# Testing for passive transfer of immunity in foals, and an evaluation of the African horse sickness vaccination schedule

Linnet Jean Isobel Crow

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Animal Science and Poultry Science
School of Agricultural Sciences and Agribusiness
Faculty of Science and Agriculture
University of KwaZulu-Natal
Pietermaritzburg
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I hereby certify that this research is the result of my own investigation. Where use was made of the work of others it has been duly acknowledged in the text. The results in this dissertation have not been submitted, in whole or in part, for a degree at any other University.

LCrow.

**Linnet Jean Isabel Crow**
August 2005

I hereby release this thesis for examination in my capacity as supervisor.

MBYoung

**Ms M B Young**
August 2005

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# PREFACE

This thesis comprises an introductory review of the literature, followed by reports of two experiments which are presented in the form of scientific papers. For this reason, there may be some repetition between chapters, particularly in terms of experimental procedure. To avoid unnecessary repetition, a single list of references is given at the end of the thesis. For the sake of completeness, several appendices are attached to Chapters Two and Three which would not ordinarily be included in a scientific paper.

The literature review looks at the passive transfer of immunity from the mare to the foal, the consequences of failure of passive transfer of immunity and different methods of testing whether the transfer of passive immunity has occurred. The review concludes with a discussion of vaccination programmes against African horse sickness.

Trial One evaluated different tests for determining whether the transfer of passive immunity from mare to foal has occurred in order to determine which of these tests should be used preferentially. A single radial immunodiffusion test was used as the reference standard. A series of samples was taken from a group of foals and tested using four methods: single radial immunodiffusion, glutaraldehyde coagulation, zinc sulphate turbidity and protein refractometer tests.

Trial Two explored African horse sickness vaccination programmes, focusing on when to vaccinate foals for the first time. A series of samples was taken from a group of foals from birth until two months after their second set of African horse sickness vaccinations (one year old). The samples were tested for the presence of African horse sickness antibodies for each of the nine serotypes to determine when maternal immunity fades and to evaluate the effect of each vaccination on the level of immunity.

# CHAPTER 1

# LITERATURE REVIEW

## 1.1 Introduction

Unlike cattle and sheep, which have been selected for reproductive efficiency for many generations, horses are selected solely on performance characteristics; for example, movement for dressage, jumping ability, racing ability and looks. This has made horse breeding reproductively inefficient and makes the foals that are born all the more valuable. It is therefore surprising that it was not until the 1980's that interest in equine neonatology increased, and the contention that a weak foal is a dead foal, or worth no more than a dead foal, was questioned (Koterba *et al.*, 1990). Equine neonatology can be described in simple terms as intensive care of foals, but also includes methods of early identification of problems and treatment to prevent the need for intensive care. This includes the identification of cases where the transfer of passive immunity may be problematic and the early identification of foals in which failure of passive transfer of immunity (FPT) has occurred (Koterba *et al.*, 1990). Passive transfer of immunity is defined as "a form of acquired immunity induced by the transfer of immune serum containing specific antibodies or of sensitized lymphoid cells from an immune to a non-immune recipient host" (Cruse & Lewis, 1995).

Jeffcott (1972) summarised the history of immunology as follows. Von Behring and Ehrlich pioneered immunology in the early 1890's. In 1892, Ehrlich recognized the important differences between active and passive immunity. Before 1900, colostrum was considered either detrimental to the newly born foal, or to have beneficial purgative qualities (Blaine, 1832). In 1900, Ransom indicated the importance of colostrum, when he found that the first milk contains the most antibodies. The significance of this, in the transfer of immunity, was only recognized in 1912 by Famulener. He showed that goats have no antibodies in their serum at birth but that there is a rapid rise in serum antibody levels after the ingestion of colostrum. He also showed that the absorption of antibodies only occurs in the first few days of the goat's life. In 1922, Smith and Little further demonstrated the importance of colostrum to the survival of the newborn. In an experiment, they found that 75 to 80% of calves deprived of colostrum died, while all the control calves, which received colostrum, survived.

In 1923, Kuttner and Ratner (cited in Jeffcott, 1972) suggested that there is a correlation between placental structure and permeability to antibodies. This explained a number of discrepancies in the early reports of placental permeability to antibodies. In 1938, Schneider and Szathmary (cited in Jeffcott, 1972) described four groupings of animals,

based on placental structure. They placed horses, pigs, cattle and goats in the first group, which has no transfer of antibodies through the placenta. Of the three routes of passive transfer of immunity (absorption by the endodermal cells of the yolk sac, absorption through the placenta, and colostrum), the only one that operates in the horse is postnatal transfer through the colostrum. This was confirmed by Ikeda (1924), Bardelli (1930) Leme'tayer *et al.* (1946a, b) (all cited in Jeffcott, 1972). Mason *et al.* (1930) found no detectable placental transmission of immunoglobulins in the mare.

The equine placenta is epitheliochorial (Morel, 1993). This means that the placenta consists of six cell layers - three on each of the maternal and foetal sides (Figure 1.1) with an intervillous space between the foetal and maternal layers (Mullins, pers. comm., 2006). This structure means that the mare's placental epithelium remains intact. The allontois chorion lies against the mare's epithelium and villi and microvilli project into crypts in the endometrium (Mullins, pers. comm., 2006). Although metabolite exchange between the mare and foal circulations does occur, blood components, particularly large molecules, such as maternal antibodies, are unable to pass through the equine placenta (Morel, 1993). The foal therefore attains very little immunity, if any, *in utero* (Morel, 1993). The mare is able to give the newborn foal passive immunity, or a level of temporary protection against common microorganisms which have challenged her immune system, through the colostrum (Jeffcott, 1974a). Passive transfer of immunity through the colostrum plays an important protective role against infectious diseases in the foal (McGuire *et al.*, 1975; Clabough, 1990 cited in Brown *et al.*, 1991 and Stoneham *et al.*, 1991). Therefore colostrum containing low or borderline levels of immunoglobulin G (IgG) poses a significant health risk to the foal (Zou *et al.*, 1998). The colostrum supplies immunity from birth until the foal's own immune system is functioning at its maximum capacity at an age of three to four months (Morel, 1993). Combined immunodeficiency disease is a genetically inherited condition. Foals born with this condition are unable to produce immunoglobulins of their own (Hayes, 1996). Foals with combined immunodeficiency disease illustrate the protective effects of the maternal antibodies quite clearly. The foals appear clinically normal for the first month, while maternal antibodies are present, and then become highly susceptible to fatal infections (Poppie & McGuire, 1976).

