing page **55**

FIGURE 4 - 1. Comparison of ELISA activities at different dilutions of serum and cojugate against EHV-2 viral antigen, control antigen, and a "no antigen" control.


Conjugate dilution
1/400
Viral antigen
1/4000
1/12000
1/400
Control antigen
1/40000
1/4000
1/12000
1/40000
1/400
No antigen
1/4000
1/12000
1/40000
2.5
2.0
1.5
1.0
0.5
0.0
1.0
0.5
0.0
Absorbence values at 490 nm
1/50
1/100
1/200
1/400
1/800
1/1600
1/3200
Serum dilution

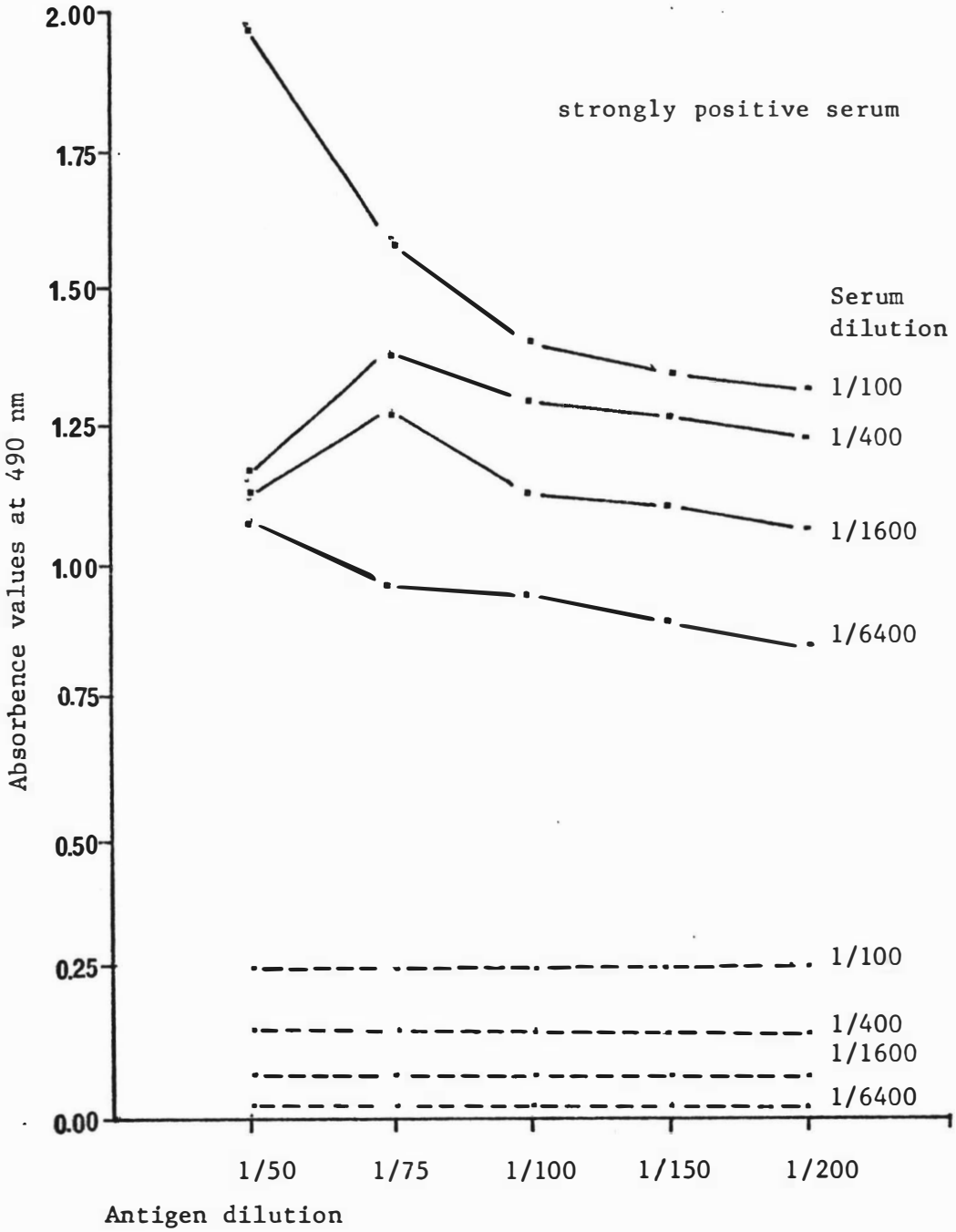


FIGURE 4 - 2. Comparison of ELISA activities at different EHV-2 viral (•——•) and control (•- - - -•) antigen concentrations against different serum dilutions.

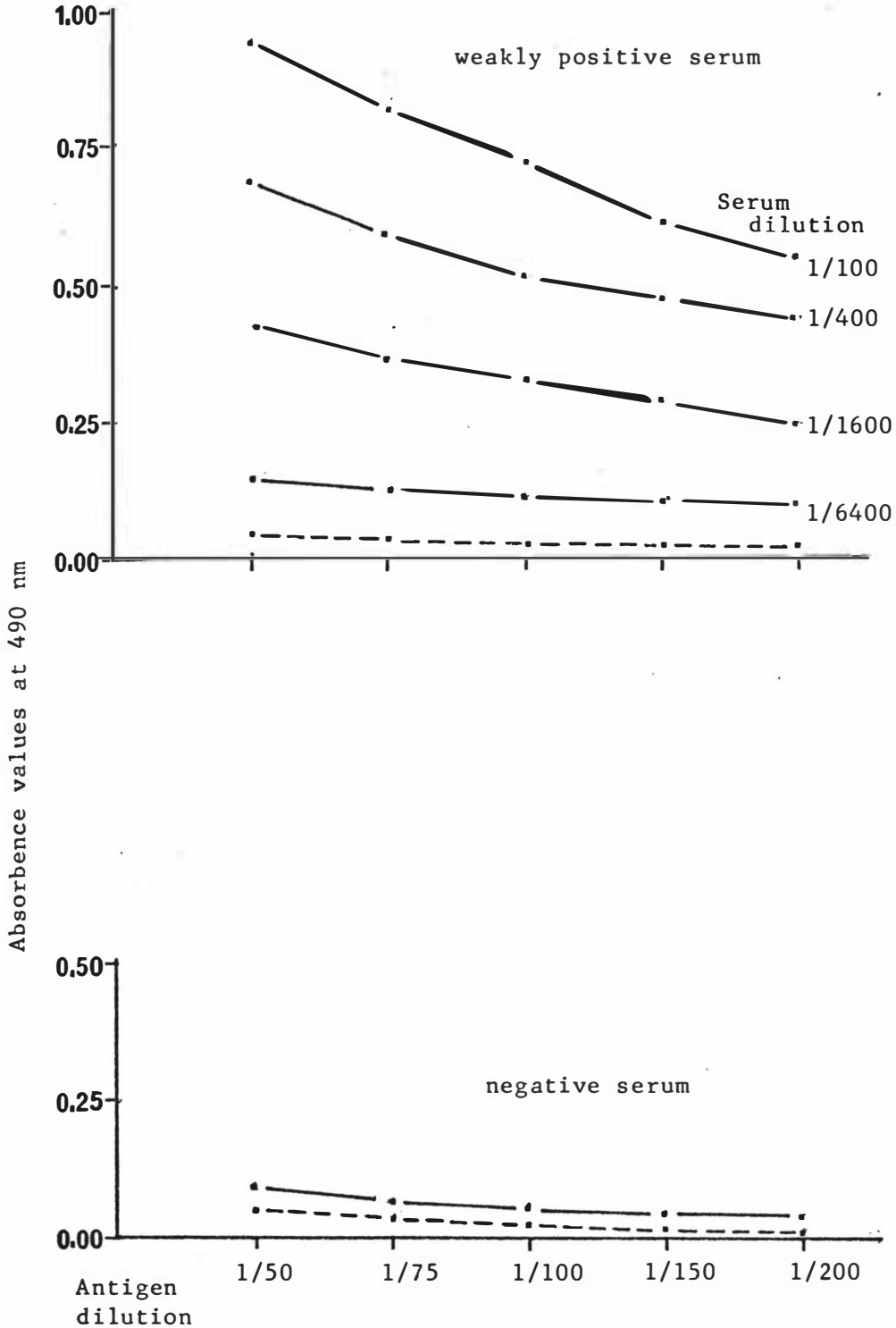
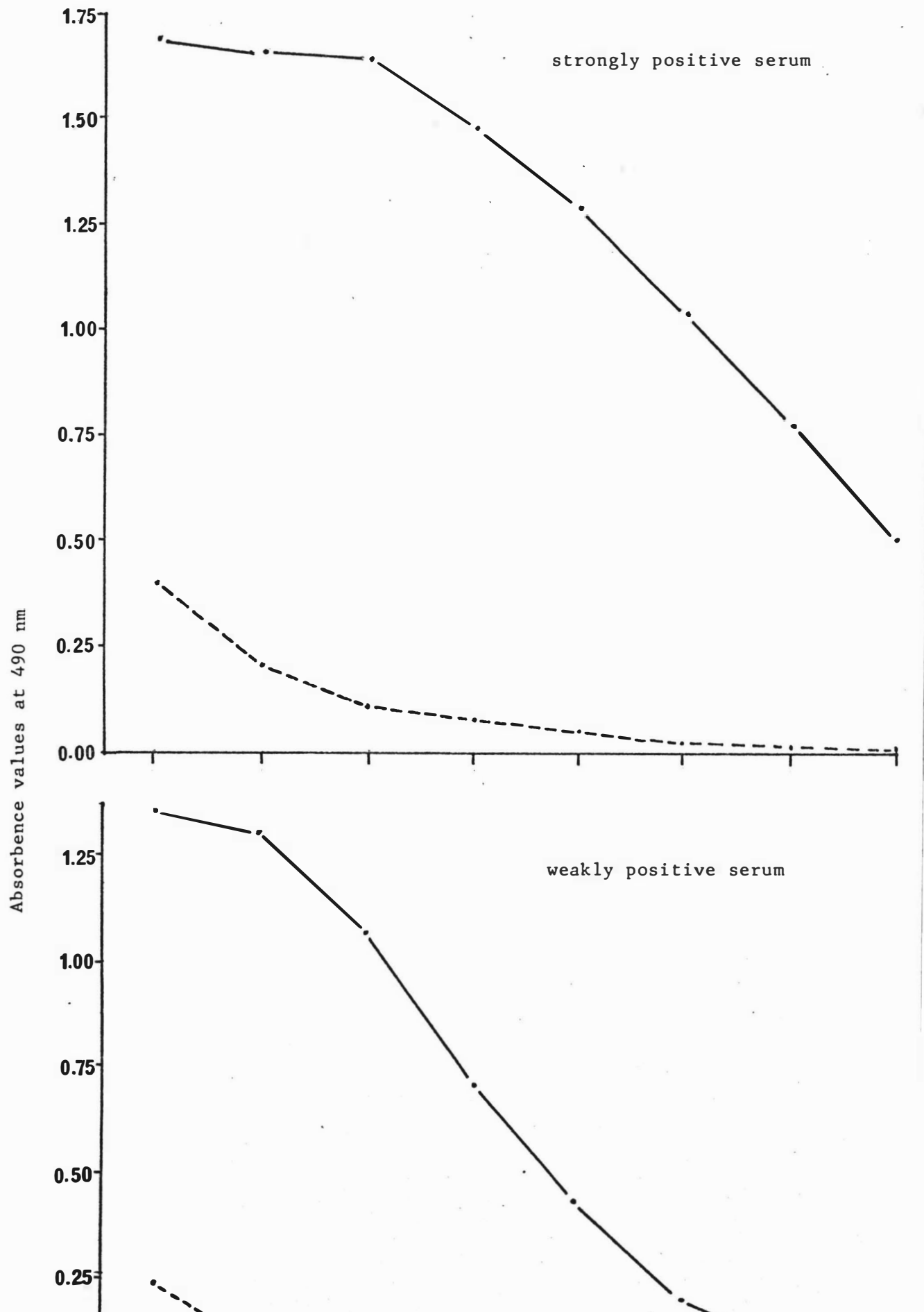


FIGURE 4-3. Comparison of ELISA activities at different EHV-2 viral (•———•) and control (•- - - -•) antigen concentrations against different serum dilutions.

Facing page **58**

FIGURE 4 - 4. Representative examples of serum titration curves against EHV-2 antigen (———.) and control antigen (·-·-·-·).


strongly positive serum
weakly positive serum
Absorbence values at 490 nm
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
Serum dilutions
1/50
1/150
1/450
1/1350
1/4050
1/12150
1/36450
1/1093

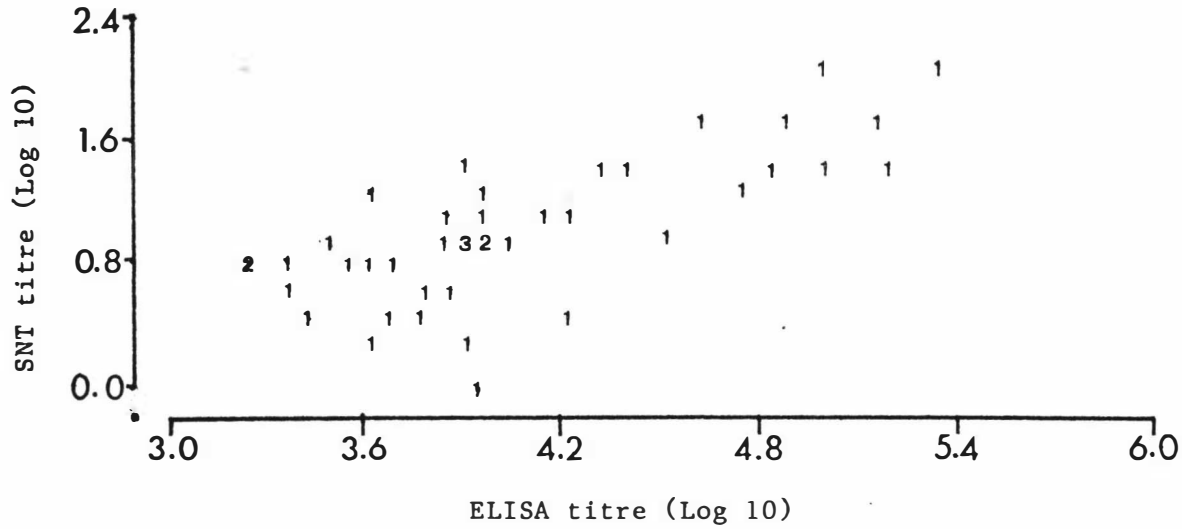


FIGURE 4 - 5. Comparison of antibody titres against EHV-2 measured by SNT and ELISA.

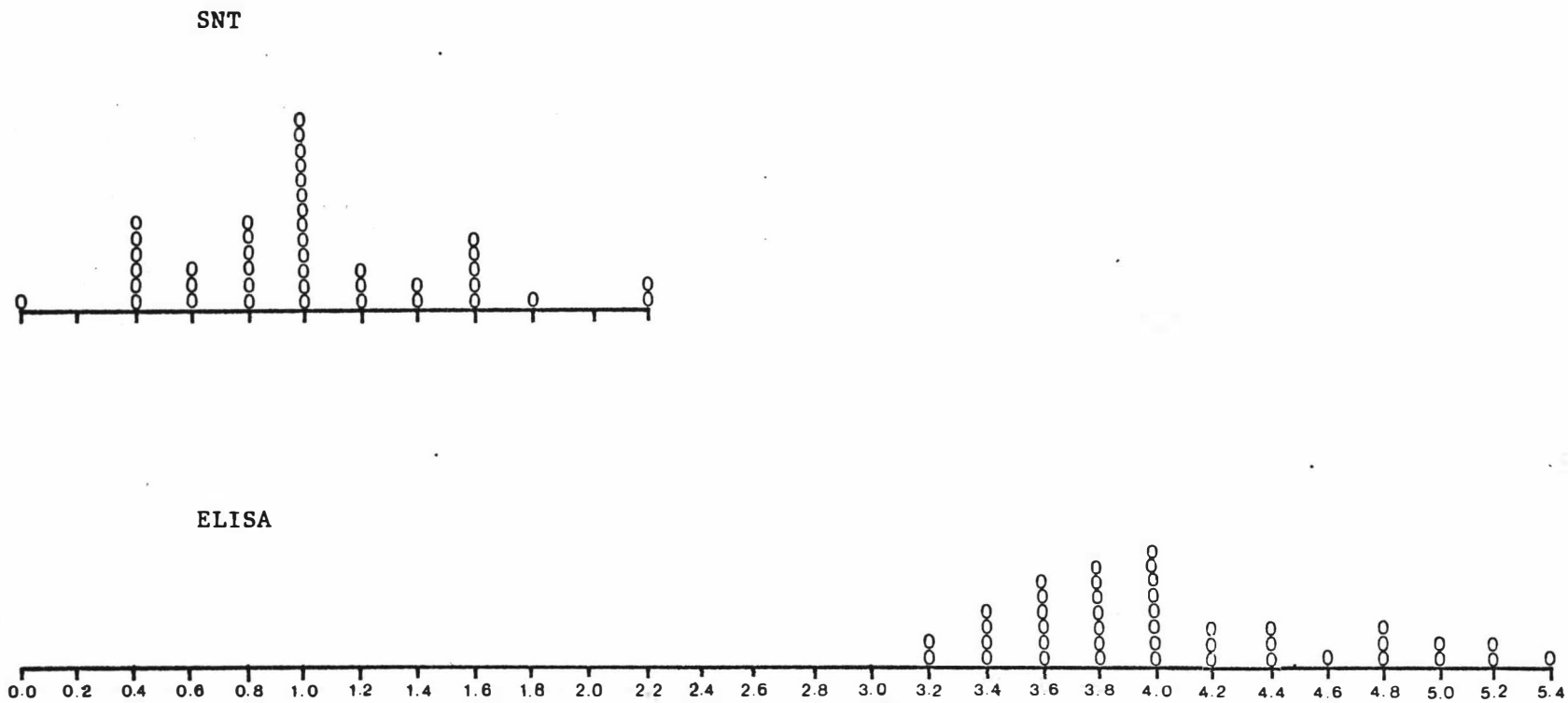


FIGURE 4 - 6. The frequency distribution of EHV-2 antibody titres obtained by SNT and ELISA

Chapter 5

EQUINE HERPESVIRUS TYPE 1

5.1 Introduction

In the U.S.A., EHV-1 subtypes 1 and 2 have been involved in respiratory disease of horses (Allen et al., 1983), while in Australia (Studdert and Blackney, 1979) and the United Kingdom (Mumford and Bates, 1984), subtype 2 has been mainly responsible for cases of upper respiratory disease (URD). Both of the subtypes have been identified as being present in New Zealand (Horner et al., 1976; Studdert et al., 1984), but their role in respiratory disease of horses in this country is not clear. The subtype 1 strain was isolated from aborted fetuses (Hutton and Durham, 1977) and subtype 2 was recovered from foals with respiratory disease (Horner et al., 1976; Horner, 1981).

An inactivated vaccine (pneumabort-k) derived from the Army-183, a subtype-1 virus has been claimed to induce sufficient immunity to prevent abortion in mares (Bryans 1978; 1980; Moore and Koonse, 1979). However, in New Zealand, abortions associated with EHV-1 have only been reported in 1976 and 1977 (Hutton and Durham, 197 ; Jolly, 1983), while respiratory disease in young horses caused by this virus is very common (Jolly, 1983). Even though the strain of EHV-1 causing URD was not known, Pneumabort-K has been recommended for use since 1981 on the stud being investigated. Since no systematic work has been carried out to evaluate the efficacy of the vaccine on this property, this survey was undertaken to establish:

1. The response of mares to the vaccine and subsequent transferrence of maternal antibody against EHV-1 to their foals.

2. The duration of passively acquired antibody in the foals and its effect on the response of the foals to the vaccination.

3. The response of vaccinated foals to, and the protection afforded by the vaccine when natural infection occurs.

4. The strain of EHV-1 associated with respiratory disease.

5. The relationship between EHV-1 infection and respiratory disease in these foals.

5.2 Materials and Methods

Pneumabort-K (Fort Dodge Laboratories, Fort Dodge, Iowa), was supplied by Jones and Hoskyns Ltd, Rolleston, New Zealand.

Sixteen mares and their foals were selected for this study. These mares were inoculated with the vaccine at three months and again at one month before parturition, and their foals were immunized when they were 30 and 60 days of age.

Nasal swabs for virus isolation and blood for serology were taken from all the mares before foaling and at monthly intervals after foaling. Wherever possible the foals were bled before and after the consumption of colostrum, and then every month after that for a period of ten months. The mares were not sampled beyond the time of weaning. These animals were also observed for signs of respiratory disease at each visit and by the veterinarians attending this stud during the intervening periods.

Extracts from the nasal swabs from these animals were passaged on RK-13 cells to detect viruses, and their sera were tested for antibodies against EHV-1 by an indirect ELISA test developed by Jolly (1983). The detailed ELISA procedure for EHV-1 was the same as that for EHV-2 described in chapter four. The virus used as antigen was also grown on RK-13 cells and treated by the polyethylene glycol (PEG) precipitation method (see chapter 4).

The procedures of collecting and processing these blood and nasal samples are given in chapter three.

5.3 Results

5.3.1 Passive Immunity

Table A-1 and A-2 (Appendix II) list the antibody titres to EHV-1 in these foals and mares as measured by the ELISA test against an EHV-1 subtype 1 viral antigen. The few presuckling sera collected were all negative for antibody to EHV-1. The available foals sera obtained soon after consumption of colostrum had antibody levels similar ($<\log10^{0.24}$ difference) to those of corresponding mares (table 5-1).

All the other foals which were not available immediately after they had ingested colostrum also had serum antibody levels against EHV-1 similar to those of their dams ($<\log10^{0.32}$ difference) at one month of age (table 5-2).

5.3.2 Response to Vaccination in Thoroughbreds

A. Response of Mares to Vaccination

The first serum samples from most of the mares were collected in either August or September on the same day as they were receiving their second injection of vaccine. Most of them had very high titres at the first collection (geometric mean titres of $\log10^{3.7\pm0.4}$) (table A-2). The second collection from most of these mares was not taken until 2-3 months later. Four mares (No. 2, 4, 8, 10) showed a rise in antibody titre (mean 2.71 fold increase) in their second serum samples which were collected somewhat after the second dose of vaccine. The second serum samples of all the other mares had very similar antibody levels to their first serum samples.

All the mares maintained high levels of antibody to EHV-1 up to the time of weaning (table A-2, figure 5-1).

B. Response of Foals to Vaccination

As shown in figure 5-1, the foals were vaccinated at one and

two months of age. However, there was no serological evidence to suggest that there was a response to the vaccine in these foals, since no rise in antibody titre to EHV-1 was detected in the sera collected at two and three months of age (table A-1, figure 5-1).

5.3.3 Evidence of Infection with EHV-1

A. EHV-1 Infection in Foals

Figure 5-1 shows the sequential ELISA antibody titres of the 16 foals for the first ten months of their life. The maternally acquired antibody titres resulting from the consumption of colostrum fell steadily, reaching the lowest point at 3-4 months of age, and were soon replaced by antibody produced as a result of natural infection. The antibody levels usually rose again by 4-5 months of age. Since this rise in antibody titres did not occur until 2-3 months after the second dose of vaccination, this was considered to be due to natural infection of the foals with EHV-1 rather than to the vaccination.

As showed in table A-1, two out of the 16 foals died of respiratory disease at about 2 months of age. At this stage there was no serological evidence suggesting infection with EHV-1, and the virus was not isolated from these animals. Among the other 14 subjects, 12 (85%) contracted EHV-1 infection between the 4th and 9th month, with half (6) being infected at the 5th month of age around the time of weaning. Three of these 12 (25%) animals showed further serological evidence of infection between 8-10 months of age.

The time when infection of the foals with EHV-1 occurred seemed to depend largely on their pre-infection antibody titres. Most of the foals had antibody titres to EHV-1 which rose after the maternally derived antibody levels fell below $\log_{10}^{3}$ ($\log_{10}^{2.5\pm0.4}$) or became negative (table A-1). The three animals which became reinfected also showed this trend since two had antibody titres of about $\log_{10}^{3}$ while the other one was negative.

Nearly half of the foals became infected for the first time in February and sporadic cases occurred thereafter. In June and July another peak was observed, three cases occurring in each month. Among the three reinfections one occurred in May and the other two in July.

All the initial increases in antibody titres were at least four fold (8.44 ± 3.25) with one foal (No. 13) having a 46.9 fold increase and two (No. 7 and 10) rose from negative to $\log10^{3}$ and $\log10^{4.2}$ respectively. Of the three reinfected foals, two (No. 9 and 11) showed an increase in antibody titre of only 3.1 and 3.6 fold with a third (No. 14) going from negative to $\log10^{4}$ at this time.

No EHV-1 virus was isolated froms nasal swabs during the investigation period despite the obvious serological response of these foals as detected by the ELISA test.

B. EHV-1 Infection in Mares

There was neither serological nor virological evidence to suggest that infection with EHV-1 was occurring in the mares (table A-2, figure 5-1). The four increases in titres (mare No. 2, 4, 8, 11) occurred in October and November and were most likely due to vaccination (see above). During the period of sampling these mares maintained reasonably high levels of antibody.

C.Effect of Maternally Derived Antibody on response to Vaccination and the Occurrence of EHV-1 Infection in Thoroughbred Foals

Among the six animals which contracted infection, initial maternal antibody titres against EHV-1 varied from $10^{2.9}$ to $10^{3.9}$. Two other foals (No. 12 and 13) with high maternal antibody titres $\log10^{3.97}$ and $\log10^{3.7}$ only become infected later (at 7 month of age). The two foals (No. 5 and 15) which did not become infected during the investigation period had initial maternal antibody levels of only about $\log10^{3.5}$ and $\log10^{3.2}$, respectively.

5.3.4 Relationship between EHV-1 Infection and Respiratory Disease

Serological evidence indicated that most of these foals became infected with EHV-1 around weaning time. From February to May most foals on this property developed a very mild respiratory disease. The main clinical signs observed were a profuse muco-purulent nasal discharge and a slight elevation of body temperature. Most of these foals were untreated and recovered in 5-7 days. The severely affected foals were succesfully treated with tetracycline and electrolyte solution for 3 days. Other foals on this stud which were not included in this survey also developed this condition around weaning time. Unfortunately, no detailed record was kept of individual animals so that the relationship between the clinical reaction and the serological response to EHV-1 could not be precisely determined.

5.4 Discussion

None of the available sera which were collected before the ingestion of colostrum showed any evidence of antibody to EHV-1. Sera from all the foals sampled after suckling had antibody levels against EHV-1 similar to their dams ($\log_{10}^{0.23}$ difference). This is in agreement with the findings of other workers (Jeffcott, 1972 ; Thomson, 1978; Neely and Hawkins, 1978) that in the horse maternal antibody can not cross the placenta so that the sole protection for foals against infection is offerred by colostral antibody. Furthermore, none of these foals after sucking had higher ELISA titres than their dams, and this is in agreement with the reports of Doll and Bryans (1963b) and Thomson (1978).

The maternal antibody titres in these foals declined steadily for 3 to 4 months after consumption of colostrum, and then were replaced by actively produced antibody titres presumbly stimulated by natural infection with EHV-1. This sequence of changes in antibody titres to EHV-1 has also been reported by Thomson (1978).

Maternally derived antibody on one hand provides protection to

the young against infectious diseases, but may also interfere with the development of active immunity. The latter effect has been well documented in different species of animals with a variety of agents (Seamer and Snape, 1972; Brar et al., 1978). Doll and Bryans (1963b) observed that foals with maternal SN titres against EHV-1 of $\log_{10}^{4}$ or over, failed to respond to vaccination with a hamster-adapted live EHV-1 vaccine. However, after vaccination of foals aged either up to 3 weeks or 3 to 4 months of age with a live attenuated EHV-1 vaccine, Frymus (1980) found both age groups showed increases in SN and CF antibody titres. The younger group developed higher CF antibody titres than did the older group despite the latter group having a waning immunity to EHV-1. The author assumed that moderate titres of maternal antibody levels increased the humoral response of the foals immunized up to 3 weeks of age. Recently, Mumford and Bates (1984) vaccinated a group of 8 to 12 months old ponies with a killed vaccine (Pneumabort-K) and found that all the animals responded serologically. These animals all had low prevaccinal titres of both SN and CF antibodies ($<\log 10^{1.0}$). In the present study, two doses of vaccine administrated intramuscularly at 30 and 60 days of age, respectively, did not induce any humoral response as detected by the ELISA test. Foals at these stages had geometric mean ELISA antibody titres of $\log 10^{3.4}$ and $\log 10^{3.1}$ respectively. Few firm conclusions can be drawn as to what extent maternal antibody inhibits the production of active antibodies, but this interference seems to be the most likely explanation in this case. Age and immunocompetence did not seem to be important since these foals were capable of responding serologically to EHV-2 infection at this time (see chapter 5).

From the findings reported above, vaccination of foals at 30 and 60 days of age as carried out in this study would appear to have little or no value due to the interference of the high levels of maternally derived antibody. If their mares are vaccinated before parturition, the following vaccination protocol with EHV-1 vaccine could be reccommended. Foals should receive their first dose of vaccine around 80 to 90 days of age when their maternal antibody has declined to below inhibitory levels. In this study, the mean titres of antibody of the foals at 90 days of age was only $\log 10^{2.8}$, and

$\log_{10}^{3.0}$ appeared to be the approximate threshold of inhibition. Doll and Bryans (1963b) also observed that only 13% of foals under 80 days old responded serologically to vaccine compared to 80% of foals over 80 days did so. The second dose should then be given preferably 2 to 3 weeks later. In order to induce sufficient immunity in foals around weaning time, they should be injected for the third time two weeks before weaning, which should protect them from EHV-1 infection and/or respiratory disease during the weaning period. Since immunity to EHV-1 is short-lived, only lasting for about 3 to 4 months (Doll and Bryans, 1963b), and reinfection with EHV-1 of foals commonly occurs in their first year of life, another dose of vaccine, therefore, should be given at around one year of age. This should then protect foals when they are brought together for yearling sales.

Although these foals ingested high levels of maternal antibody from their vaccinated mares, this passive immunity did not last any longer than that reported in other foals in which the dams had not been immunized before parturition (Doll and Bryans, 1963b; Thomson, 1978). In the USA, outbreaks of rhinopneumonitis in foals were reported as occurring in the autumn and winter when they were brought together in confined quarters (Doll and Bryans, 1963a). Thomson (1978) also reported that passive immunity waned around weaning time and this was when foals contracted infection with EHV-1; he also reported that one foal with no maternal antibody to EHV-1, as measured by the SN test, became infected with the virus at two weeks of age. In this survey, most of the foals were infected with EHV-1 in February to May (autumn and winter in the southern hemisphere) at the time when the maternal antibody in foals declined to low and presumbly unprotective levels ($<\log_{10}^{3}$).

The source of infection with EHV-1 could not be determined since there was no evidence of infection in the mares before and/or at the time of weaning. In September, a stallion was imported from Australia and developed respiratory disease when it was in the quarantine centre. A blood sample was taken and an SN titre of 1:16 was measured against a EHV-1 subtype-1 virus. Unfortunately no virus isolation attempts were carried out (G.W. Horner, personal

communication). This stallion was introduced on to the property being investigated soon afterwards. It is not known whether this horse was the source of infection for the foals on this stud. Aternatively, since latent EHV-1 infection is believed to occur (Burrows and Goodridge, 1979, 1984) and might be reactivated in some mares (Doll and Bryans, 1963a), they might have passed the infection on to their foals. If this ocurred, excretion was probably transient since EHV-1 was not isolated from the mares at any of the visits. Presumbly, if the mares were excreting virus, those foals with a low or no maternal antibody titre ($<\log10^3$) would contract infection, while others which still had maternal antibody levels $>\log10^3$ would resist infection, but become infected with this virus later.

Thomson (1978) reported that all those foals which were sampled for a full year showed infection with EHV-1 in their first year of life and 38% were reinfected. In this present survey, 12 out of 14 (85%) became infected with 3 (25%) being reinfected. However, this investigation was done only for ten months. If this study had been carried out for a full year, the same results as reported by Thomson (1978) might have been obtained since the maternal antibody titres in the two uninfected foals had only just reached the level of $\log10^3$ by the time of the last sampling, and had therefore just reached a susceptible state when the study was concluded.