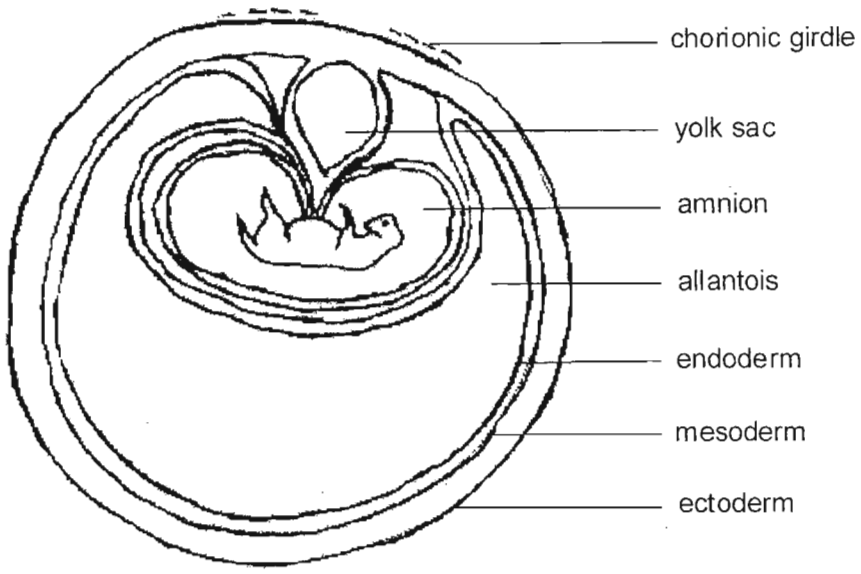


**Figure 1.1** Figure showing the structure of the equine placenta. Note the six cell layers (Morel, 1993).

## 1.2 Transfer of passive immunity

Active immunity is defined as the immunity resulting from the administration of an antigen, i.e. the antibodies are produced by the animal itself (Tizard, 2000), as opposed to passive immunity which is defined as the immunity acquired by the transfer of pre-formed antibodies (Davies, 1997). Passive transfer of immunity provides the foal with temporary protection against microorganisms to which the mare has been exposed. In horses, this protection is supplied through the colostrum. The mammary gland selectively concentrates immunoglobulins from the blood (Figure 1.2). ImmunoglobulinG (IgG) is present in the colostrum from the time that the colostrum is first available (Pearson *et al.*, 1984). The immunoglobulin levels in the milk of healthy mares are insignificant by 24 hours *post partum* (Jeffcott, 1974a). The immunoglobulins are absorbed by epithelial cells in the foal's small intestine. These cells are replaced by mature epithelial cells within 38 hours (Jeffcott, 1973, cited in Jeffcott, 1974a). By 24 hours *post partum*, the new cells are unable to absorb the large immunoglobulin molecules so it is important that the foal receives the colostrum early (Jeffcott, 1974a).

Immunoglobulins are absorbed by the epithelial cells of the small intestine, by pinocytosis. Each cell absorbs as much as it can, before discharging the immunoglobulins (and other molecules) into the intracellular space. The molecules pass into the lymphatic system and from there into systemic circulation (Figure 1.3) (Jeffcott, 1972 and 1974a).

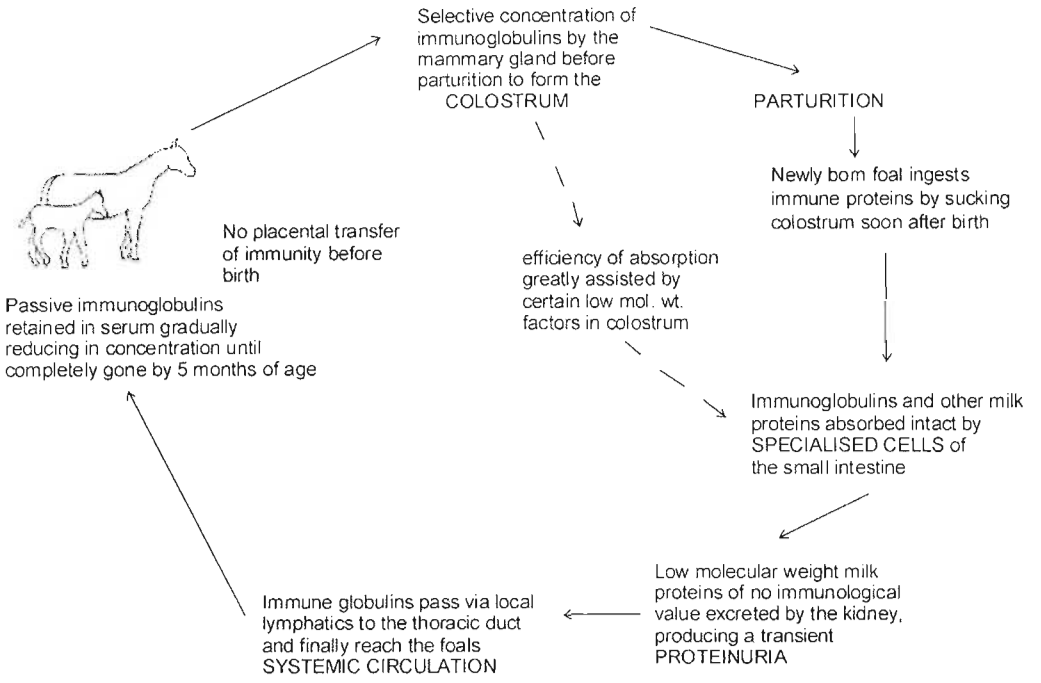


**Figure 1.2** Pathway of transfer of maternal immunity to the foal (Jeffcott, 1974a)

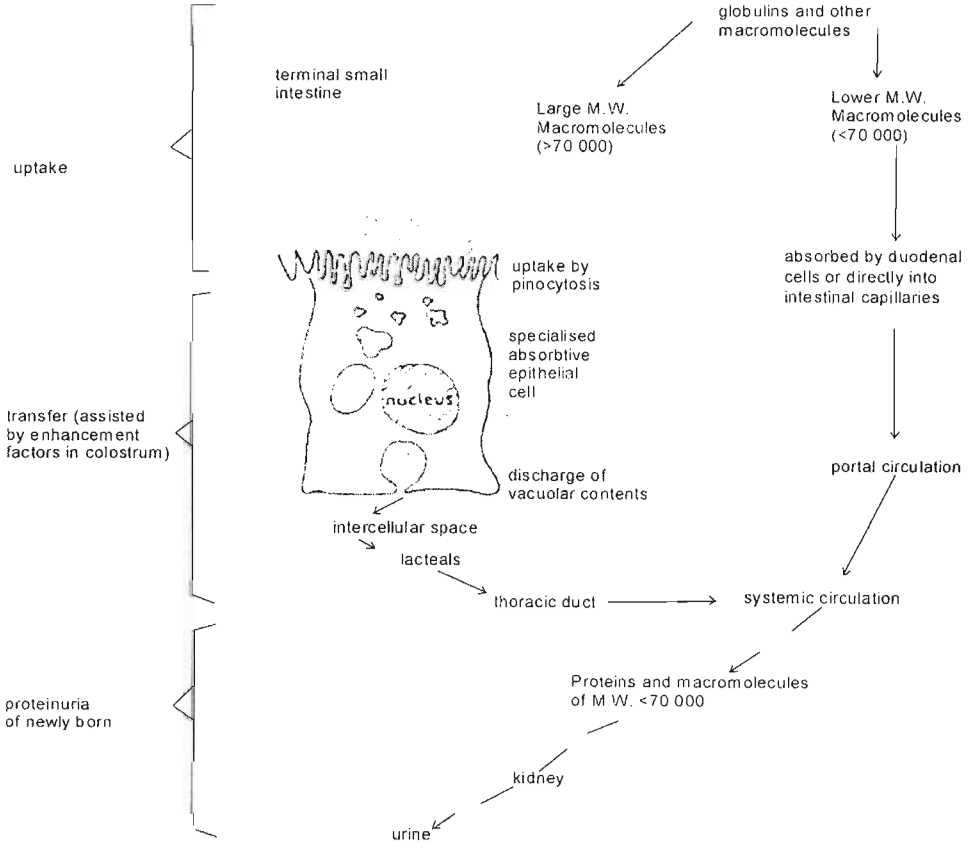


**Figure 1.3** Schematic representation of absorption of globulins and other macromolecules (Jeffcott, 1972)

In foal serum, IgG levels have reached a peak by 12 to 18 hours after the foal has suckled for the first time. The lowest level is reached four to five weeks later, and the levels then rise again to a second peak at six to eight months, remaining steady until the end of the first year (Jeffcott, 1974b). In a trial by Jeffcott (1974b), one group of foals was allowed to suckle normally (Group 1), a second group was prevented from suckling but fed supplementary colostrum and hyperimmune serum (Group 2), and the third group was deprived of colostrum and fed milk substitutes (Group 3). The pattern in IgG levels for the first two groups was as described above and the only difference between the groups was that the second group had higher levels of serum IgG. In contrast, the third group had no serum IgG until the second week, followed by a steep rise until seven or eight weeks with the level then steady until the end of the first year (Figure 1.4).