In this study, since all the foals which became infected or reinfected had preinfection antibody titre below $\log10^3$, this titre was considered as an approximate threshold for protection. However, this relationship between preinfection titres and infection in this study ($\log10^3$) could not be compared with those observed by others. Burrows and Goodridge (1973) found that an SN titre of $\log10^{1.4}$ would be protective, and Bryans (1969) reported that titres of $\log10^2$ or greater would form a barrier to infection located in the mucosa of the pharynx. Later, Bryans and Prickett (1970) suggested that there was an association between resistence to infection and SN titres above $\log10^2$. Mumford and Bates (1984) concluded that CF antibody titres had a better correlation with infection than the SN antibody levels. The level of protection of

$\log_{10}^{3}$ reported in this study was measured by an ELISA test which was apparently much more sensitive than either the SN or CF tests. A further complication could be that the ELISA test was carried out using a subtype 1 virus as antigen, while the virus which infected the foals may have been EHV-1 subtype 2. Although these two subtypes are reported to cross react to some extent in the SN test (Kawakami et al., 1962; Burrows and Goodridge, 1973), their degree of cross reactivity has not been determined in the ELISA test. Furthermore, no experimental infection with EHV-1 has been done to evaluate the protection afforded by different titres of antibodies detected by ELISA.

During this survey, EHV-1 virus was not isolated from either the mares or their foals, although the serological and clinical evidence suggested an EHV-1 infection in the foals. This is in contrast to the finding of Thomson (1978) who recovered EHV-1 from foals with clinical signs which subsequently showed a rise in antibody titres, and also from foals without serological evidence of infection. A possible explanation for the failure to recover EHV-1 from the foals in the present study could be that the interval between collections was too long (30 days), since Shimizu et al. (1961) have reported that EHV-1 is only excreted for up to 14 days after primary infection. Because no virus was isolated, the virus strain which caused the respiratory disease in the foals could not be determined. However, abortions associated with EHV-1 have never been found to occur on this property, and EHV-1 previously isolated from this stud was identified as EHV-1 subtype 2 (Horner, 1981; Studdert et al., 1984). Therefore it seems likely that EHV-1 subtype 2 was responsible for the respiratory disease and the serological response observed in this particular property.

Table 5-1 Reciprocal ELISA antibody titres against EHV-1 in mares and in their foals after consumption of colostrum

Horse No.	Mare	Foal
1	11945	8758
2	4789	2472
3	2704	2285
4	16349	4407
5	12590	9286
GMT	9675	5441
log10	3.90	3.66

NB: GMT -- Geometric mean titres

Table 5-2 Reciprocal ELISA antibody titres against EHV-1 in mares, and in their foals at one month of age

Horse No.	Mares	Foals
1	4908	1828
2	11754	1748
3	955	706
4	4792	1670
5	5510	3578
6	23793	7613
7	ND	2474
8	2685	ND
9	2537	1232
10	5613	5312
11	7042	2798
12	9809	8456
13	5434	5074
14	6484	3781
15	2816	1408
16	5482	789
GMT	5012	2455
log10	3.7	3.4

NB: ND -- not done

GMT -- Geometric mean titres

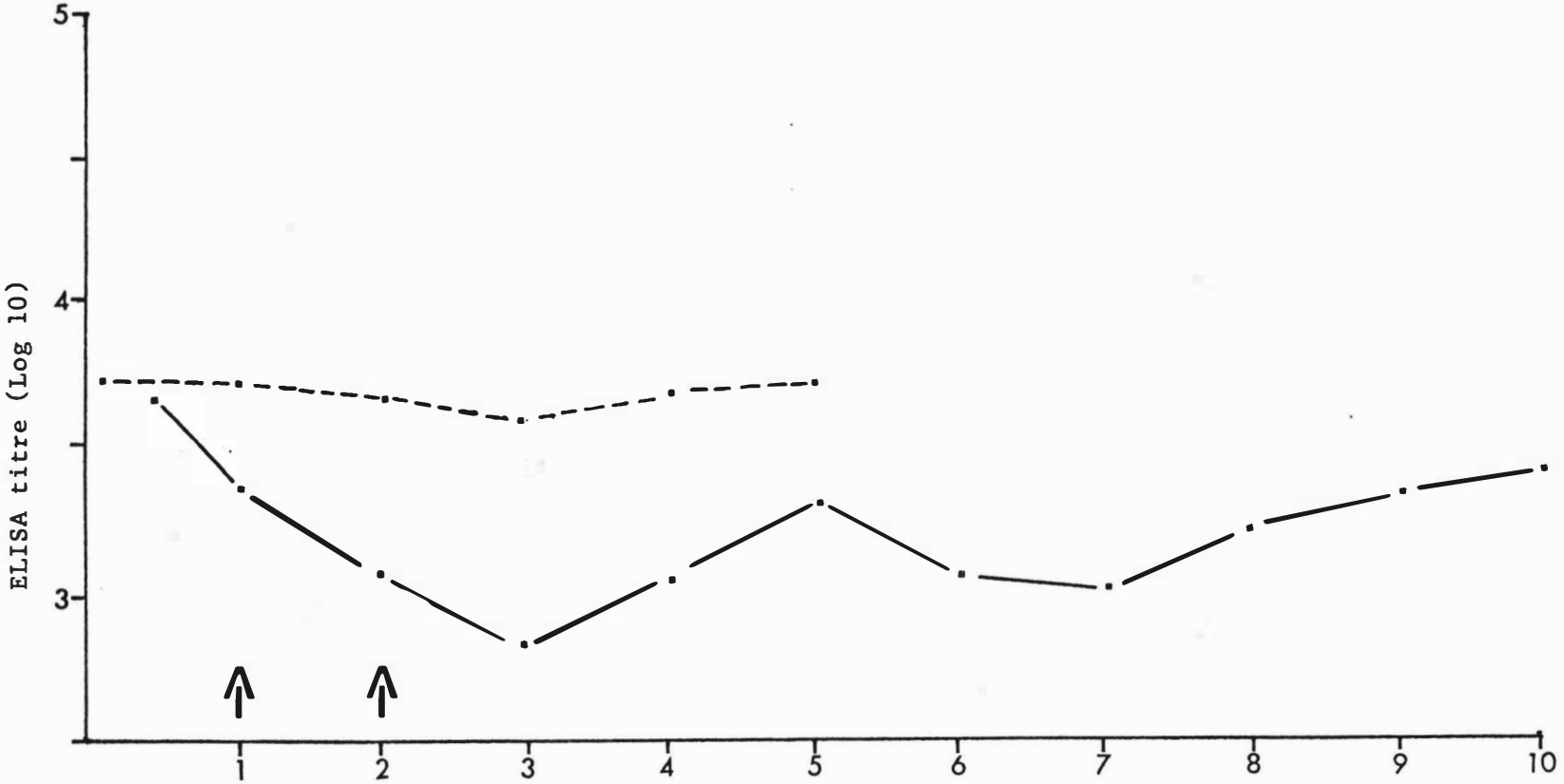


FIGURE 5 - 1. ELISA antibody titres against EHV-1 in mares (•——•) and in their foals (•- - - - -•) over a period of ten months (⇑ vaccination of foals).

Chapter 6

EQUINE HERPESVIRUS 2

6.1 Introduction

There has been no proven association between EHV-2 infection and any clinically recognized disease in horses, and experimental infections have only resulted in very mild reactions (Blakeslee *et al.*, 1975). However, outbreaks of respiratory disease associated with EHV-2 infection have been reported in young foals (Pálfi *et al.*, 1978; Sugiura *et al.*, 1983; Horner, 1985) and immunosuppression has also been demonstrated both *in vitro* and *in vivo* after infection with the virus (Blakeslee *et al.*, 1980; Aller *et al.*, 1980).

In the stud under investigation, respiratory disease occurred every year in 2-4 month-old foals, some of which died of pneumonia and/or other complications either soon after the primary symptoms or after a prolonged illness. EHV-2 was isolated from most of these infected subjects (Horner *et al.*, 1976; Horner, 1985). It was considered that EHV-2 might play a role in this respiratory disease and in the subsequent deaths of the foals. Therefore, a survey was conducted:

1. To monitor antibody titres against EHV-2 in foals and their dams during the first ten months of the foals lives.

2. To determine whether or not these animals become infected with EHV-2, and if so at what age.

3. To attempt to establish a relationship between the serological and virological evidence and the clinical problem encountered on this property.

The animals sampled and the procedures used in this study were

described in detail in chapters three and four.

6.2 Results

6.2.1 Maternally Derived Antibody

All the sera collected were subjected to measurement of antibody levels against EHV-2 using the ELISA test described in chapter four. Sera collected from the foals before ingestion of colostrum were all negative. Soon after colostrum consumption, these foals had similar serum antibody levels in the sera to those of their dams ($<\log10^{0}$ .3 difference; table 6-1). Before foaling, mares had relatively lower mean antibody titres to EHV-2 ($\log10^{3.5\pm0.5}$) than to EHV-1 ($\log10^{3.7\pm0.4}$).

6.2.2 Virus Isolation

Tables 6-2 and 6-3 summarise the total of 59 viral isolates recovered from these animals during this study, with 50 being from the foals and 9 from the dams.

Unfortunately, all the nasal swabs collected from the mares before parturition were heavily contaminated with bacteria and fungi and no virus isolations were made. After foaling EHV-2 was only isolated from 5 (No. 2, 5, 6, 7, 14) out of the 16 mares. Four of these five mares excreted the virus on more than one occasion.

In contrast, EHV-2 was recovered from all the foals under 4 months of age, with most of them (10/16) yielding the virus in their first two months of life. Figure 6-1 shows the incidence of EHV-2 infection in these foals by age and time respectively. Over the 10 months period, virus was isolated from each of these foals between 2 and 6 times, except for two foals (No. 4 and 17) which died. Virus shedding persisted for 2-7 months with virus being recovered either consistantly or intermitently. The prevalence of EHV-2 infection in these foals (figure 6-2) reached a peak around 3-4 months of age and declined thereafter. By 9 months of age the virus could no longer be isolated (figure 6-2).

6.2.3 Serological response

A. Serological Response of Foals to EHV-2 Infection

Figure 6-3 shows the decline of maternally acquired antibody and the subsequent development of active antibody production to EHV-2 in the mares and in their foals during their first ten months of life. The antibody titres against EHV-2 in the foals and in their dams are summarised in table A-3 and A-4, respectively.

All but one foal responded serologically around the time of natural EHV-2 infection, with a rise in antibody titre either at the time when the virus was first isolated, or at the next serum collection (table A-3). The exception was the foal (No. 7) which had the highest passively acquired antibody titre and yielded virus at 3 months of age, but had no serological response until two months later (Figure 6-4). The mean increase in antibody titres in these foals were 7$\pm$ 2.4 fold. The serological response of a representative foal (No. 11) is shown in figure 6-5.

The striking feature of the humoral immune response to EHV-2 in foals was the steady and prolonged increase in antibody titres following infection (figures 6-3, 6-4, 6-5). The peak response was reached 3-4 months after the first viral isolation. As soon as the peak of antibody production was reached, the isolation rate of virus decreased until eventually no virus was isolated.

No relationship was observed between the initial maternal antibody levels against EHV-2 in the foals and the occurrence or course of the virus infection. The foal (NO. 7) which had the highest maternal antibody level contracted the virus infection at the same time as the other foals and the duration of virus shedding in this foal was also no shorter than the others, although its serological response was apparently delayed.

B. Serological Response of Mares to EHV-2 Infection

Of the five mares from which EHV-2 was isolated, 4 subsequently showed a slight increase in antibody titres (mare No. 2, 5, 6, 14) (table A-4). Only mare No. 7 which had the highest initial antibody level did not respond serologically. A rise in antibody titres against EHV-2 was also demonstrated in mare No. 3 from which the virus was not isolated. All these increases in antibody titre were less than two fold and whether these should be considered as a seroconversion is questionable; however, the steady rise and the persistence of these slightly increased antibody titres suggested that there was an increased response to the viral infection.

6.2.4 Clinical Observations Associated with EHV-2 Infection in the Foals

In November and December 1983, respiratory disease occurred in the foals, and the clinical signs were generally very mild. In most cases only nasal discharge, swollen lymph nodes and an elevation of body temperature were seen. However, two severely affected animals (foal No.4 and 16) died in November. Isolation of EHV-2 was made 15 to 20 days before their deaths. At post-mortem, EHV-2 was recovered from one foal, and a number of bacteria were also isolated from both animals. Of the 16 foals, ten showed clinical signs at around the time when the virus was isolated. Nasal samples from 4 animals were sent to RAHL, and from one of these samples an equine adenovirus was also recovered (G.W. Horner, personal communication).

The pathological changes in the two dead foals were typical of those seen in foals which had previously died on this stud. Pneumonia, chronic pharyngitis and depletion of lymphocytes were observed in both foals, while widespread petechial haemorrhages were also seen in one foal. These changes were considered as consistent with a primary viral infection with secondary bacteraemia (G.W. Horner, personal communication). No signs other than serous nasal discharge were observed in any of the mares during the sampling period.

6.2.5 Characterization of Viral Isolates

Virus isolations from nasal swabs were made in RK-13 cells. Most of the isolates produced a CPE in the first passage. The time required for the first appearance of a CPE varied from one isolate to another, but usually took from 6 to 14 days. Generally, it took longer for the CPE to appear in cells infected with viruses isolated from mares compared to those from foals. The time to produce a CPE on subsequent passages decreased, and ranged from 2 to 7 days. The CPE produced by all the isolates was similar in appearence and consisted of a rounding of cells, syncitial formation and detachment of cells from the culture flasks (plates 6-1 and 6-2). After staining with haematoxylin and eosin, striking Cowdry type A intranuclear inclusions were seen with a distinct halo separating the inclusion from the nuclear membrane (plate 6-3). Under the electron microscope, the virus showed typical herpesvirus morphology (plate 6-4). Chloroform sensitivity test revealed that all the viral isolates were chloroform sensitive. From these characteristic features, these isolates were identified as EHV-2.

6.3 Discussion

This survey demonstrated that all the foals in this group became infected with EHV-2 by 4 months of age, with most of them becoming infected in their first 2 months of life regardless of the maternally derived antibody level, and probably independent of the time of the year. The evidence to support this claim is seen in figure 6-1 which shows that the age distribution of virus isolations is closer to a normal curve than is the distribution according to the time of the year. The earliest isolation of this virus was made from a foal (No. 9) at 33 days of age. Wilks and Studdert (1974) also reported that the earliest they had recovered EHV-2 virus was from a 30 day old foal, and no EHV-2 was isolated from foals sampled at 1 and 3 days of age . These authors postulated that foals might become infected with EHV-2 at any time between 3 and 30 days of age. In this present study most of the foals were sampled for the first time when they were around one month of age, and four foals yielded

EHV-2 at their first collection. This supports the view held by Wilks and Studdert (1974).

Persistency and latency are both common features of herpesvirus infections. Persistent infections, however, are distinct from latent infections because in the former, virus is released continously while in the latter, virus may not be detectable for a long period (Rapp and Jerkofsky, 1973). In this present survey, all the foals shed EHV-2 for 2 to 6 months, although the virus was recovered from their mares much less frequently. This finding confirms the persistent nature of infections with EHV-2 in horses which have been reported by others. Thus Turner et al. (1970) consistently isolated EHV-2 from the nasal cavity of 3 horses over a period of 418 days and Blakeslee et al. (1975) also reported that after experimental exposure two young foals excreted virus for 98 and 118 days respectively. This virus was also recovered from leucocytes of 71 out of 80 apparently normal horses (Kemeny and Pearson, 1969).

Because of the persistent nature of EHV-2 infection some horses may act as carriers in a horse population. It is therefore possible that carrier mares provided the source of infection for their foals in this study. Thus, the 5 mares from which EHV-2 was recovered all excreted the virus before and/or at the same time as the isolation was made from their foals. The other foals whose dams apparently were not excreting EHV-2 might have become infected either from a transient or undetected period of shedding from their dams, or more likely from other infected foals and mares since these horses were running together as a group in a paddock.

All of the foals that were sampled received a relatively low concentration of colostral antibody against EHV-2. The duration and rate of decline of the passively acquired antibody could not be accurately determined in these foals because of the rapid production of antibodies by these animals. After infection with EHV-2, all the foals responded serologically by increasing their antibody titres at least 3 fold. The peak antibody level was not reached until 3 to 4 months after the initial response, and then was maintained for a

long period.