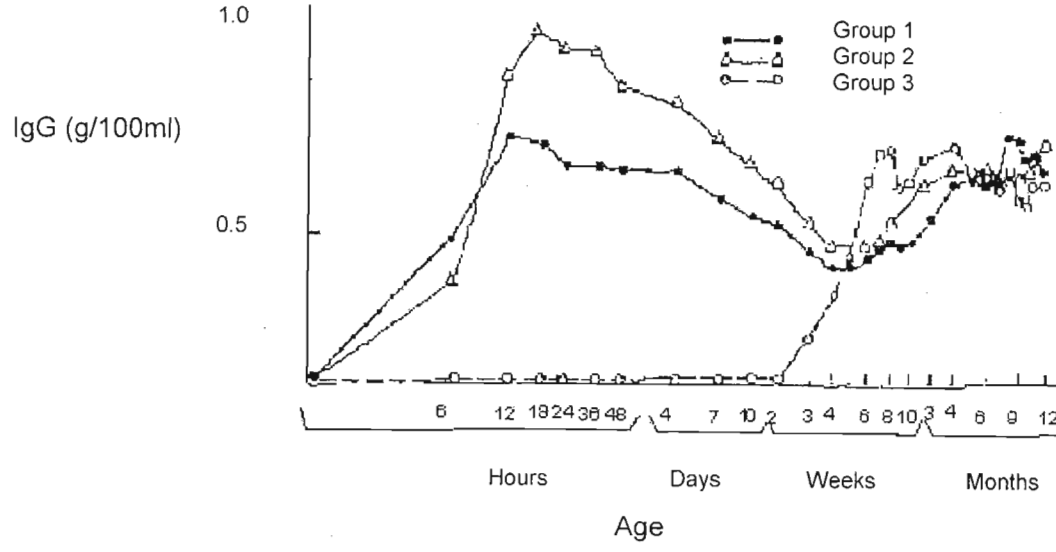


**Figure 1.4** Immunoglobulin G levels in colostrum-fed and colustrum-deprived foals from birth to 1 year (Jeffcott, 1974b); Group 1 = normal suckling; Group 2 = no suckling and supplementary colostrum and hyperimmune serum; Group 3 = no colostrum.

## 1.3 Failure of passive immunity (FPT) and factors affecting passive transfer of immunity.

The general definition of failure of passive transfer (FPT) is that FPT occurs when the foal has inadequate blood levels of IgG (Brown *et al.*, 1991). A more specific definition of FPT is a blood count of less than 200 mg IgG per 100 ml serum. Partial failure of passive transfer (PFPT) may be defined as less than 400 mg IgG per 100 ml serum (McGuire *et al.*, 1977; Rumbaugh *et al.*, 1979 and Perryman & Crawford, 1979; Crawford

& Perryman, 1980 and Perryman, 1981 all cited in Pearson *et al.*, 1984). In 1990, Ley *et al.* wrote "the level of IgG transfer that is considered adequate is open to debate". Their reasoning was that, although 400 mg per 100 ml serum was generally considered adequate, high risk foals would benefit from higher levels of IgG. In addition the half-life of maternal antibody is reported to be 21 to 24 days (Jeffcott, 1974a), so that a foal with a 24 hr IgG level of 400 mg per 100 ml serum will have 200 mg IgG per 100 ml serum at three weeks of age and a predisposition to humoral immunodeficiency at one month of age until the foal's own immune system matures. Ley *et al.* (1990) suggested that more than the immediate neonatal period of the foal needs to be considered when defining FPT, as a foal with an initial IgG of 800 mg per 100 ml serum will be less predisposed to developing infectious diseases at one to two months of age than foals with a lower initial IgG level. More recently, the definition has been changed to less than 400 mg IgG per 100 ml serum for FPT and less than 800 mg IgG per 100 ml serum for PFPT (LeBlanc *et al.*, 1992; Wilkins & Dewan-Mix, 1994; Raidal, 1996 and Tyler-McGowan *et al.*, 1997).

Failure of passive transfer results in an increased likelihood of the foal developing infectious diseases (McGuire *et al.*, 1977 and Clabough *et al.*, 1991). Raidal (1996) found a significant association between the foal's immune status and the incidence of infectious disease. In contrast, no significant association was found between the incidence of diarrhoea and the immune status of the foal. The reason for this is probably that diarrhoea is usually associated with non-infectious causes.

### 1.3.1 Inadequate ingestion of colostrum

There are many factors that affect the occurrence of FPT. The most important of these is inadequate ingestion of colostrum. Premature lactation by the mare is one of the most common causes of FPT as the foal may not receive enough colostrum (Jeffcott, 1972; Jeffcott, 1974a; Rumbaugh *et al.*, 1979; Kruse-Elliot & Wagner, 1984 cited in LeBlanc *et al.*, 1992; Varner & Vaala, 1986 cited in Lee *et al.*, 1992 and Jeffcott, 1987 cited in Lee *et al.*, 1992). Possible reasons for premature lactation are placentitis, placental separation or twinning (Jeffcott, 1974a).

If the IgG concentration in the colostrum is low, then FPT may still occur even if the foal receives a large amount of colostrum (Jeffcott, 1972; Kruse-Elliot & Wagner, 1984 cited in LeBlanc *et al.*, 1992; Varner & Vaala, 1986 cited in Lee *et al.*, 1992 and Jeffcott, 1987 cited in Lee *et al.*, 1992).

The quantity of colostrum ingested and the time after birth that it is ingested are also important. The quantity ingested affects the amount of IgG the foal will receive. However, this is not a linear relationship as other factors including colostrum quality also

affect the IgG absorption (Jeffcott, 1972; Kruse-Elliot & Wagner, 1984 cited in LeBlanc *et al.*, 1992; Varner & Vaala, 1986 cited in Lee *et al.*, 1992 and Jeffcott, 1987 cited in Lee *et al.*, 1992).

The time between birth and drinking the colostrum is important because the intestines become impermeable to large molecules such as milk proteins and immunogloblins (Jeffcott, 1972; Jeffcott, 1974a; Rumbaugh *et al.*, 1979; Varner & Vaala, 1986 cited in Lee *et al.*, 1992 and Jeffcott, 1987 cited in Lee *et al.*, 1992). If the foal suckles late, then FPT is likely to occur. Reasons for the foal suckling late include a foal that is born weak (Jeffcott, 1972), with abnormalities (Varner & Vaala, 1986 cited in Lee *et al.*, 1992; Jeffcott, 1987 cited in Lee *et al.*, 1992 and Raidal, 1996) or suffering from neonatal maladjustment syndrome (NMS) (Varner & Vaala, 1986 and Jeffcott, 1987, both cited in Lee *et al.*, 1992). Neonatal maladjustment syndrome is a non-infectious, central nervous system disorder of newborn foals that is associated with gross behavioural abnormalities (Koterba *et al.*, 1990).

Another important factor in FPT is gestation length. As gestation length falls below 335 days or exceeds 345 days, so colostrum and foal serum IgG concentrations decrease (Clabough *et al.*, 1991 and Kruse-Elliot & Wagner, 1984 cited in LeBlanc *et al.*, 1992). Longer gestation lengths may lead to low serum levels of IgG because of premature closure of the foal's gastrointestinal tract to macromolecules. If gestation is short, low serum levels may result because of inadequate amounts of IgG in the colostrum (Clabough *et al.*, 1991 and Kruse-Elliot & Wagner, 1984 cited in LeBlanc *et al.*, 1992).