An interesting observation was that a steady increase in antibody titres to EHV-2 occurred during the 2-6 months period of persistent viral infection. This is in contrast to other persistent herpesvirus infections. In humans infected with herpes simplex virus, Andrewes and Pereira (1967) found that after the initial antibody response,recurrent clinical episodes did not stimulate further rises in antibody titres. In another persistent herpesvirus infection of man, Epstein-Barr (EB) virus infection, antibody titres first appear with the onset of symptoms and reach a peak by the end of this phase or at the start of convalescence, and then remain constant for life (Epstein and Achong, 1977). The same phenomenon has also been observed in subclinical infections with EB virus (Henle and Henle, 1979). In cattle infected with infectious bovine rhinotracheitis virus (IBR), Snowdon (1965) observed a rise in antibody titre in one cow on several occasions immediately following the isolation of the virus. The IBR virus was also recovered intermittently over an extended period (up to 718 days) from other animals in this group, but no rise in antibody titres at the time of virus isolation was recorded. However, in the present study, all the foals showed a steady increase in antibody titres during the period when EHV-2 virus was being constantly recovered. Presumbly this steady increase was in response to continued antigenic stimulation from the persisting virus infection. It is not clear why there were not separate primary and anamnestic components to the antibody response, but one possibility could be an interference by the virus on thymus derived lymphocytes with suppressor function (T-s cells). This has been reported for other virus infections (Woodruff and Woodruff, 1975), and EHV-2 has itself been shown to interfere with T cell function *in vitro* (Blackeslee *et al*., 1980). Recently, Sugiure *et al*. (1983) reported that among 7 foals from which EHV-2 was isolated, only two showed a serological response, and they believed that these foals had a limited ability to produce antibody because of their age.

In this present study, a serological response was demonstrated in all the foals following infection with EHV-2, however, its role

in eliminating EHV-2 infection was questionable. Nevertheless, when the antibody levels reached their peak, the virus isolation rate decreased, and finally no EHV-2 was isolated from any of these foals. Possible explanations for this sequence of events would include:

1, Antibodies induced by this virus infection might only have been able to suppress multiplication of the virus and not completely abrogate infection until sufficiently high titres were eventually reached. Evidence for the protective role of antibody in EHV-2 infections has been given by Belák et al. (1980) who demonstrated that passive immunization of foals with hyperimmune serum against EHV-2 delayed the occurrence of EHV-2 infection and decreased the severity of the clinical reaction.

2, A delayed appearance of IgA in the pharyngeal region might have eventually reacted with the virus, and decreased virus shedding. The ontogeny of immunoglobulin production following natural infection or immunization with a variety of viral agents is in a sequential manner, namely IgM, IgG and IgA (Ogra et al., 1975). IgA either from local secretion or of serum origin is important in protecting the mucosal membranes from infection, or at least reducing the infecting dose (Thomson, 1978). Although the ELISA test used in this study might have detected mainly IgG antibody, particularly since the conjugate used was anti-IgG (whole molecule), detection of other antibody classes was also possible due to the common light chains in all classes of immunoglubulin. The increase in antibody titres of these foals around 6 months of age might have been mainly due to an increase of IgA antibody. The effect of IgA in the pharyngeal mucosa could explain why the recovery of EHV-2 from the nasal cavity was less frequent from the foals after 6 months of age. The infrequent recovery of this virus from the nasal mucosa of mares might also be explained by a high IgA level in the mucosal secretions, though it may still be isolated from the leucocytes of a high proportion of adult horses (Kemeny and Pearson, 1969).

3, Cell mediated immunity (CMI) could develop slowly after

infection and eliminate virus-containing cells, reducing the virus titres in the nasal cavity. The CMI response is believed to destroy virus which is protected from antibody by its intra-cellular location (Bloom and Rager-Zisman, 1975). It has been shown in vitro by Blakeslee et al. (1980) that EHV-2 is capable of suppressing lymphocyte transformation. If this suppression occurs following EHV-2 infection of foals, then the immune system of these animals might become compromised. The effect of immunosuppression would probably be relatively short-lived and variable in its severity. Horses that experienced immunosuppression would recover and could then actively control the viral infection. This protective role of CMI would explain why mares with low antibody levels but a well developed CMI, shed the virus less frequently than foals with high antibody levels.

In the light of the above findings, an association seems likely between EHV-2 infection and the respiratory disease observed in foals during November and December, although the virus was also isolated from foals without any overt clinical reactions. There are also other reports which support the view that EHV-2 could be a pathogen in young foals. Pálfi et al. (1978) reported a respiratory disease of young foals 6 to 10 weeks of age, some of which also died. EHV-2 was the only virus isolated from all these cases and no rise in antibody titres could be demonstrated against respiratory viruses other than EHV-2. Moreover, the administration of hyperimmune serum against EHV-2 to foals delayed the infection and prevented the annual respiratory disease which had been observed on the same properties previously. Foals in non-participating studs still became infected and developed clinical signs early in their life (Belák et al., 1980). Recently, Sugiure et al. (1983) also reported that only EHV-2 and no other respiratory viruses could be recovered from foals with pneumonitis. However, this virus can also be frequently isolated from clinically normal horses (Kemney and Pearson, 1969) and from degenerated equine leucocytes used for routine cell culture preparations (Hsiung, 1969). Furthermore, experimental infection of foals with the LK strain of EHV-2 only resulted in a mild reaction (Blakeslee et al., 1975). Experimental infection with other strains of EHV-2 have not been attempted.

Recently, Blakeslee *et al.* (1980) reported a dose-related immunosuppression following *in vitro* infection of lymphocytes with EHV-2, and this effect was also seen *in vivo* using the rabbit as a model (Aller *et al.*, 1980). It is therefore possible that foals become infected with EHV-2 early in their life and the virus initiates a respiratory disease, but under normal circumstances of infection, the clinical signs are mild and may go unnoticed The most common manifestation may be a chronic pharyngitis as reported by Blakeslee *et al.* (1975). However, if the foals are under stress or infected with a large dose of virus, the virus may invade the cells of the immune system and causes immunosuppression. This suppressed immune system could make the foals more susceptible to secondary infections with other viral and bacterial agents. The immune system of the two foals which died in this study might have become compromised after infection with EHV-2 so that these animals could not mount sufficient immunity to combat the secondary bacterial infection. Indeed, the pathological changes observed in the lymphoreticular system of the two foals ranged from severe depletion of lymphocytes (thymus, spleen) to hyperplasia (tonsil, mandibular lympoh nodes) with presence of blast cells. Bacteria including *Pasteurella spp.* and *Streptococci spp.* were also isolated from these animals (G.W. Horner, personal communication).

In older horses, EHV-2 infection may be subclinical. Thus, after experimental infection with EHV-2 Blakeslee *et al.* (1975) did not induce any overt symptoms in older horses, although two young foals developed a mild respiratory disease. In the present investigation 10 out of 16 foals showed clinical reactions around the time that EHV-2 was isolated, but no signs were observed in any of the mares including those from which the virus was recovered.

In conclusion, although direct evidence demonstrating a definite association between EHV-2 and the clinical problem being experienced on this study under investigation could not be obtained, there is some circumstantial evidence that EHV-2 was associated with the respiratory disease in foals.

Table 6-1 Reciprocal ELISA antibody titres against EHV-1 in mares, and in their foals after ingestion of colostrum

Horse No.	Mare	Foal
1	9264	8586
2	24833	23561
3	2134	1710
4	872	198
GMT	4549	2305
log10	3.7	3.4

NB: GMT -- Geometric mean titres

Table 6-2 Recovery of EHV-2 from the Foals during their first ten months of life

Foal No.	Age in days 30	60	90	120	150	180	210	240	270	300
1	-	ND	+	ND	+	+	-	-	-	-
2	+	-	ND	-	+	-	-	+	-	ND
3	-	+	ND	ND	+	+	-	-	-	-
4	+	DEAD								
5	-	-	ND	+	-	+	+	-	-	ND
6	-	ND	+	+	+	SOLD				
7	-	ND	+	+	+	+	+	-	-	ND
8	-	+	+	ND	-	+	+	-	-	-
9	+	+	ND	+	+	+	+	-	-	ND
10	-	+	ND	+	+	-	+	+	-	ND
11	-	+	+	ND	+	-	-	-	-	-
12	-	ND	+	+	-	-	-	ND	ND	ND
13	-	+	ND	+	+	-	-	-	-	ND
14	-	-	ND	+	+	+	-	-	-	ND
15	-	+	ND	+	-	-	-	-	-	ND
16	+	DEAD								

NB: + -- EHV-2 Isolation
- -- Negative
ND -- Not Done

Table 6-3 Recovery of EHV-2 from mares over the first five months of their foal's life

Mare No.	Bp	15-45	46-75	76-105	106-135	136-166
1	ND	-	-	ND	ND	-
2	+	-	-	ND	-	-
3	ND	-	-	ND	ND	ND
4	ND	-	ND	ND	ND	ND
5	ND	-	+	ND	-	+
6	+	-	ND	+	-	ND
7	+	-	ND	+	-	ND
8	ND	-	-	ND	ND	ND
9	ND	-	-	ND	ND	ND
10	ND	-	-	ND	ND	ND
11	ND	-	-	-	ND	ND
12	ND	-	ND	-	ND	ND
13	ND	-	-	-	ND	ND
14	ND	+	-	ND	+	ND
15	ND	-	-	ND	ND	ND
16	ND	-	ND	ND	ND	ND

NB: Bp -- Before paturition

ND -- Not Done

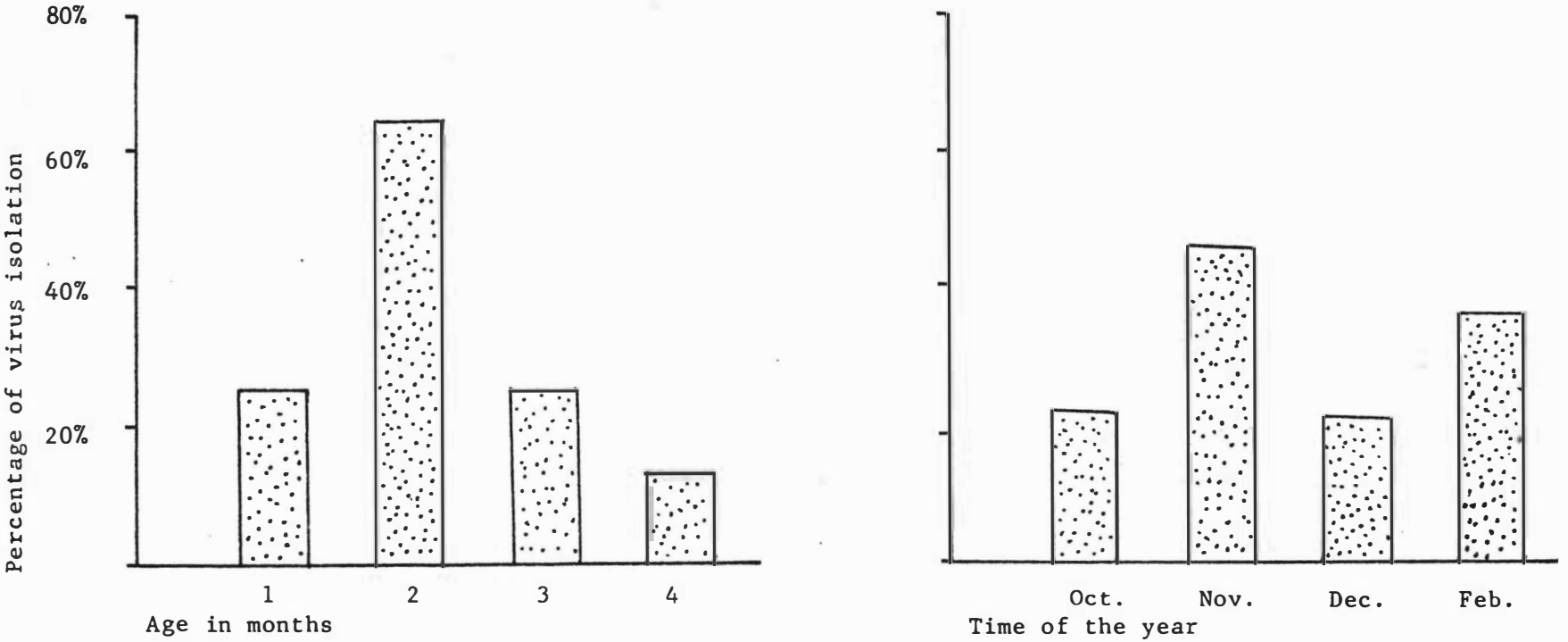


FIGURE 6 - 1. Incidence of EHV-2 infection in 16 foals with age or with month of the year.

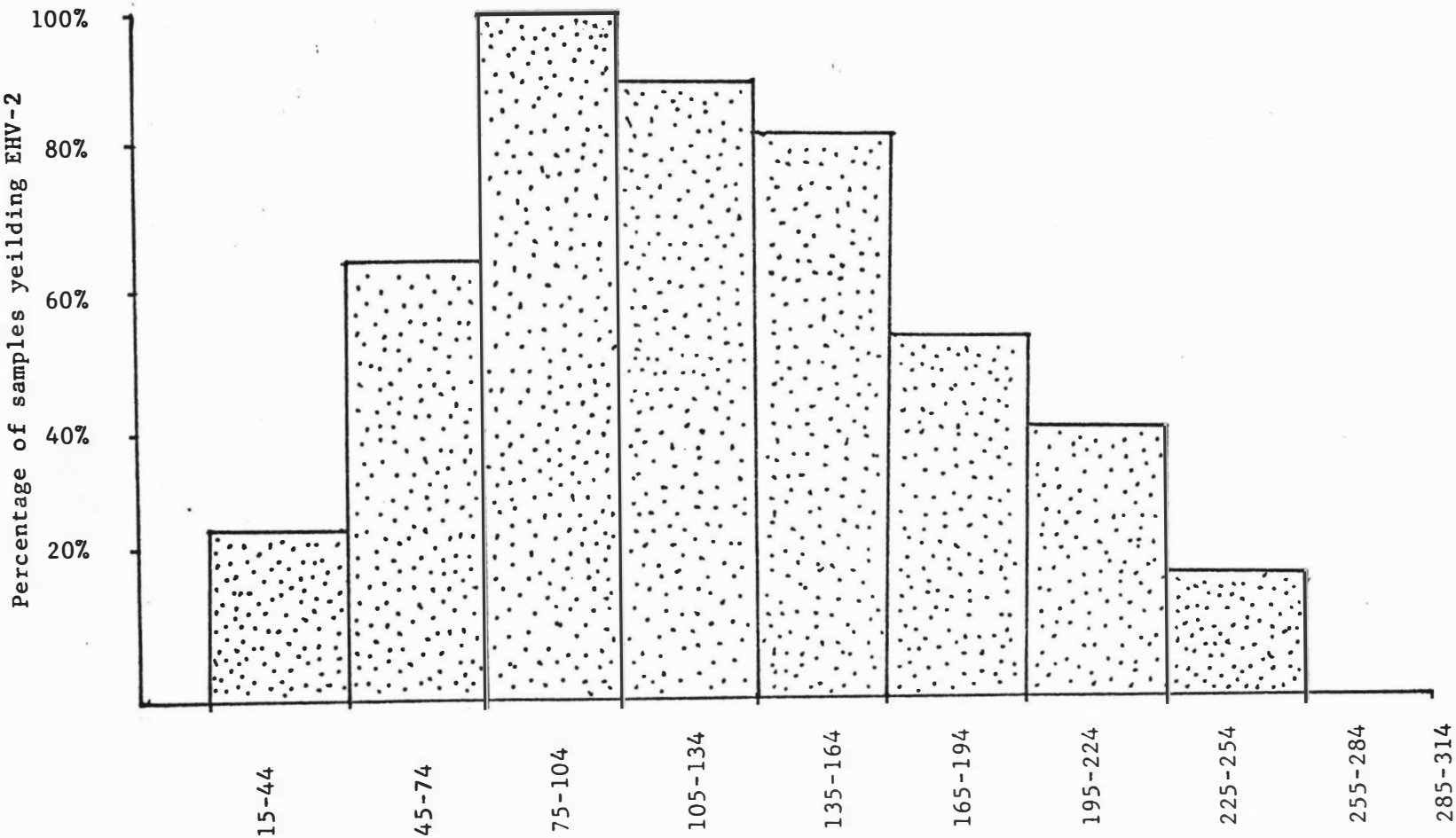


FIGURE 6 - 2. Histogram of prevalence of EHV-2 infection in 16 foals during the first ten months of life.