### 1.3.2 Other factors

A foal may receive a seemingly sufficient quantity of high quality colostrum and FPT may still result. This would occur when the small intestine fails to absorb the immunoglobulins (Varner & Vaala, 1986 and Jeffcott, 1987 both cited in Lee *et al.*, 1992). McGuire *et al.* (1975) suggested that failure to absorb ingested immunoglobulins could be due to a factor in the mammary secretions affecting absorption, or an intrinsic defect in the foal's absorption mechanisms. Another possible reason is stress at birth (Morris, 1968 cited in Jeffcott, 1974a and Rumbaugh *et al.*, 1979). This may explain the findings of Clabough *et al.* (1991) and Raidal (1996) that the type of delivery (normal or assisted (dystocia)) affects the incidence of FPT.

The incidence of FPT is greater in premature or dysmature foals than in normal foals (Jeffcott, 1974a; Rumbaugh *et al.*, 1979; Varner & Vaala, 1986 cited in Lee *et al.*, 1992 and Jeffcott, 1987 cited in Lee *et al.*, 1992). A premature foal is a foal born at a gestational age of younger than 320 days, which displays immature physical

characteristics. A dysmature foal is a full term but immature foal that is usually undersized (Koterba *et al.*, 1990). The time in the season when the mare foals affects the incidence of FPT (Clabough *et al.*, 1991 and Raidal, 1996). A possible explanation for this may be that serum IgG levels increase with total solar radiation (LeBlanc, 1990). Mare age and parity also affect FPT (Clabough *et al.*, 1991). LeBlanc *et al.* (1992) found that FPT occurs more frequently in foals whose dams are greater than 15 years old.

Failure of passive transfer may be deliberately caused by human intervention when the foal is not allowed to drink from the mare because isosensitization is possible (Varner & Vaala, 1986 and Jeffcott, 1987 both cited in Lee *et al.*, 1992). Isosensitization is a condition similar to the rhesus negative condition in humans. The pregnant mare becomes sensitized to the foetal red cell antigens inherited from the stallion and produces antibodies, which are transmitted to the foal via the colostrum. Failure of passive transfer is therefore a consequence of preventing the severe and often fatal haemolytic anaemia through haemagglutination and haemolysis of erythrocytes (Jeffcott, 1974a).

Other less important factors in causing FPT have been identified. Very rarely, the mare does not produce colostrum containing IgG (Jeffcott, 1974a). If the mare is clinically ill, then FPT may occur (Varner & Vaala, 1986 and Jeffcott, 1987 both cited in Lee *et al.*, 1992). The sex of the foal may affect the transfer of passive immunity. Raidal (1996) found that male foals have lower circulating IgG levels than females. Induction of parturition may adversely affect the transfer of passive immunity but research is inconclusive here (Townsend *et al.*, 1983).

## 1.4 Testing for transfer of passive immunity

Testing for the transfer of passive immunity can be done on the mare's colostrum or the foal's blood.

### 1.4.1 Testing colostrum samples

One of the methods of testing for the transfer of passive immunity is to test the mare's colostrum. The colostrum needs to be sampled soon after the foal suckles as the protein levels in the colostrum fall steadily after suckling until negligible amounts are reached between 18 and 24 hours later (Jeffcott, 1974b).

Pearson *et al.* (1984) recommended collecting colostrum within eight hours of parturition. This allows the foal time to acquire passive transfer of immunity, but the colostrum has

not yet been diluted by milk. These researchers suggested using single radial immunodiffusion, to test the quality of the colostrum as described by Mancini *et al.* (1965) and discussed further in Chapter 1.4.2.

LeBlanc *et al.* (1986) described an alternative method of testing the colostrum. The colostral specific gravity was found to be a good indicator of foal serum levels, 24 hours after parturition. The specific gravity of the colostrum is measured using a modified hydrometer (Figure 1.5). The hydrometer consists of a specimen compartment with a graduated scale (pycnometer), in a water-filled column. The specific gravity is determined by the depth at which the graduated scale of the pycnometer settles in the water column. This instrument is known as a colostrometer and is calibrated so that distilled water at 24°C has a specific gravity of 1.0 at room temperature (24°C). The colostrum needs to cool before being tested, and on very hot or cold days the colostral reading needs to be adjusted for an accurate measurement of the specific gravity. If the colostral specific gravity is less than 1.06, the foal is more likely to have serum IgG levels of less than 800 mg per 100 ml serum (FPT) than if the colostral specific gravity is greater than 1.06 (Tyler-McGowan *et al.*, 1997).

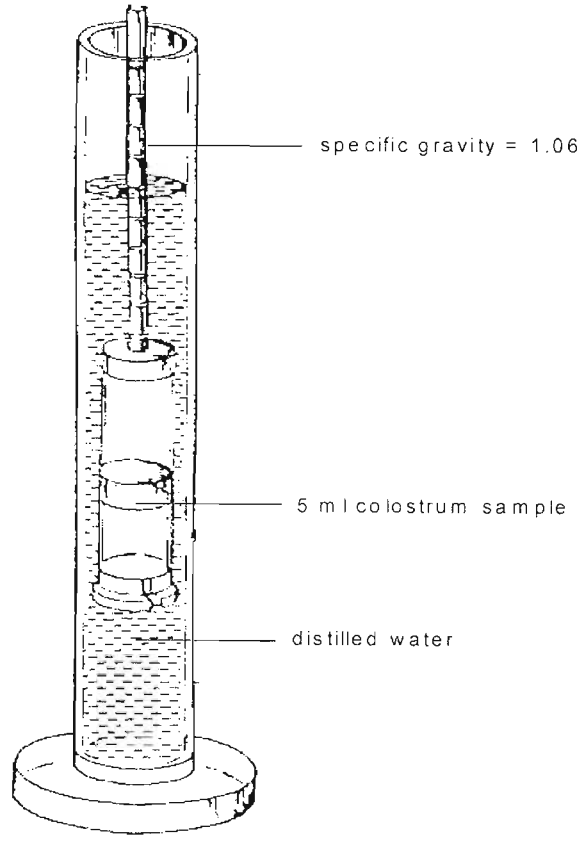


**Figure 1.5** Colostrometer used for determining the specific gravity of colostrum (LeBlanc *et al.*, 1986).

### 1.4.2 Testing blood samples

Although colostrum quality is an important factor in the development or prevention of FPT, the involvement of other factors, such as the time between birth and first suckling, the amount of colostrum that the foal drinks, failure to absorb immunoglobulins, etc., suggest that it may be better to test the foal directly. This would involve measuring the blood serum levels of IgG (Clabough *et al.*, 1991).

Total serum protein (TSP) refractometry is a method of measuring the total protein in the serum (McBeath *et al.*, 1971). This test uses the principle that the refractive index of a solution is determined by its concentration. Proteins are the major components of blood serum and so affect the concentration (McBeath *et al.*, 1971), thereby affecting the refractive index of the serum. A few drops of a serum sample are placed onto the face of the prism and the serum protein scale read (Figure 1.6) (McBeath *et al.*, 1971). McBeath *et al.* (1971) found a positive correlation (r= 0.723, t > 0.1) between the refractometer reading and serum IgG in calves.