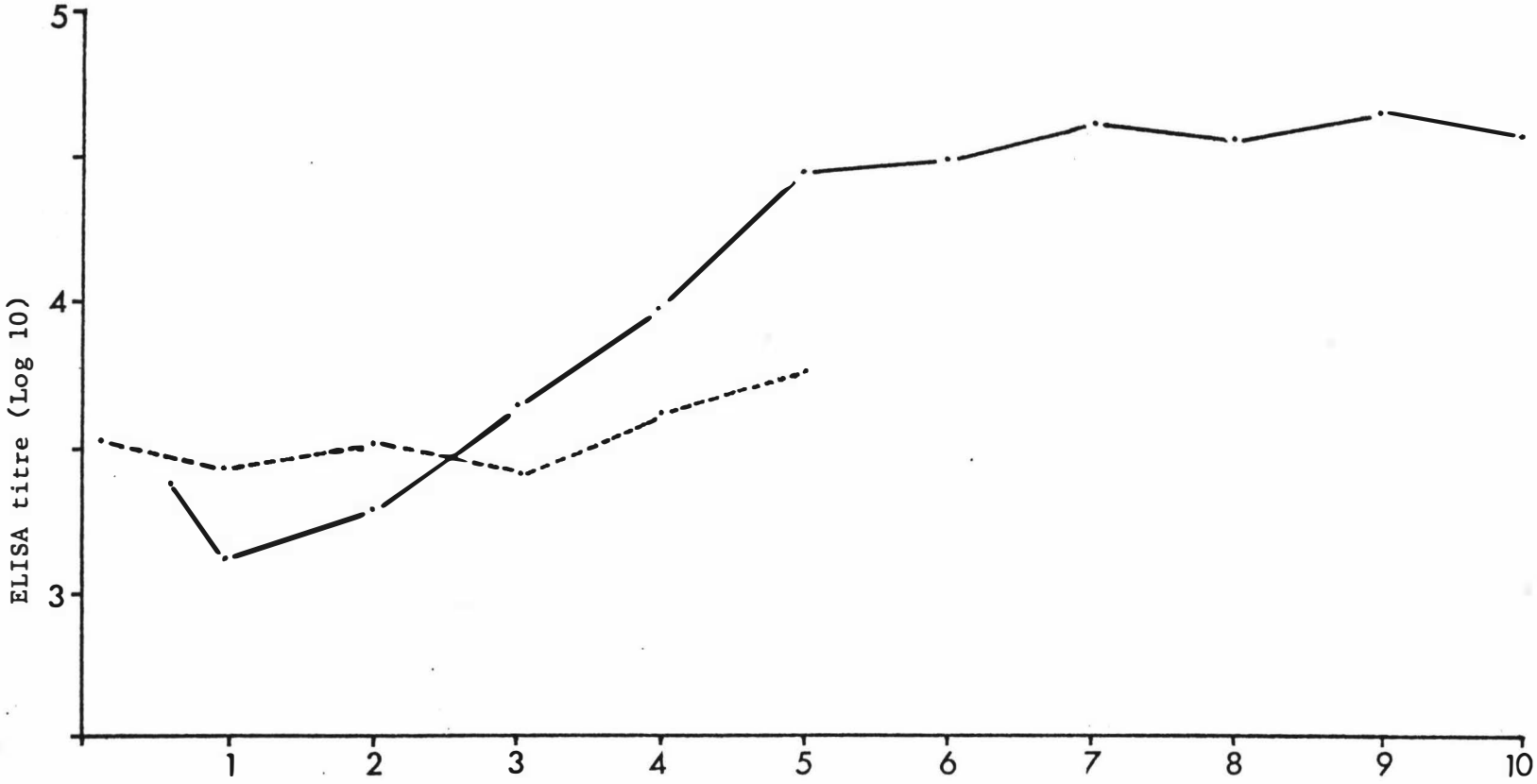


FIGURE 6 - 3. ELISA antibody titre against EHV-2 in mares (·——·) and in their foals (·- - - -·) over a period of 10 months.

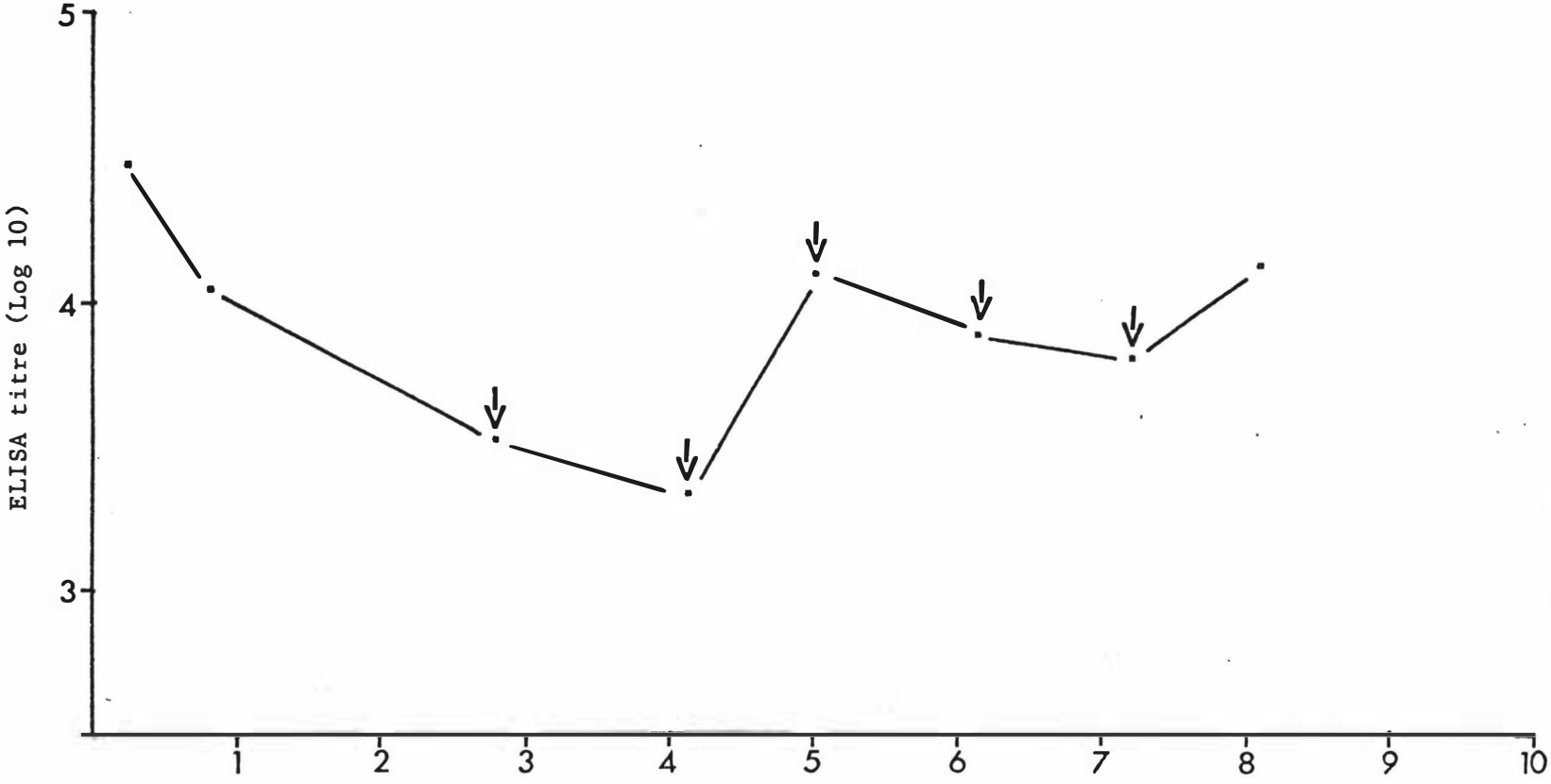


FIGURE 6 - 4. Serological response of Foal No. 7 to EHV-2 infection (↓ virus isolation).

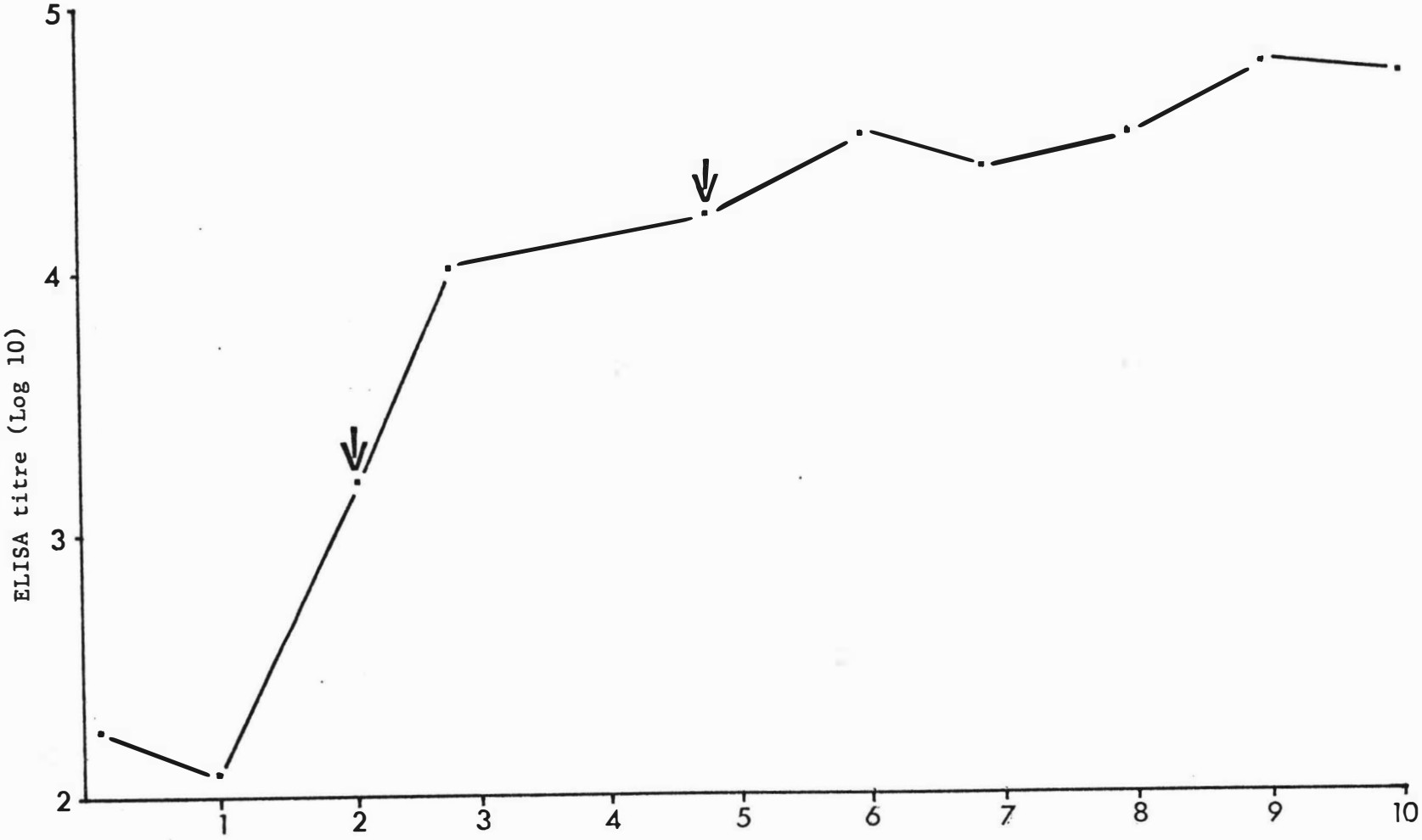
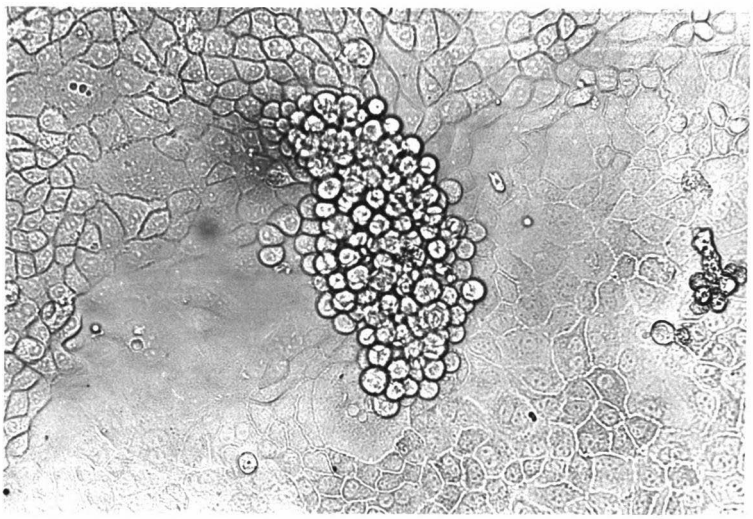


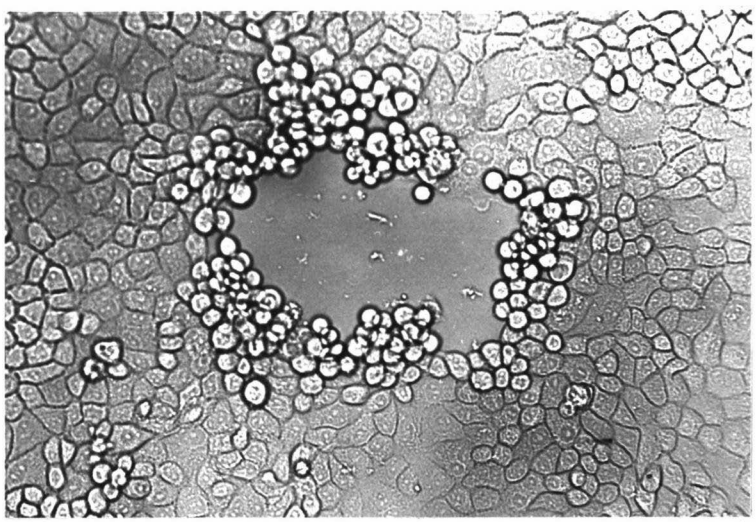
FIGURE 6 - 5. Serological response of Foal No.11 to EHV-2 infection (↓ virus isolation).

Facing page 92

Plate 6 - 1 Characteristic CPE of EHV-2 in RK -13 cells rounding of cells (3 days post infection).

Plate 6 - 2 Characteristic EPE of EHV-2 in RK-13 cells destruction of cell sheet (5 days post infection).

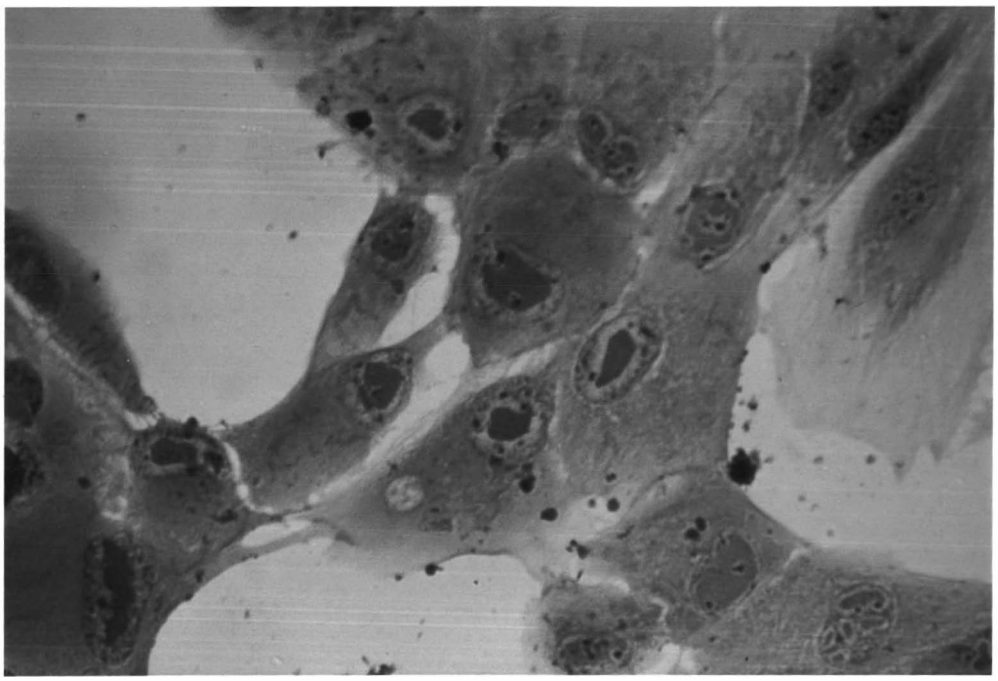


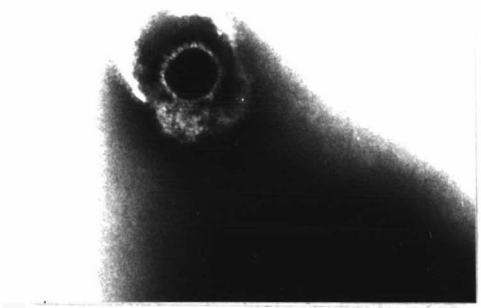


Facing page 93

Plate 6 - 3 Cowdry type A inclusion bodies in EHV-2 infected RK-13 cells (stained with H & E).

Plate 6 - 4 Typical herpesvirus morphology of equine viral isolate under the electron microscope.
Enveloped, empty partical (Magnification X 85,500).





Chapter 7

GENERAL CONCLUSION

The present investigation confirmed the presence of EHV-1 and EHV-2 on a particular stud farm in which respiratory disease had previously been a problem in foals, and gave information about the epidemiology of these infections on the property.

Serological evidence suggested that most of the foals (85%) became infected with EHV-1, and a small proportion (38%) were apparently reinfected during their first ten months of life. The infection with EHV-1 occurred at around weaning time when maternally derived antibody had declined to a non-protective level, and again at the time when the foals were approaching the end of their first year of life. This was similar to the pattern of EHV-1 infections in foals in other countries.

The contribution of EHV-1 infections to respiratory disease of these foals was, however, less than was anticipated when compared with other reports. The clinical response was limited to a profuse muco-purulent nasal discharge in most of the foals, and a few showed an elevation of body temperature at around the time of infection. Despite a large increase in antibody titres to EHV-1, the virus itself was not isolated from the foals, and this was probably because the foals ingested high levels of colostral antibodies to EHV-1 which interfered with virus recovery.

Vaccination of the foals with Pneumabort-K at 30 and 60 days of age proved to be of little value on this property, again probably due to the interference of high levels of passively acquired antibody. Since the epidemiolgical investigation revealed that EHV-1 mainly affected foals at around the time of weaning, immunization commencing at 90 days of age would, therefore, have been desirable. By this time the maternally acquired antibody should have declined to an uninhibitory level, and this vaccine

would then be more likely to induce sufficient immunity to protect them against EHV-1 infection.

In order to study the epidemiology of EHV-2 infection, an ELISA system was developed to measure serum antibodies to this virus. The test proved to be 1500 times more sensitive than the standard serum neutralisation test. It was also highly specific and had the advantage of being cheap and easy to perform.

Using this test, rises in titres against EHV-2 were demonstrated in foals at 2-4 months of age; this occurred shortly after EHV-2 was first isolated from all these animals. The infection with EHV-2 proved to be persistent, with virus being recovered over a 4-6 month period. During this time, an unusual continuous increase in antibody titres against EHV-2 was observed.

An outbreak of respiratory disease, similar to that seen in previous years, occurred in the foals in November and December when they were 2-4 months of age. It was at around this time that EHV-2 was first recovered from the foals, and a serological response to EHV-2 was first noted shortly afterwards.

From the above findings it was concluded that EHV-1 infection was probably associated with the respiratory disease which occurred at around the time of weaning. It seemed possible that EHV-2 had a contributory role in the respiratory disease regularly occurring on the stud in foals of 2-4 months of age. Further study is required to confirm and extend these observations.

APPENDICES and REFERENCES

Appendix I

COMPOSITION OF BUFFERS AND SOLUTIONS

Antibiotic-Trypsin-Versene (ATV)

0.5	gm	Trypsin (Difco 1:250)
0.2	gm	Versene (EDTA Sequestric acid)
8.0	gm	NaCl
0.4	gm	KCl
1.0	gm	Dextrose
0.58	gm	$NaHCO_3$
2×10^5	IU	Penicillin
100.0	mg	Streptomycin
0.02	gm	Penol red

Made up to 1 L with deionised distilled water. Sterilise by filtration.

Coating Buffer (0.05 M)

1.59	g	Na_2CO_3
2.93	g	$NaHCO_3$

Made up to 1 L deionised water. Final pH 9.6.

Citrate-phosphatase Buffer Stocks

A. Citric Acid Solution (0.1 M)

2.1 g Citric acid

Made up to 100 ml with deionised water.

B. Phosphate Solutioin (0.2 M)

2.84 g Na_2HPO_4

Made up to 100 ml with deionised water.

NaCl-Tris-EDTA Buffer (NTE)

5.84 g NaCl
1.21 g Tris
0.37 g EDTA

Made up to 1 L deionised water. Final pH 7.2.

PBS-Tween 20 (0.02 M)

8.0 g NaCl
0.2 g KH_2PO_4
2.9 g Na_2HPO_4 (12 H_2O)
0.2 g KCl
0.5 ml Tween 20

Made up to 1 L with deionised water. Final pH 7.4.

PBS-Tween-4%BSA

4.0 g BSA

Made up to 100 ml with PBS-Tween 20.

Penicillin, Streptomycin, Kanamycin (PSK)

10.0 gm Streptomycin
10.0 1-mega vial Penicillin
10.0 gm Kanamycin

Made up to 1 L PBS. Sterilise by filtration.

Phosphate Buffered Saline (PBS)

8.0 gm NaCl
0.2 gm KCl
1.15 gm Na_2HPO_4
0.2 gm KH_2PO_4

Made up to 1 L with deionised distilled water. Sterilise by autoclaving (15lbs for 15 minutes). Final pH 7.2-7.4.

Substrate Solution

9.72 ml 0.1 M Citric acid solution
10.28 ml 0.2 M Phosphate solution
8.0 gm OPD
8.0 ul H_2O_2
Mix above reagents together immediately before use.

Transport Medium

10.0 ml PSK
5.0 ml Amp B
0.2 g BSA
Made up to 100 ml T199. Sterilise by filtratration. Final pH 7.2. Dispense in small volumes.

Tricine Buffered 199 (T199)

10.0 gm Powered media
1.8 gm Tricine
1.5 gm $NaHCO_3$ (closed vessel)
Made up to 1 L with deionised distilled water. Sterlise by filtration. Stored at -4 ^{o}C.

Trypan Blue

0.2 gm Trypan blue powder
100.0 ml PBS
Dispense in 1.8 mls.

Appendex II

Table A-1 Reciprocal of ELISA antibody titres against EHV-1 in foals during their first ten months of life

Foal No.	Age in months										
	AB	1	2	3	4	5	6	7	8	9	10
1	ND	1828	ND	485	ND	5673	5320	4719	3445	1468	3285
2	-	1748	1216	ND	4976	ND	14876	3081	3996	4369	ND
3	-	706	362	ND	ND	3503	5054	2267	1219	2198	2444
4	ND	1670	440	DEAD							
5	ND	3578	4467	ND	3487	3035	1687	1725	1491	1108	ND
6	8785	7613	ND	1697	82	780	SOLD				
7	4798	2474	ND	369	182	-	-	1047	1650	ND	ND
8	2285	ND	451	362	ND	4817	1537	1003	3581	2528	2813
9	ND	1232	1728	ND	727	128	832	929	629	1289	ND
10	ND	5312	1298	ND	2042	556	172	81	ND	15418	ND
11	4407	2981	1070	544	ND	5664	4570	1280	1028	962	2990
12	9286	8456	ND	1362	3153	1306	652	4267	1654	ND	ND
13	ND	5074	3270	ND	3472	2208	691	396	18562	6655	ND
14	ND	3781	2009	ND	472	2085	70	-	-	10942	ND
15	ND	1408	1607	ND	3072	1756	891	385	283	343	ND
16	ND	789	DEAD								

NB: AB After birth
ND Not done
\- Negative

Table A-2 Reciprocal of ELISA antibody titres against EHV-1 in mares during the first five months of their foal's life

Mare No.	Bf	1	2	3	4	5
1	5405	4908	ND	4608	ND	6216
2	6778	11754	12108	ND	ND	ND
3	1322	955	889	ND	ND	ND
4	1015	4792	4291	ND	ND	ND
5	10814	5510	4041	ND	5842	5428
6	11945	23793	9479	ND	6721	ND
7	2472	ND	3795	1517	1591	ND
8	2704	2685	3564	4548	ND	ND
9	3186	2537	2757	ND	ND	ND
10	4066	5613	7991	ND	ND	ND
11	16349	7042	5187	7910	ND	ND
12	12590	9809	ND	4883	4645	3412
13	9421	5434	4530	ND	ND	ND
14	7583	6484	9053	ND	7219	6320
15	3088	2816	2783	ND	ND	ND
16	11823	5482	ND	ND	ND	ND

NB Bf -- Before foaling
ND -- Not done

Table A-3 Reciprocal of ELISA antibody titres against EHV-2 in foals during their first ten months of life

Foal No.	Age in days 15	30	60	90	120	150	180	210	240	270	300
1	ND	1053	ND	8267	ND	55046	43211	28232	32443	61877	45061
2	ND	4436	31870	ND	68382	53961	30019	13802	23226	22965	ND
3	ND	1864	1539	ND	ND	21990	33988	43504	19814	77146	21045
4	ND	6684	6321	DIED							
5	ND	3458	5204	ND	83264	131772	144201	890935	359050	202127	ND
6	8586	5518	ND	2759	10379	33033	ND	ND	ND	ND	ND
7	23561	11484	ND	3358	1876	12649	7564	6187	13636	ND	ND
8	1710	ND	786	4236	ND	49387	65113	35417	38868	37071	49947
9	ND	600	1759	ND	15761	45063	45984	195190	96272	107469	ND
10	ND	3323	2663	ND	3470	17519	24307	20345	13988	19904	ND
11	189	128	1653	11878	ND	16895	35519	24910	37255	65520	58108
12	994	622	ND	2070	15067	42297	64597	57882	76308	ND	ND
13	ND	391	286	ND	2890	7914	24807	46125	100315	36796	ND
14	ND	562	335	ND	1722	4633	4695	9119	4400	6366	ND
15	ND	453	1291	ND	18692	116759	58180	225900	73446	112405	ND
16	ND	184	DIED								

NB: ND -- Not done

Table A-4 Reciprocal of ELISA antibody titres against EHV-2 in mares during the first five months of their foal's life

Mare No.	BF	30	60	90	120	150
1	3440	3719	ND	2069	ND	3678
2	5753	7574	8270	ND	ND	ND
3	5913	6411	12151	ND	ND	ND
4	16066	13300	14462	ND	ND	ND
5	4667	7031	2163	ND	5183	3239
6	9264	8978	ND	10854	8862	4041
7	15320	13993	ND	10247	14029	ND
8	2134	1719	1217	1726	ND	ND
9	771	695	832	ND	ND	ND
10	3397	5475	4102	ND	ND	ND
11	872	761	788	1039	ND	ND
12	896	1080	964	ND	ND	ND
14	779	550	535	ND	659	ND
15	2175	2982	4039	ND	ND	ND
16	2110	1114	ND	ND	ND	ND

NB Bf -- Before foaling

ND -- Not done

REFERENCE

Abraham,A.; Sivanandan,V.; Newman,J.A.; Maheswaran, S.K. (1984): Rapid purification of avian influnza virus for use in enzyme-linked immunosorbent assay. Am. J. Vet. Res., 45: 959-962.

Allen, G.P.; Turtinen, L.W. (1982): Assessement of the base sequence homology between the two subtypes of equine herpesvirus 1. J. of Virology, 44: 249-255.

Allen, G.P.; Yeargan, M.R.; Turtinen, L.W.; Bryans, J.T.; McCollum, W.H. (1983): Molecular epizootiological studies of equine herpesvirus-1 infections by restrion endonuclease fingerprinting of viral DNA. Am. J. Vet. Res., 44: 263-271.

Aller, M.S.; Blakeslee, J.R. Jr. (1980): Research and Review. Immunosupression induced by equine herpesvirus II. Equine Practioner, 1: 2.

Andrewes, C.; Pereira, H.G. (1967): Virus of Vetebrates. 2nd ed. p. 283 (Bailliere, Tindall & Cassell, Ltd.).

Bagust, T.J. (1971): The equine herpesviruses. Vet. Bull., 41: 79-92.

Bagust, T.J.; Pascoe, R.R. (1970): Studies on equine herpesviruses : 1. Characterisation of a strain of equine rhinopneumonitis virus isotated in Queensland. Aust. Vet. J. 46: 421-427.

Bartha, A. (1970):The virology of equine herpesvirus 1 infection. Proc. 2nd Int. Conf. Equine Infec. Dis. Paris, 1969, pp. 18-25 (Karger, Basel).

Bass, E.P. (1979): Immunization with a modified live virus equine rhinopneumonitis vaccine and an aluminium hydroxide adsorbed equine influenza vaccine. Proc. 24th ann. Conf. Am. Ass. Equine Pract., 1978, pp. 65-74.

Belák, S.; Pálfi,V.; Tuboly, S.; Bartha, L. (1980): Passive immunization of folas to prevent respiratory disease caused by equine herpesvirus type 2. Zbl. Vet. Med. B 27: 826-830.

Blakeslee. J.R.Jr.; Olesen, R.G.; McAllister, E.S.; Fassbender, J.; Dennis, R. (1975) : Evidence of respiratory tract infection induced by equine herpesvirus, type 2, in the horse. Can. J. Microbiol., 21: 1940-1946.

Blakeslee, J.R.Jr.; Tarr, M.J.; Aller, M.S.; Lewis, N.J.; Murdick, D.; Olsen, R.(1980) Immundogical, epidemiological, and biochemical studies of equine herpesvirus 2. Obtained from Dr. M.J. Tarr, Department of veterinary pathology, Ohio State University, Columbus, Ohio.

Bloom, B.R.; Rager-Zisman, B. (1975): Cell-mediated immunity in viral infections. (chapter 7). Virul Immunology and Immunopathology. pp. 113-136. (Academic press).

Bolton, D.C.; Chu, H.J.; Ardans, A.A.; Kelly, B.; Zee, Y.C.; (1981): Evaluation of the critical parameters of a sensitive ELISA test -using purified infectious bovine rhinotracheitis virus antigens. Vet. Microbiol., 6: 265-279.

Brar, J.S.; Johnson, D.W.; Muscoplat, C.C.; Shope, R.E.; Meiske, J.C. (1978): Maternal immunity to infectious bovine rhinotracheitis and bovine diarrhea viruses: duration and effect on vaccination in young calves. Am. J. Vet. Res., 39: 241-244.

Bryans, J.T. (1969): On immunity to disease caused by equine herpesvirus 1. J.A.V.M.A., 155: 294-309.

Bryans, J.T. (1978) : Immunization of pregnant mares with an inactivated equine herpesverus 1 vaccine. Proc. 4th Int. Conf. Equine Infec. Dis., Lyon (1976), pp. 83-92 (Veterinary Publications).

Bryans, J.T. (1980a): Application of management procedures and prophylactic immunization to the control of equine rhinopneumonitis. Proc. 26th Ann. conf. Am. Ass. Equine Pract., pp 259-272.

Bryans, J.T.(1980b): Serological responses of pregnant Thoroughbred mares to vaccination with an inactivated equine herpesvirus 1 vaccine. Am. J. Vet. Res. 41: 1743-1746.

Bryans, J.T.; Doll.E.R. (1959): Multiplication of equine rhinopneumonitis virus in syrian hamstrers. Cornell Vet., 49: 525-529.

Bryans, J.T.; Prickett, M.E. (1970): A consideration of the pathogenesis of abortigenic disease caused by equine herpesvirus 1. Proc. 2nd Int. Conf. Equine Infect. Dis., Paris (1969) , pp 34-40 (Karger, Basel).

Bryans, J.T.; Swerczek, T.W.; Darlington, R.W.; Crowe, M.W. (1977) : Neonatal foal disease associated with perinatal infection by equine herpesvirus I. J. Equine Med. Surg., 1: 20-26.