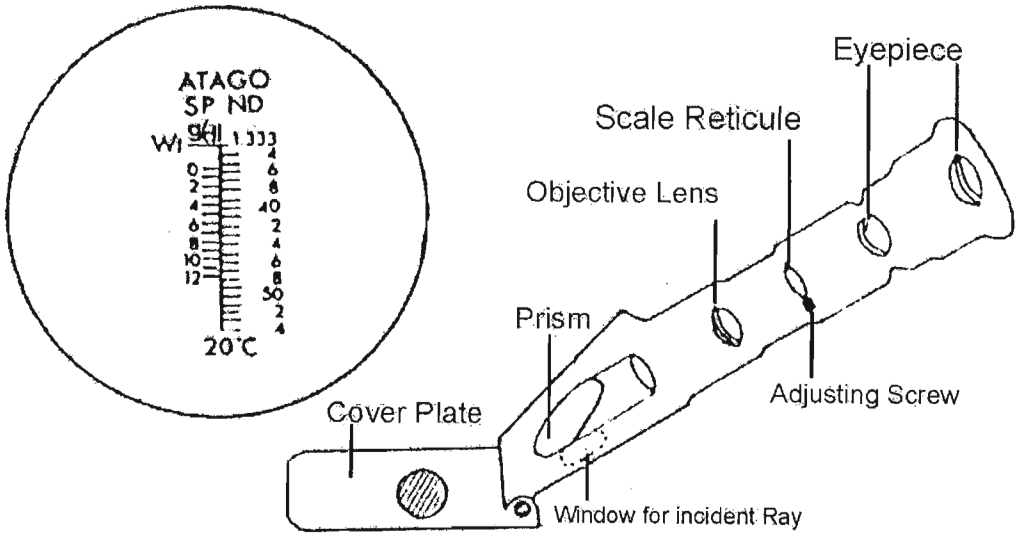


**Figure 1.6** Refractometer for measuring the total protein in serum. The inset shows the serum protein scale on the left (McBeath *et al.*, 1971).

Reid and Clifford (1974) found a positive correlation (r= 0.774, p < 0.001) between the refractometer and another method of evaluation, the zinc sulphate turbidity (ZST) test (discussed below). Their conclusion was that, for lambs, the refractometer is an effective although indirect method of measuring serum IgG. Reid and Martinez (1975) found that, as the serum albumin levels of young calves and lambs are fairly constant,

the refractometer gives an acceptable indication of the amount of globulin present in the serum. They found that a refractometer reading of 7.0 or higher indicates successful passive transfer of immunity. Rumbaugh *et al.* (1978) worked with mares and foals and found a correlation of 0.58 between the refractometer and single radial immunodiffusion (discussed below). However, the points were widely dispersed in relation to the line (Figure 1.7). This means that serious errors could result if total protein values are used to estimate the neonatal foal's IgG status. The measurement of total serum assumes that a low total serum protein after suckling equates to a failure of passive transfer of immunity, and further assumes that the serum protein concentration is constant from foal to foal. Serum protein concentration varies greatly from foal to foal, and some foals have a higher value pre-suckling than others have post-suckling. This overlapping of values makes it difficult to interpret the TSP results and makes it an unreliable test (Rumbaugh *et al.*, 1978). LeBlanc (1990) evaluated the total serum protein or refractometer test as follows: "Total protein is not useful in the foal for evaluating the IgG level. Total protein varies greatly between individuals and is influenced by too many factors to accurately estimate the IgG level."

The zinc sulphate turbidity (ZST) test is a simple and rapid method of testing serum IgG levels. The ZST test is a measure of the total serum immunoglobulin. It is based on the formation of a visible precipitate when immunoglobulin combines with zinc ions. Serum (0.1 ml) is added to 6.0 ml of a $ZnSO_4$ solution (208 mg/l) (McEwan *et al.*, 1970, cited in Jeffcott, 1974a). Results can be estimated visually or with a spectrophotometer. Serum IgG levels are estimated to be greater than 400 mg/100 ml serum when newsprint cannot be read through the test tube. The advantages of this test are that it is inexpensive and the results are available within an hour. A disadvantage is that there is a high incidence of false positive results, possibly because of haemolysis in the serum sample or unknown factors (non-specific precipitation) (LeBlanc, 1990). For this reason, the test is best used as an initial screening test and a more dependable, but slower test, used as verification (LeBlanc, 1990).

The latex agglutination test (LAT) is based on the observation of an agglutination reaction when latex particles are mixed with serum or blood containing IgG. The advantages of this method are that serum or whole blood can be used and the incidence of false positives is much lower than with the ZST test (LeBlanc, 1990). However, the test is affected by temperature and must be administered according to manufacturers instructions (LeBlanc, 1990). This test is not readily available in South Africa.

The glutaraldehyde coagulation test involves the addition of 50 µl of 10% glutaraldehyde to 0.5 ml of serum. The serum is then observed for clotting (Beetson *et al.*, 1985 and Clabough *et al.*, 1989). The clot is formed by cross-linking of the immunoglobulin molecules by glutaraldehyde (Jones & Brook, 1995).

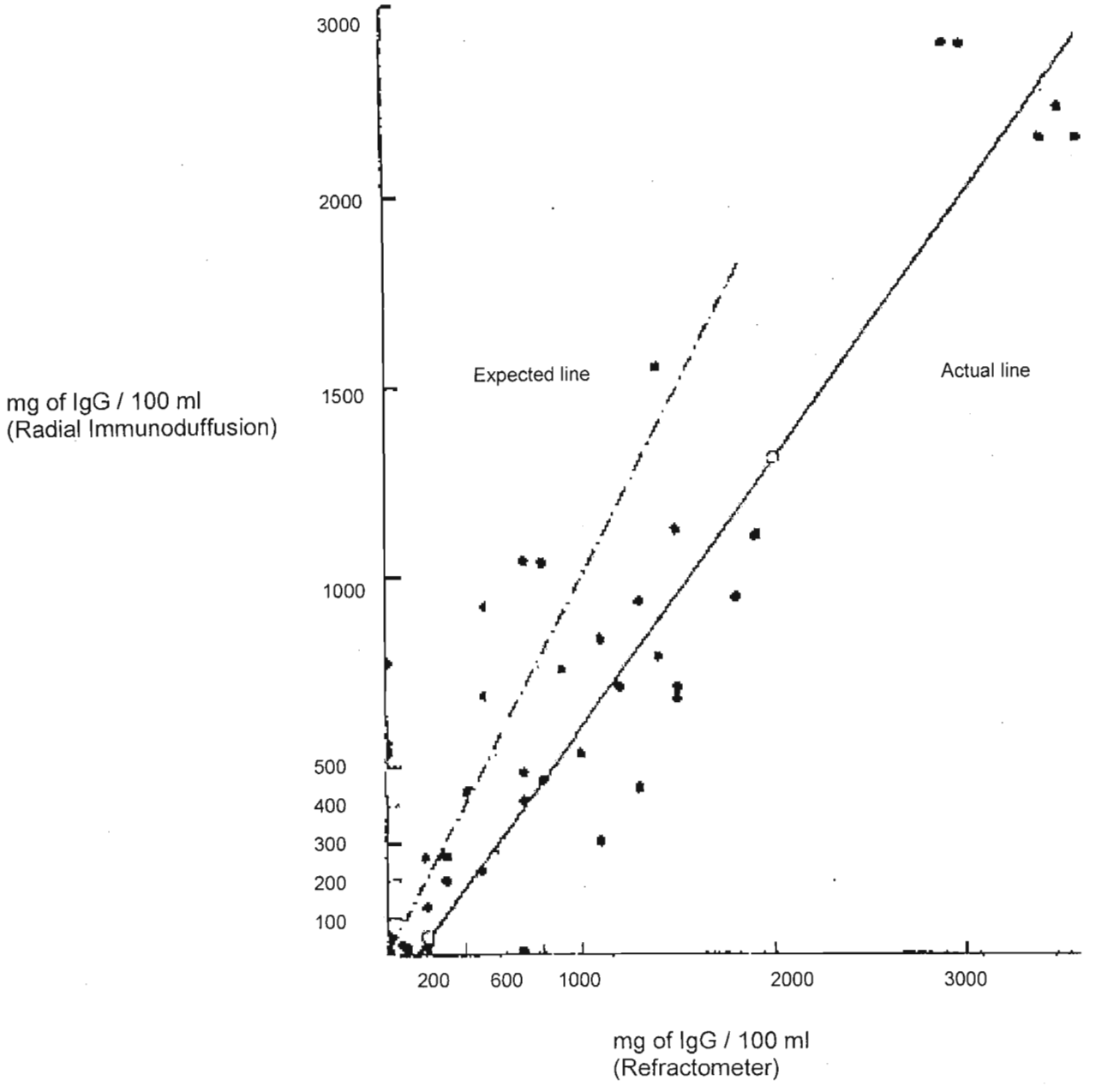


**Figure 1.7** Correlations of immunoglobulin concentration for neonatal foals measured by radial immunodiffusion and estimated from total protein (refractometer readings), (Rumbaugh *et al.*, 1978)

With the glutaraldehyde coagulation test, the results show an inverse correlation between the time taken for coagulation and the amount of immunoglobulin present (Figure 1.8) (Sandholm, 1974 and 1976). This test is highly specific and sensitive at the

critical immunoglobulin values (400 and 800 mg/100 ml serum). A positive coagulation reaction is said to occur when a solid gel, which does not move when tilted, forms (Figure 1.9). Negative reactions produce little or no change in the consistency of the serum, or are soft gels that do not solidify within one hour of the addition of the glutaraldehyde (Beetson *et al.*, 1985). Failure of passive transfer is diagnosed if there is no positive reaction after 60 minutes. Partial failure of passive transfer is diagnosed when the positive reaction takes between 10 and 60 minutes. Normal transfer has occurred if the positive reaction occurs within 10 minutes (Clabough *et al.*, 1989).

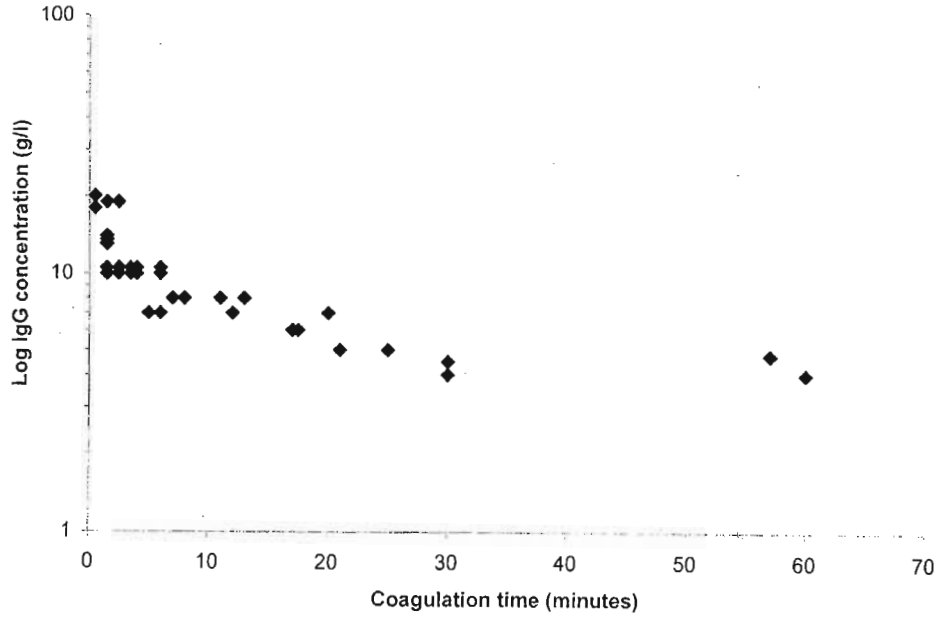


**Figure 1.8** Correlation of the log serum IgG concentration (g/l) of samples giving a positive glutaraldehyde coagulation test reaction (using a 10% glutaraldehyde solution) and their coagulation time (minutes) (Beetson *et al.*, 1985)

LeBlanc (1990) briefly described three other tests for blood serum. They are the quantitative IgG test, the concentration immunoassay technology (CITE) test and the radial immunodiffusion (SRID) test. The quantitative IgG test is a turbometric procedure using automated chemistry equipment with anti-IgG. It is rapid and accurate but is expensive and requires specialised chemistry equipment.

Workman (1987) described the concentration immunoassay technology (CITE) test as an enzyme immunoassay. Two reactants (antigen and antibody) are brought together with an enzyme attached to one component of the assay to measure the extent of the reaction. A substrate or chromogen system is also available for the enzyme in order to quantify the extent of the reactions. The sample is its own negative control because of the test format. The CITE test is a rapid, foal-side (the test is performed almost

immediately on the farm or in the veterinary clinic) semi-quantiative IgG test, producing consistent and reliable results (Ley *et al.*, 1990). LeBlanc's (1990) appraisal of the CITE test is that it is rapid and easy to perform but is expensive.