Burrows, R. (1968): Rhinopneumonitis virus neutralizing antibody levels of British Thoroughbred mares. Proc. 1st Int. Conf. Equine Infect. Dis., Stresa (1966), PP. 122-130 (Grayson, Foundation, Kentucky).

Burrows, R.; Goodridge, D. (1973): In vivo and in vitro studies of equine rhinopneumonitis virus strains. Proc. 3rd Int.

Conf. Equine Infect. Dis., Paris 1972, pp. 306-321 (Karger, Basel).

Burrows, R.; Goodridge, D. (1979): Equid herpesvirus 1 (EHV-1): Some observations on the epizootiology of infection and on the innocuity testing of live virus vaccine. Proc. 24th ann. Conf. Am. Ass. Equine Pract., 1978, PP. 17-29.

Burrows, R.; Goodridge, D.(1984): Studies of persistent and latent equid herpesvirus 1 and herpesvirus 3 infections in the Pirbright pony herd. Letent herpesvirus infections in Veterinary Medicine. PP. 307-319, (Martinus Nijhoff Publishers, 1984).

Burrows, R.; Goodridge, D.; Denger, M. S. (1984): Trials of an inactivated equid herpesvirus 1 vaccine: Challenge with a subtype 1 virus. Vet. Rec. 114: 369-374.

Buxton, A.; Fraser,G. (1977): Animal Microbiology. pp 747-748. (Blackwell Scientific Publications).

Campbell, T.M.; Studdert, M.J. (1983): Equine herpesvirus type 1 (EHV-1). Vet. bull., 53: 135-146.

Castellano, G.A.; Hazzard, G.T.; Madden, D.L.; Sever, J.L. (1977): Comparison of the enzyme-linked immunosorbent assay and the indirect hemagglutination test for detection of antibodies to cytomegalovirus. J. infect. Dis., 136: suppl., pp. S337-S340.

Darlington, R.W.; James,C. (1966): Biological and morphological aspects of the growth of equine abortion virus. J. Bacteriol., 92: 250-257.

Denoyel, G.A.; Gaspar, A.; Nouyrigat,C. (1980): Enzyme immunoassay for measurement of antibodies to herpes simplex virus infection: Comparison with complement fixation,immunofluorescent-antibody, and neutralization techniques. J. clin. Microbiol., 11: 114-119.

Dimock, W.W. (1943): Equine abortion. J.A.V.M.A., 122: 282-284.

Dimock, W.W.; Edwdards, P.R. (1933): Is there a filterable virus of abortion in mares? Suppl. Res. Bull., No. 333, Ky. agr. exp. sta., Cited by G.R. Thomson in "The role of equid herpesvirus 1 in acute respiratory disease of horses and the use of inactivated antigens to immunise against the infection". (1978), Ph D thesis, University of London, London.

Dimock, W.W.; Edward, P.R.(1936): The differential diagnosis of equine abortion with special reference to a Hitherto undescribed form of epizootic abortion of mares. Cornell Vet. 26: 231-240.

Dinter, Z.; Klingebon, B.(1976): Serological study of an outbreak of paresis due to equid herpesvirus 1 (EHV-1). Vet. Rec. 99:10-12.

Dixon, R.J.; Hartley, W.J.; Hutchins, D.R.; Lepherd, E.E.; Feilen, C.; Jones, R.F.; Love, D.N.; Sabine, M.; Wells, A.L. (1978): Perinatal foal motality associated with a herpesvirus. Aust. vet. J. 54: 103-105.

Doll, E.R. (1953): Intrauterine and intrafetal inoculations with equine abortion virus in pregnant mares. Cornell Vet., 43: 112-123.

Doll, E.R. (1961): Immunization against viral rhinopneumonitis of horses with live virus propagated in hamsters. J. A. V. M. A., 139: 1324-1330.

Doll. E.R. (1972): Viral pneumonitis. Equine Medicine and Surgery. 2nd edition., pp. 28-35 (American Veterinary Publications).

Doll, E.R.; Bryans, J.T. (1962a): Incubation period for abortion in equine rhinipneumonitis. J.A.V.M.A., 141: 351-354.

Doll, E.R.; Bryans, J.T. (1962b):Development of complement-fixing and virus-neutralizing-antibodies in viral pneumonitis of horses. Am. J. Vet. Res., 23: 843-846.

Doll, E.R.; Bryans, J.T. (1963a): Epizootiology of equine viral rhinopeumonitis. J.A.V.M.A., 142: 31-37.

Doll, E.R.; Bryans, J.T. (1963b): Immunization of young horses against viral rhinopnemonitis. Cornell Vet., 53: 24-41.

Doll, E.R.; Bryans, J.T. (1963c): A planned infection program for immunizing mares against viral rhinopneumonitis. Cornell Vet., 53: 249-262.

Doll, E.R.; Bryans, J.T.; McCollum, W.H.(1959b): A procedure for evaluating the antigenicity of killed virus vaccines for equine rhimopneumonitis. Cornell Vet. 49: 212-220.

Doll, E.R.; Bryans, J.T.; McCollum, W.H.; Crowe, M.E.W. (1957): Isolation of a filterable agent causing arteritis of horses and abortion by mares, its differentiation from the equine abortion (influenza) virus. Cornell Vet., 47: 3-42.

Doll, E.R.; Kintner, J.H. (1954): A comparative study of the equine abortion and equine influenza viruses. Cornell Vet. 44: 355-367.

Doll, E.R.; McCollum, W. H.; Bryans, J.T.; Crowe, M.E.W. (1959a): Effect of physical and chemical environment on the viability of equine rhinopneumonitis virus propagated in hamsters. Cornell Vet., 49: 75-81.

Doll, E.R.; Richards, M.G.; Wallace, M.E. (1953): Adaption of the equine abortion virus to Syrian hamsters. Cornell Vet. 43: 551-558.

Doll, E.R.; Richards, M.G.; Wallace, M.E. (1954): Cultivation of the equine influenza virus in suckling Syrian hamsters. Its

similarity to the equine abortion virus. Cornell Vet., 44: 133-138.

Doll, E.R.; Richards, M.G.; Wallace, M.E.; Bryans, T.J. (1952): The influence of an equine fetal tissue vaccine upon hemagglutination activity of mare serums: Its relation to hemotytic icterus of new born foals. Cornell Vet. 42: 495-505.

Dutta, S.K.; Campbell, D.L. (1978): Pathogenicity of equine herpesvirus in vivo pesistance in equine tissue macropheges of herpesvirus types 2 detected in monolayer macrophage cell culture. Am. J. Vet. Res., 39: 1422-1427.

Dutta, S.K.; Myrup,A.C.(1983): Infectious center assay of intracellular virus and infective virus titre for mononuclear cells infected in vivo and in vitro with equine herpesvirus. Can. J. Comp. Med., 47: 64-69.

Dutta, S.K.; Myrup, A.; Bumgardner, M.R. (1980): Lymphocyte Responses to virus and mitogen in Ponies during experimental infection with Equine herpesvirus 1 Am. J. Vet. Res. 41: 2066-2068.

Dutta, S.; Shipley, W.D.(1975) : Immunity and the level of neutralization antibodies in foals and mares vaccinated with a modified live-virus rhinopneumonitis vaccine. Am. J. Vet. Res., 36:445-448.

Dutta, S.K.; Talbot, N.C.; Myrup, A.C.(1983): Detection of equine herpesvirus-1 antigen and the specific antibody by enzyme-linked immunosorbent assay. Am. J. Vet. Res., 44: 1930-1934.

Engvall, E. (1977): Quantitative enzyme immunoassay (ELISA) in microbiology. Med. Biol., 55:193-200.

Engvall, E.; Perlmann,P. (1971): Enzyme-linked immunosorbent

assay (ELISA): Quantitative assay of immunoglobulin. G. Immunochemistry 8: 871-874.

Epistein, M.A.; Achong, B.G.(1977): Pathogenesis of infections mononucleosis. Lancet, ii, 1270-1273.

Erasmus, B.J. (1968): The activation of horses virus infections of the respiratory tract in horses by immunization against African Horse Sickness. Proc. 1st Int. Conf. Equine Infec. Dis., Stresa (1966), pp. 117-123. (Grayson, Foundation, Kentucky).

Erasmus, B.J. (1970): Equine Cytomegalovirus. Proc. 2nd Int. Conf. Equine Infec. Dis., Paris (1969). pp. 46-55. (Karger, Basel)

Feldman, H.A.; Wang, S.S. (1961): Sensitivity of various viruses to chloroform. Proc. Soc. exp. Biol. Med., 106: 736-738.

Flammini; C.F.; Allegic, G.(1972): herpesvirus from equine kidney tissue culture. Achivio Veterinario Italino. 23: 93-99.

Fouad, M.T.A. (1965): Studies on equine rhinopneumonitis virus with particular reference to some physico-chemical and cytopathological characteristic when propagated in porcine kidney cell tissue cultures. Dissertation abstracts vol. 25, no. 8, pp.4360.

Frymus, T. (1980): Humoral and cell-mediated immune response of foals vaccinated with attenuated equine herpesvirus type 1 (EHV-1). Zbl. Vet. Med. B 27: 742-758.

Gerber, J.D.; Marron, A.E.; Bass, E.P.; Beckenhauer, W.H.(1977) : Effect of age and pregnancy on the antibody and cell-mediated immune responses of horses to equine herpesvirus 1. Can, J. Comp. Med., 41: 471-478.

Gilman, S.C.; Docherty, J.J.(1977): Detection of antibodies specific for herpes simplex virus in human sera by the

enzyme-linked immunosorbent assay. J. infect. Dis., 136: suppl., pp.S286-S293.

Girard, A., Greig, A.S.; Mitchell, D. (1968): A virus associated with vulvitis and balanitis in the horse - A preliminary report . Can. J. Comp. Med., 32: 603-604.

Gleeson, L.J.; Coggins, L. (1980): Response of pregnant mares to equine herpesvirus 1 (EHV-1). Cornell Vet., 70: 391-400.

Gleeson, L.J.; Studdert, M.J. (1977): Equine herpesviruses. Experimental infection of a foetus with type 2. Aust. Vet. J., 53: 360-362.

Greenwood, E.S.; Simson, A.R.B. (1980): Clinical report of a paralytic syndrome affecting stallions, mares and foals on a Thoroughbred studfarm. Equine Vet. J., 12: 113-117.

Harden, T.J.; Bagust, T.J.; Pascoe, R.R.; Spradbrow, P.B. (1974): Studies on equine herpesvirus . 5. Isolation and characterisation of slowly cytopathic equine herpesviruses in Queensland. Aust. Vet. J., 50: 483-488.

Hartley, W.J.; Dixon, R.J. (1979): An outbreak of foal perinatal mortality due to equid herpesvirus type 1: Pathological observations. Equine vet. J., 11: 215-218.

Henle, W; Henle, G. (1979): Seroepidemiology of the virus. The Epstein-Barr Virus. pp. 61-78 (Springer, Berlin, Heidelberg & New York).

Herrmann, J.E.; Hendry, R.M.; Collins, M.F. (1979): Factors involved in enzyme-linked immunoassay of viruses and evaluation of the method for identification of enteroviruses . J. clin. Microbiol., 10:210-217.

Horner, G.W.; (1981): Serological relationship between abortifactient and respiratory strains of equine herpesvirus

type 1 in New Zealand. N.Z. Vet. J., 29: 7-8.

Horner, G.W.(1985): A disease syndrome in Thoroughbred foals resembling infectious mononucleosis of man associated with equine herpesvirus type 2. Equine Vet. J.,in press.

Horner, G.W.; Hunter, R.; O'Flaherty, J.D.; Dickinson, L.G. (1976): Isolation of equine herpesviruses from horses with respiratory disease. N.Z. Vet. J., 24: 171-176.

Hsiung, G.D.; Fischman, H.R.; Fong, C.K.Y.; Green, R.H. (1969): Characterization of a cytomegalo-like virus isolated from spontanously degenerated equine kidney cell culture. Proc. Soc. exp. Biol. Med., 130: 80-84.

Huttons, J.B.; Durham, P.J.K. (1977): Lett. ed. , N.Z. vet. J., 25: 42.

Ishizaki, R.; Shimizu, T.; Matumoto, M.(1962): Sequential development of antigens of equine rhinopneumonitis virus in cultured horse kidney cells as studied with flourescent antibodies. Arch. ges. Virusforsch. 12: 346-362.

Jackson, T.; Kendrick, J.W. (1971): Paralysis of horses associated with equine herpesvirus 1 infection. J.Am. Vet. Med. Ass., 158: 1351-1357.

Jeffcott, L. B. (1972): Passive immunity and its transfer with special reference to the horse. Biological Reviews, 47: 439-464.

Jelev, W. (1957): [Histopathology of foetuses in equine virus abortion, with special reference to differential diagnosis. Arch. exp. VetMed., 11: 906-920. Abstract in Vet. Bull., 28: 501.

Jolly, P.D. (1983): The development of an enzyme linked immunosorbent assay for the detection of antibodies to equid

herpesvirus type 1 in horse sera. B. Phil. Thesis.Massey University. New Zealand.

Jolly, P.D.; Robinson, A.J.; Fu, Z.F. (1985): Disease caused by EHV-1 in horses - A review. N.Z. Vet. J., In press.

Kaji, T.(1963): Epizootiological investigation of equine rhinopneumonitis by complement fixation test. III. Chronological change of infection. Nat. Inst. Anim. Hlth.Quat., Tokyo, 3: 115-120.

Karpas, A.(1966): Characterization of a new herpes-like virus isolated from foal kidney. Annales Inst. Pasteur, Paris, 110:688-696.

Kawakami, Y.; Kaji, T.; Ishizaki, R.; Shimizu, T.; Matumoto, M.;(1962): Etiology study on an outbreak of acute respiratory among colts due to equine rhinopneumonitis virus. Jap. J. exp. Med.,32:211-229.

Kawakami, Y.; Shimizu, T.(1978): Combined immunizing effects of live and inactivated equine herpesvirus 1 in horses. Proc. 4th Int. Conf. Equine Infec. Dis., Lyon (1976), pp. 75-82 (Veterinary Publications).

Kawakami, Y.; Shimizu, T.; Murase, N.(1974): Vaccination of mares against abortion due to equine rhinopneumonitis virus. Bull. Nat. Inst. Anim. Hlth., No.69:13-17. Summary in Nat. Inst. Anim. Hlth. Quat. Tokyo, 14: 209.

Kawakami, Y.; Tokui, T.; Nakano, K.; Kume, T.; Hiramune, T.; Murase, N.(1970): An outbreak of abortion due to equine rhinopneumonitis virus among mares in the Hidaka district, Hokkaido. I. Epizootiological survey and virus isolation. Bull. Nat. Inst. Anim. Hlth. No. 61, 9-16 (1970). Summary in Nat. Inst. Anim. Hlth. Quat. Tokyo, 10:173-174.

Kemeny, L.; Pearson, J. E.(1970): Isolation of herpesvirus from

equine leukocytes: Comparison with equine rhinopeumonitis virus. Can. J. Comp. Med., 34:59-65.

Kemp, M.C.; Perdue, M. L.; Rogers, H.W.; O'Calleghan, D.J.; Randall, C. C.(1974): Structural polypeptides of the hamster strain of equine herpesvirus type 1: Products associated with purification. Virology, 61:361-375.

Kendrick, J. W.; Stevenson, W.(1979): Immunity to equine herpesvirus 1 infection in foals during the first year of life. J. of Reproduction and Fertility. Suppl., 27: 615-618.

Kester, W. O.(1976): Equine pharyngitis report. Am. Ass. Equine Pract. News Letter, No. 3: 19-25.

Kono,Y.; Kobayashi,K.(1964): Cytopathogenic equine orphan (CEO) virus in horse kidney cell culture. I. Isolation and properties. Nat. Inst. Anim. Hlth. Quat. Tokyo, 4:10-20.

Little, P.B.; Thorsen , J. (1976): Disseminated necrotizing myeloencephalitis: A herpes-associated neurological disease of horses. Vet. Pathol., 13: 161-171.

Lowry, O.H.; Rosebrough, N.J.; Farr, A.L.; Randall, R.J.(1951): Protein measurement with the Folin phenol reagent. J. Biol. Chem., 193:265-275.

Matumoto, M.; Ishizaki, R.; Shimizu, T. (1965): Serologic survey of equine rhinopneumonitis virus infection in various countries. Arch. ges. Virusforsch., 15: 609-624.