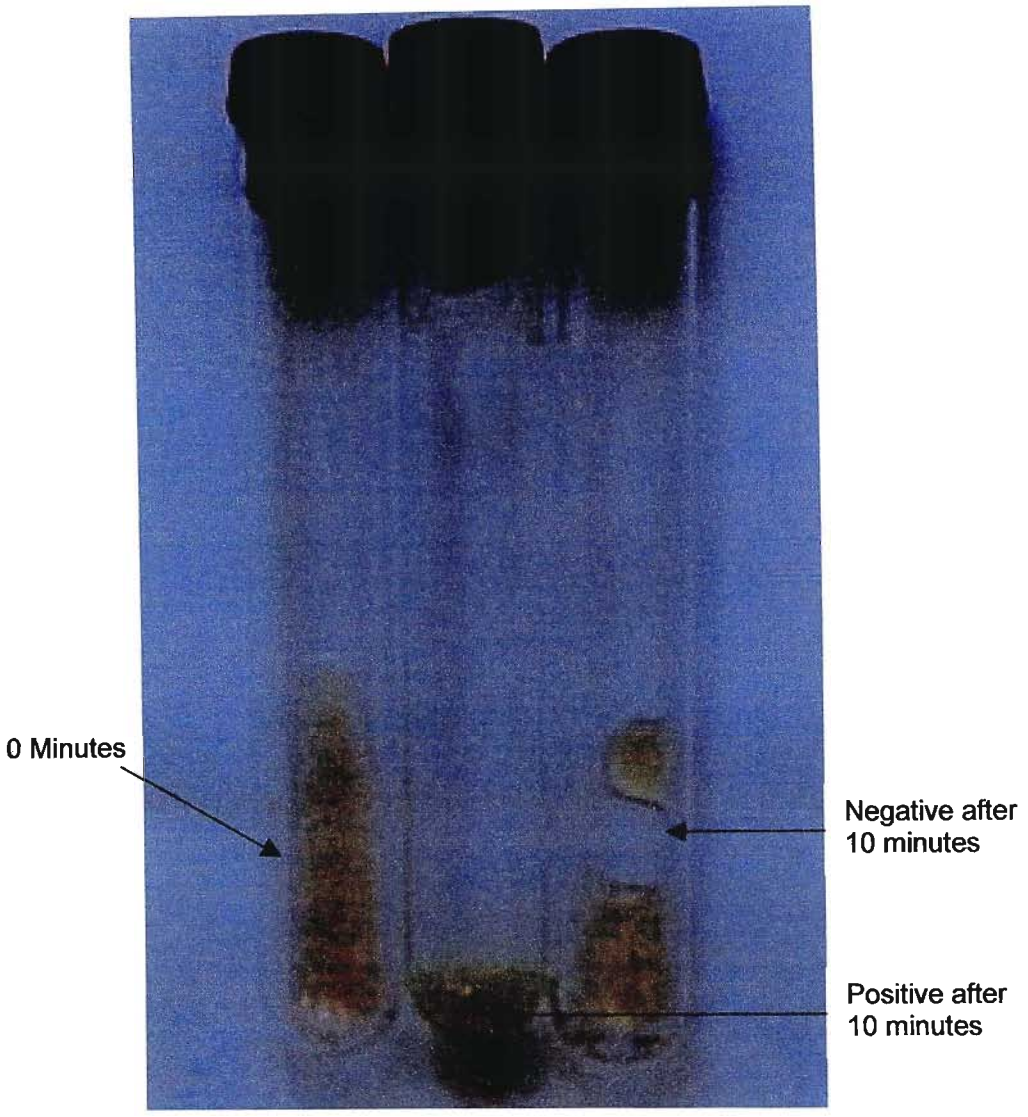


**Figure 1.9** Example of glutaraldehyde coagulation results (own photograph).

The test that is regarded as the standard for IgG testing is the single radial immunodiffusion (SRID) test (Ley *et al.*, 1990). A single diffusion type of precipitate reaction is performed by incorporating one reactant (usually antibody) into an agar gel at a uniform concentration. The other reactant is introduced into a well from which it is allowed to diffuse into the gel where it reacts with the internal reactant (Mancini *et al.*, 1965). Radial immunodiffusion is when the gel is spread on a surface with the diffusion taking place radially starting from a circular well. These two methods in combination form the SRID test (Mancini *et al.*, 1965). The final area reached by the precipitate is

directly proportional to the amount of antigen employed and is inversely proportional to the antibody concentration, provided enough time is allowed for all the antigen to combine. Temperature has no effect on the result (Mancini *et al.*, 1965). The SRID test as described by LeBLanc (1990) is accurate and allows specific quantitation of IgG. This test can also be used on colostrum. The disadvantages of this test are that it is expensive and the results are only available in 18 to 24 hours. Ley *et al.* (1990) used the SRID as the comparison test when comparing other methods and stated that it is the accepted standard for IgG tests.

## 1.5 The importance of testing for transfer of passive immunity

A foal is highly dependent on passive immunity for the first three to four weeks of its life. The tests for the transfer of passive immunity concentrate on measuring the IgG concentration because it has been shown that this is the immunoglobulin present in the greatest amount in the colostrum and in the foal's blood serum (McGuire & Crawford, 1973). It is also the last immunoglobulin to reach the minimum level. Serum levels of IgG reach a maximum level within 24 hours of the first drink and reach a minimum one to two months after birth (McGuire & Crawford, 1973).

Brewer and Mair (1988), citing anecdotal reports, questioned the association between low serum IgG levels and the subsequent development of illness that other researchers had found. However they came to no conclusions themselves. Since this, Cohen (1994) found that pneumonia is the major cause of death in foals of all ages and septicaemia is the major cause of death in foals less than seven days old. He also found that the assessment of passive immunity leads to decreased morbidity from septicaemia and pneumonia. Raidal (1996) found that confirmation of the transfer of passive immunity gives an important indication of whether a foal will have health problems. Carter and Martens (1986; cited in Tyler-McGowan *et al.*, 1997) found that FPT is the most important contributory cause of disease in foals, especially septicaemia. Tyler-McGowan *et al.* (1997) confirmed this. Cohen (1994), Raidal (1996) and Tyler-McGowan *et al.* (1997) all agreed that the early recognition of FPT, by monitoring IgG levels, is important for foal health.

## 1.6 Prevention and treatment of failure of passive transfer of immunity

For any disease condition, prevention surpasses treatment in efficacy. Colostrum is the most important aid that vets and stud managers have in preventing FPT. There are a number of precautions that can be taken to prevent FPT. The mare should arrive on the farm where she is to foal at least a fortnight before foaling (Jeffcott, 1974a). The general

recommendation is that the mare be on the farm where she is to foal for six weeks before foaling. This allows the mare time to produce immunoglobulins to local microorganisms and for these to be included in the colostrum (Morel, 1993). Conditions at parturition should be as hygienic as possible (Jeffcott, 1974a). The mare should be attended at parturition, to prevent problems and to ensure that the foal suckles normally and in good time. A colostrum bank should be kept on the stud farm if possible and care must be taken with the collection, freezing and storage of the colostrum (Jeffcott, 1974a). Colostrum (180 to 250 ml) should be labelled, dated and moved to a freezer as soon as possible (-15°C to -20°C). The colostrum should be quality-checked before storage (Jeffcott, 1974a). If the colostrum is stored as described above, it must be used within a year. If the colostrum is to be stored for more than a year it must be stored below -25°C (Jeffcott, 1974a). LeBlanc (1990) stated that the foal should receive adequate colostrum within the first six hours of life, while Koterba *et al.* (1990) recommended that the newborn foal should be bottle fed if it has not had its first drink within two to three hours of birth. These authors also suggested that the colostrum quality and the foal's serum IgG level be checked.

Stoneham *et al.* (1991) recommended several management techniques to maximise the foal's colostrum intake, in order to improve the transfer of passive immunity. When there is a previous history of unexplained FPT, the foal should be given a minimum of 500 ml donor colostrum orally, before it suckles its dam. When premature lactation occurs, at least 550 ml of colostrum should be stripped from the mare and stored at 20°C. When the foal is born, the colostrum should be thawed in a warm water bath and given orally to the foal before it suckles the dam.

The degree of mammary development in the mare and the colostrum should be visually assessed. Jeffcott (1974a) described high quality colostrum as golden yellow, thick and viscous. Stoneham *et al.* (1991) recommended that, if the dam's colostrum does not meet this criterion, it should be supplemented with at least 550 ml of donor colostrum. They further recommended the routine assessment of serum IgG levels between 12 and 72 hours after birth to identify foals at risk.

LeBlanc *et al.* (1992) recommended that the colostral specific gravity be measured as a preventative method. A specific gravity of less than 1.06 would indicate that supplemental colostrum should be given within 12 hours of birth. The recommendations of Lee *et al.* (1992) support this. The foal may be slow to nurse but the changes in the intestinal tract continue, so the time between birth and drinking must be monitored. If the foal has not drunk within three hours, it should be assisted to drink or be fed additional colostrum and then tested so that more colostrum can be given if necessary (Lee *et al.*, 1992).

If FPT occurs, Jeffcott (1974a) suggested feeding the foal frozen or freshly collected colostrum or administering the dam's plasma or an IgG preparation to it. The IgG solution is subcutaneously administered and is not as effective as the dam's plasma (Jeffcott, 1974a). The administration of the dam's plasma is not an option in cases where the foal has been prevented from drinking the colostrum because the mare is isosensitised to the foal. Stoneham *et al.* (1991) recommended a plasma transfusion, of plasma with a minimum IgG of 15 g/l. The plasma donor should be the dam whenever possible. If this is not possible, then compatibility tests need to be performed and the donor should have been exposed to the same pathogens to which the foal will be exposed (Stoneham *et al.*, 1991).

LeBlanc (1990) concurred with these recommendations but also mentioned the disadvantages of the treatments. Oral IgG supplementation (colostrum or lyophilized equine IgG) is the first treatment that is advised, because it is the natural route of transfer of immunity and is the easiest treatment to administer. However, there is a time limit to its effectiveness and there is a limited availability of high-quality banked colostrum. The other possible treatment is IgG supplementation by plasma transfusion. This has serious disadvantages as a large volume of plasma is needed and the administration of this volume of plasma may result in fluid overload when the foal's circulation system is unable to cope with the extra fluid. Purchased plasma is expensive and may not have the protection required in the area of use. Plasma collection is time consuming and advance planning is required to harvest plasma on the farm. An alternative treatment of a purified IgG solution is recommended by Liu *et al.* (1991). The advantage of this treatment is that it does not need to be administered as early as colostrum. The amount that needs to be delivered intravenously is much less than when administering plasma (200 to 300 ml *vs* 2 to 3 litres). The solution is prepared from a large plasma pool and it therefore has a larger antibody spectrum and will supply better protection than plasma from a single donor. Some adverse reactions have been observed but these are no worse than reactions observed with the administration of plasma (Liu *et al.*, 1991).