Maurer, F. D.; Jones, T.C.(1943): The blood picture in equine influenza. Am. J. Vet. Res., 4: 257-264.

Mayr, A.; Bohm, H. O.; Brill, J.; Woyciechowska (1965): [Characteristics of an equine abortion virus from Poland, as compared with those of a kowmn equine rhinopneumonitis.] (English summary). Arch. ges. Virusforsch, 17: 216-230.

Mayr, A. (1970): Vaccination of horses against equine herpesvirus 1 infection. Proc. 2nd Int. Conf. Equine Infec. Dis., Paris (1969), PP. 13-17 (Karger, Basel).

Mayr, A.; Pette, J.; Petzoldt, K.; Wagener, K.(1968): [Development of a live vaccine against equine rhinopneumonitis (abortion of mares)]. Zbl. VetMed. B15: 406-418. Abstract in Vet. Bull., 38: 686.

McAllister, E.S.; Blakeslee, J.R.Jr.(1977): Clinical observations of pharyngitis in the horses. J. A. V. M. A., 170:739-741.

McCollum, W.H.; Doll, E.R.; Wilson, J.C.; Johnson, C.B. (1962): Plaque formations by equine rinopneumonitis virus on monolayer cell cultures of equine, ovine, and porcine kidneys. Cornell Vet., 52: 534-542.

McGee, W.R. (1970): Clinical aspects of disease caused by equine herpesvirus 1. Proc. 2nd Int. Conf. Equine Infec. Dis., Paris (1969), PP. 13-17 (Karger, Basel).

Miranda, Q.R.; Bailey, G.D.; Fraser. A.S.; Tenoso, H.J. (1977): Solid-phase enzyme immunoasseay for herpes simplex virus. J. infect. Dis., 136: suppl, PP. S304-S310.

Moore, B.O.; Koons, H. J. (1979): Inactivated equine herpesvirus I vaccine- Pneumabort-K. Proc. 24th ann. Conf. Am. Ass. Equine Pract., 1978, PP. 75-80.

Mumford, J.A., Bates, J. (1984): Trails of an inactivated equine herpesvirus 1 vaccine: Challenge with a subtype 2 virus. Vet. Rec., 114: 375-381.

Mumford, J.A.; Rosedale, P.D. (1980): Virus and its ralationships to the "pool performence" syndrome. Equine Vet. J., 12: 3-9.

Mumford, J.A.; Thomson, G.R. (1976): Serological methods for identification of slowly growing herpesvirus isolated from the

respiratory tract of horses. Proc. 4th. INt. Conf. Equine Infec. Dis., Lyon (1976), PP.49-52 (Veterinary Publications).,

Neely, D.P.; Hawkins, D.L. (1978): A two-year study of the clinical and serological responses of horses to a modified live-virus equine rhinopneumonitis vaccine. J. Equine Med. Surg., 2: 532-540.

O'Callaghan, D.J.; Allen, G.P.; Randall, C.C. (1978): Structure and replication of the equine herpesvirus. Proc. 4th Int. Conf. Equine Infec. Dis., Lyon (1976), PP. 1-31 (Veterinary Publications).

O'Callaghan, D.J.; Randall, C.C. (1976): Molecular anatomy of herpesviruses: recent studies. Prog. Med. Virology, 22: 152-210.

Ogra, P.L.; Morag, A.; Tiku, M.L. (1975): Humoral immune response to viral infections. (chapter 4), Viral Immunology and Immunopathology. pp. 57-77. (Academic Press).

Pachciarz, J.A.; Bryans, J.T. (1978): Cellular immunity to equine rhinopneumonotis virus in pregnant mares. Proc. 4th Int. Conf. Equine Infec. Dis., Lyon (1976), PP. 115-127 (Veterinary Publications).

Pálfi, V.; Belák, S.; Molnar, T. (1978): Isolation of equine herpesvirus type 2 from foals showing respiratory symptoms. Brief report. Zbl. Vet. Med., B 25: 165-167.

Papp-vid, G.; Derbyshire, J.B. (1978): The protective antigens of equine herpesvirus type 1. Can. J. Comp. Med.,42: 219-226.

Papp-vid, G.; Derbyshire, J.B. (1979): The virus neutralizinh activity of antibodies specific to the envelope and nucleocapsid of equine herpesvirus type 1. Can. J. Comp. Med., 43: 231-233.

Patel, J.R.; Edington, N. (1983): The pathogenicity in mice of respiratory, abortion and paresis isolates of equine herpesvirus-1. Vet. Microbiol., 8: 301-305.

Patel, J.R.; Edington, N.; Mumford, J.A. (1982): Variation in cellular tropism between isolates of equine herpesvirus-1 in foals. Archives of Virology, 74: 41-51.

Peacock, G.V. (1969): Biological requirments and control of equine rhinopneumonitis vaccine (live virus). J. A. V. M. A., 155: 310-314.

Pearce, H.G.; Alley, M.R. (1976): Failure to demonstrate equine rhinopeumonitis virus as a cause of abortion in mares in New Zealand. N.Z. Vet. J., 24: 127-131.

Perdue, M.L.; Kemp, M.C.; Randall, C.C.; O'Callaghan, D.J. (1974): Studies of the molecular anatomy of the L-M cell strain of equine herpesvirus type 1: Proteins of the nucleocapsid and intact virion. Virology, 59: 201-216.

Platt, H.; Singh, H.; Whitwell, K.E. (1980): Pathological observations on an outbreak of paralysis in broodmares. Equine Vet. J., 12: 118-126.

Plummer, G. (1964): Serological comparison of the herpes viruses. Br. J. exp. Path., 45: 135-141.

Plummer, G.; Goodheart, C.R.; Henson, D.; Bowling, C.P. (1969): A comparative study of the DNA density and behavior in tissue cultures of fouteen different herpesviruses. Virology, 39: 134-137.

Plummer, G.; Goodheart, C.R.; Studdert, M.J. (1973): Equine herpesviruses: antigenic relationships and deoxyribonucleic acid densities. Infect Immunity, 8: 621-627.

Plummer, G.; Watson, A.P. (1963): Equine herpes viruses.

Virology, 19: 412-416.

Powell, D.G. (1975): Equine infectious respiratory disease. Vet. Rec., 96: 30-34.

Powell, D.G.; Burrows, R.; Spooner, P.R.; Goodridge, D.; Thomson, G.R.; Mumford, J.A. (1976): A study of infectios respiratory disease among horses in Great Britain, 1971-1976. Proc. 4th Int. Conf. Equine Infec. Dis., Lyon (1976), pp. 451-460 (Veterinary Publications).

Prickett, M.E. (1970): The Pathology of disease caused by equine herpesvirus 1. Proc. 2nd Int. Conf. Equine Infec. Dis., Paris (1969), pp. 24-33 (Karger, Basel).

Purdy, C.R.; Ford; S.J.; Grant, W.F. (1977): Equine rhinopneumonitis virus (herpesvirus type 1): attenuation in stable monkey cell line. Am. J. Vet. Res., 38: 1211-1215.

Purdy, C.R.; Ford; S.J.; Porter. R.C. (1978): Equine rhinopneumonitis vaccine: immunogenecity and safety in adult horses including pregnant mares. Am. J. Vet. Res. 39: 377-383.

Randall, C.C.; McVickar, D.L.; Doll, E.R. (1950): A complement fixation test for equine virus abortion. Pro. Soc. exp. Biol. Med., 75: 465-468.

Rapp, F.; Jerkofsky, M.A. (1973): Persistent and latent infections. (chapter 9), The Herpesviruses. (Academic Press).

Rasmussen, P.G. (1966): Isolation and identification of herpes equine virus from horses on Danish Thoroughbred studs. Nord. VetMed., 18: 95-102.

Roberts, A.W.; Whitenack, D.L.; Carter, G.R. (1974): Recovery of adenoviruses and slow herpesviruses from horses having respiratory tract infection. Am. J. Vet. Res., 35:

1169-1172.

Roberts, R.S. (1965): A paralytic syndrome in horses. Vet. Rec., 77: 404-405.

Roeder, P.L.; Scott, G.R. (1975): The prevalence of equine herpes virus 2 infections. Vet. Rec., 96: 404-405.

Roizman, B. (1973): Provisional labels for herpes virus. J. gen. Virol., 20: 417-419.

Russell, W.C.; Crawford, L.V. (1964): Properties of the nucleic acids from some herpes group viruses. Virology, 22: 288-292.

Sabine, M.; Feilen, C.; Herbert, L.; Jones, R.F.; Lomas, S.W.; Love, D.; Wild, J. (1983): Equine herpesvirus abortion in Australia 1977 to 1982. Equine Vet. J., 15: 366-370.

Sabine, M.; Robertson, G.R.; Whallen, J.M. (1981): Differentiation of sub-types of equine herpesvirus 1 by restriction endonuclease analysis. Aust. Vet. J., 57: 148-149.

Saunders, G.C.; Clinard, E.H.; Bartlett, M.L.; Sanders, W.M. (1977): Application of the indirect enzyme-labeled antibody microtest to the detection and surveillance of animal diseases. J. Infect. Dis., 136 suppl: S258-S266.

Savigny, D. de; Voller, A. (1980): The communication of ELISA data from laboratory to clinician. J. Immunoassay, 1: 105-128.

Saxegaard, F. (1966): Isolation and identification of equine rhinopneumonitis virus (equine abortion virus) from cases of abortion and paralysis. Nord. VetMed., 18: 504-512.

Semerdjiev, B. (1962): Haemagglutination by equine abortion virus. Zbl. Bakt. 1, 185: 316-324. Abstract in Vet. Bull., 33:

188.

Shenman, J.; Thorsen, J.; Barnum, D.; Mitchell, W.R.; Ingram, D.G. (1977): Infectios causes of equine respiratory disease on Ontario Standardbred racetracks. J. Clinical. Microbiol., 5: 285-289.

Shimizu, T.; Ishizaki, R.; Kono, Y.; Ishii, S.; Matumoto, M. (1959): Propagation of equine abortion virus in horse kidney tissure culture. Jap. J. exp. Med., 27: 175-180.

Shimizu, T.; Ishizaki, R.; Kumanomido, T. (1974): Combined immunizing effects of live and inactivated equine rhinopneumonitis virus in colts. Bull. Nat. Inst. Anim. Hlth., 69: 5-8. Summary in Nat. Inst. Anim. Hlth. Quat. Tokyo, 14: 208.

Shimizu, T.; Kawakami, Y.; Kaji, T.; Ishizaki, R.; Sugimura, K.; Matumoto, M. (1961): Experimental infection of colts with equine rhinopneumonitis virus. Natn. Inst. Anim. Hlth. Quat., Tokyo, 1: 119-125.

Snowdon, W.A. (1965): The IBR-IPV virus: Reaction to infection and intermittent recovery of virus from experimentally infected cattle. Aust. Vet. J., 41: 135-145.

Soehner, R.L.; Gentry, G.A.; Randall, C.C. (1965): Some physicochemical characteristics of equine abortion virus nucleic acid. Virology, 26: 394-405.

Studdert, M.J. (1971): Equine herpesviruses. 4. Concurrent infection in horses with strangles and conjunctivitis. Aust. Vet. J., 47: 434-436.

Studdert, M.J. (1974): Comparative aspects of equine herpesviruses. Cornell Vet., 64: 94-122.

Studdert, M.J. (1983): Restriction endonuclease DNA fingerprinting

of respiratory, foetal and perinatal foals isolates of equine herpesvirus type 1. Archives of Virology, 77: 249-258.

Studdert, M.J.; Blackney, M.H. (1979): Equine herpesvirus: On the differentiation of respiratory from foetal strains of type 1. Aust. Vet. J., 55: 488-492.

Studdert, M.J.; Fitzpatrickl, D.R.; Horner, G.W.; Westbury, H.A.; Gleeson, L.J. (1984): Molecular epizootiology and pathogenesis of equine herpesvirus type 1 (equine abortion virus) and type 4 (equine rhinopneumonitis virus) isolates. Aust Vet. J., 61: 345-348.

Studdert, M.J.; Simpson, T.; Roizman, B. (1981): Differentiation of respiratory and abortigenic isolates of equine herpesvirus 1 by restriction endonucleases. Science, 214: 562-564.

Studdert, M.J.; Turner, A.J.; Peterson, J.E. (1970): Equine herpesviruses. 1. Isolation and characterisation of equine rhinopneumonitis virus and other equine herpesviruses from horses. Aust. Vet. J., 46: 83-89.

Sugiura, T.; Fukuzawa, Y.; Kamada, M.; Ando, Y.; Hirasawa, K. (1983): Isolation of equine herpesvirus type 2 from foals with pneumonitis. Bull. Equine Res. Inst., 20: 148-153.

Thein, P. (1978): The association of EHV-2 infection with keratitis and research on the occurrence of equine coital exanthema (EHV-3) of horses in Germany. Proc. 4th Int. Conf. Equine Infec. Dis., Lyon (1976), pp. 33-42 (Veterinary Publications,).

Thein, P. (1981): Infection of the central nervous system of horses with equine herpesvirus serotype 1. J. of the South African Veterinary Association, 52: 239-241.

Thein, P.; Hechler, H.; Mayr, A. (1981): [Comparative studies on the activity of paramunity induced by PIND-AVI, mitogen PHA-D

and rhinopneumonitis virus on the peripheral lymphocytes of the horses]. (English summary). Zbl. Vet. Med., B28: 432-449.

Thomson, G.R. (1978): The role of equid herpesvirus 1 in acute respiratory disease of horses and the use of inactivated antigens to immunise against the infection. Ph. D Thesis, University of London, London.

Thomson, G.R.; Mumford, J.A. (1977): In vitro stimulation of foals lymphocytes with equine herpesvirus 1. Research in Veterinary Science, 22: 347-352.

Thomson, G.R.; Mumford, J.A.; Campbell, J.; Griffiths, L.; Clapham, P. (1976): Serological detection of equid herpesvirus 1 infections of the respiratory tract. Equine Vet. J., 8: 58-65.

Thomson, G.W.; McCready, R.; Sanford, E.; Gagnon, A. (1979): An outbreak of herpesvirus myeloencephalitis in vaccinated horses. Can. Vet. J., 20: 22-25.

Turner, A.J.; Studdert, M.J.; Peterson, J.E. (1970): Equine herpesvirus 2. Persistence of equine herpesviruses in experimentally infected horses and the experimental induction of abortion. Aust. Vet. J., 46: 90-98.

Turtinen, L.W.; Allen, G.P. (1982): Identification of the envelope surface glycoproteins of equine herpesvirus type 1. J. gen. Virol., 63: 481-452.

Turtinen, L.W.; Allen, G.P.; Darlington, R.W.; Bryans, J.T. (1981): Serological and molecular comparisons of several equine herpesvirus type 1 strains. Am. J. Vet. Res., 42 2099-2104.

Voller, A.; Bidwell, D.E. (1976): Enzyme-immunoassays for antibodies in measles, cytomegalovirus infections and after rubella vaccination. Br. J. exp. Pathol., 57: 243-247.

Voller, A.; Bidwell, D.E.; Bartlett, A. (1979): The Enzyme Linked Immunosorbent Assay (ELISA): A guide with abstracts of microplate applications. Sponsored by, and available from: Dynatech Europe (Guernsey, Great Britain).

Wharton, J.H.; Henry, B.E.; O'Callaghan, D.J. (1981): Equine cytomegalovirus: Cultural characteristics and properties of viral DNA. Virology, 109: 106-119.

Wilks, C.R.; Coggins, L. (1976): Immunity to equine herpesvirus type 1 (rhinopneumonitis): In vitro lymphocyte response. Am. J. Vet. Res., 37: 487-492.

Wilks, C.R.; Coggins, L. (1977a): Protective immunity in equine herpesvirus type 1 infection of hamsters. Cornell Vet., 67: 385-403.

Wilks, C.R.; Coggins, L. (1977b): In vitro cytotoxicity of serum and peripheral blood leycocytes for equine herpesvirus type 1-infected target cells. Am. J. Vet. Res., 38: 117-121.

Wilks, C.R.; Studdert, M.J. (1974): Equine herpesviruses. 5. Epizootiology of slowly cytopathic viruses in Foals. Aust. Vet. J., 50: 438-422.

Woodruff, J.F.; Woodruff, J.J. (1975): The effect of viral infection of the immune system. (chpter 22). Viral Immunology and Immunopathology. Notkins, A.L. ed. PP. 393-418 (Academic Press).