The latest recommendations are those of Lee *et al.* (1992), which revert to the early diagnosis of FPT, followed by colostral supplementation. If the diagnosis is later than 12 hours after birth, the intravenous administration of plasma is recommended.

Foals that are already clinically ill when FPT is diagnosed will require more plasma than clinically healthy foals. Ill foals may require more IgG due to greater use of, or loss of, IgG to pathologic processes. Ill foals should be rechecked at regular intervals in case additional plasma therapy is required (Wilkins & Dewan-Mix, 1994).

## 1.7 Passive immunity and vaccination programmes

The passive transfer of immunity from mother to newborn is important in the well-being of the newborn. However, it complicates vaccination programmes that are an integral component of animal husbandry at this time.

African horse sickness (AHS) is a disease endemic to South Africa and will be used as an illustration of this problem. African horse sickness is a viral disease with nine serotypes (Murphy *et al.*, 1999). It is an *orbivirus* of the reoviridae family and is the most important and lethal virus of horses (Murphy *et al.*, 1999). It is an arthropod borne disease, the most important vector being certain species of the *Culicoides* biting midge (Mellor & Hamblin, 2004). The disease is important financially, not only in the loss of valuable horses but also in its effects on the export of horses. An effective control programme is therefore essential. Factors that affect the incidence of the disease are the number and concentration of horses on the farm, movement of horses on to and off the farm and external environmental and management influences (Wilson *et al.*, 1995). Although other factors are included in a control programme, including *Culicoides* control practices, vaccination is an important part of disease control. The other factors in disease prevention include surveillance, early detection, quarantine and hygiene measures (Lunn & Townsend, 2000).

Vaccination programmes should be tailored to each farm, taking into consideration the age, type, number and use of horses, stocking density and value of the horses (Wilson *et al.*, 1995). Other factors that should be considered are the facilities, management, geographic location and the potential exposure to the disease (Wilson *et al.*, 1995). For an effective vaccination programme to be developed, it is important to know when the foals can first be vaccinated and how effective the vaccination is (Lunn & Townsend, 2000). Vaccination can only be considered effective when most of the animals in the herd are immune. High herd immunity provides indirect protection of the susceptible animals in the population (Lunn & Townsend, 2000). Practically, the first and most important immunisation for the foal is the transfer of passive immunity from the mare (Lunn & Townsend, 2000).

Passive immunity gives immediate protection, through the transfer of pre-formed antibodies, but is short-lived when compared to active immunity (Tizard, 2000). Active immunity does not result in immediate protection (Tizard, 2000) but it ensures that a future encounter with the same antigen will trigger a faster and greater reaction than initially occurred (Figure 1.10) (Starr, 1997).

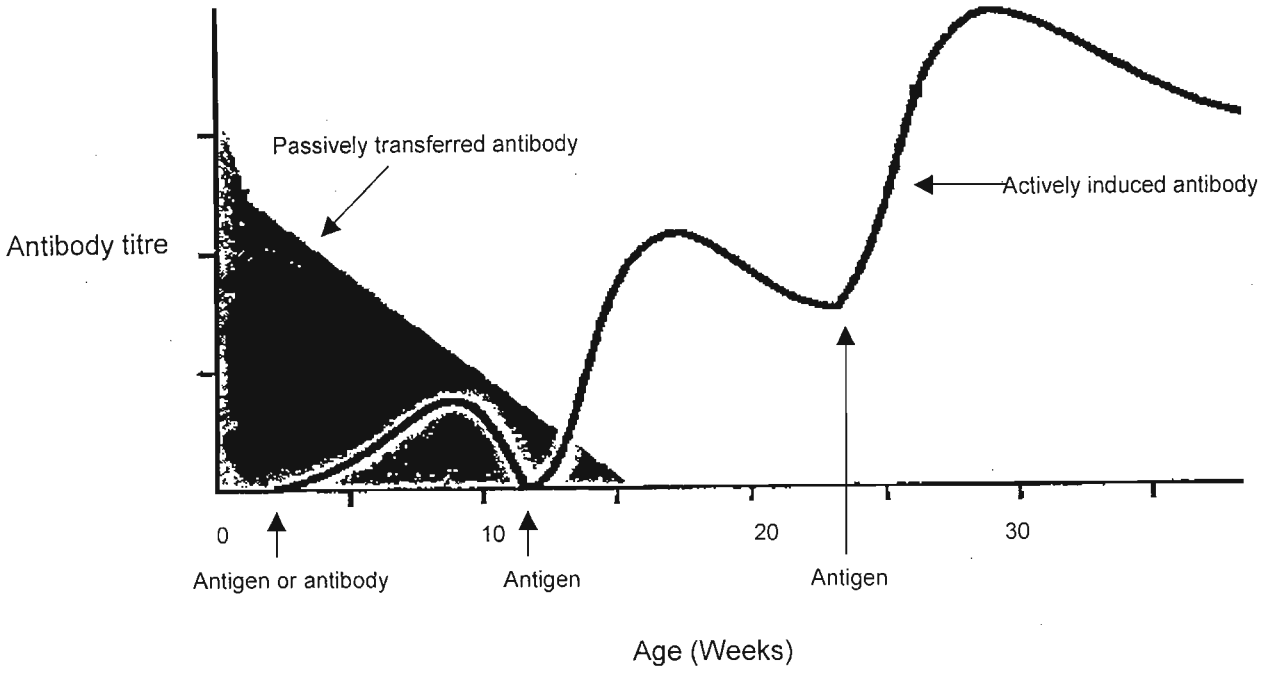


**Figure 1.10** The levels of serum antibody (degree of protection) conferred by active and passive methods of immunisation (Tizard, 2000)

Vaccination may be passive or active. Here vaccination is taken to be active. Vaccination is intended to provide an initial exposure to an antigen and most vaccination programmes require a booster to enhance the reaction (Tizard, 2000). This ensures that if the antigen is encountered naturally, the reaction is fast and effective (Tizard, 2000). The timing of vaccinations is important to ensure sufficient protection at all times. If a vaccine is administered too late, there will be a gap in protection between the fading of passive maternal immunity and the active protection from a vaccination. It is also important that a vaccination is not given too early, as the maternal immunity may negatively affect the active immune response (Tizard, 2000). According to Tizard (2000), the requirements of a vaccine are that it induces a prolonged strong immunity in both the animal and any foetus it may be carrying, produces no side effects, is cheap and stable, with a reaction that is distinguishable from natural infection.

Aside from the requirements noted above, the four critical properties of a vaccine are as follows (Ada, 1990; Tizard, 2000):

1) Stimulation of antigen-presenting cells so that the antigen is efficiently processed and the appropriate cytokines are released.

2) Stimulation of B and T cells to generate large numbers of memory cells. Both CD4 and CD8 T-cells are required so that both the MHC I and MHC II complexes can be reacted to (Lunn & Townsend, 2000).

3) Helper and effector T cells generated for several epitopes, so that variation in immune response is overcome.

4) Antigen persisting in lymphoid tissue, so that antibody-producing cells are generated for long-lasting protection.

Most vaccination failures are the result of the vaccine lacking one of the four critical properties above (Tizard, 2000). There are, however, other reasons for the failure of a vaccination (Figure 1.11). One of these reasons is the prior presence of passive immunity (Tizard, 2000).

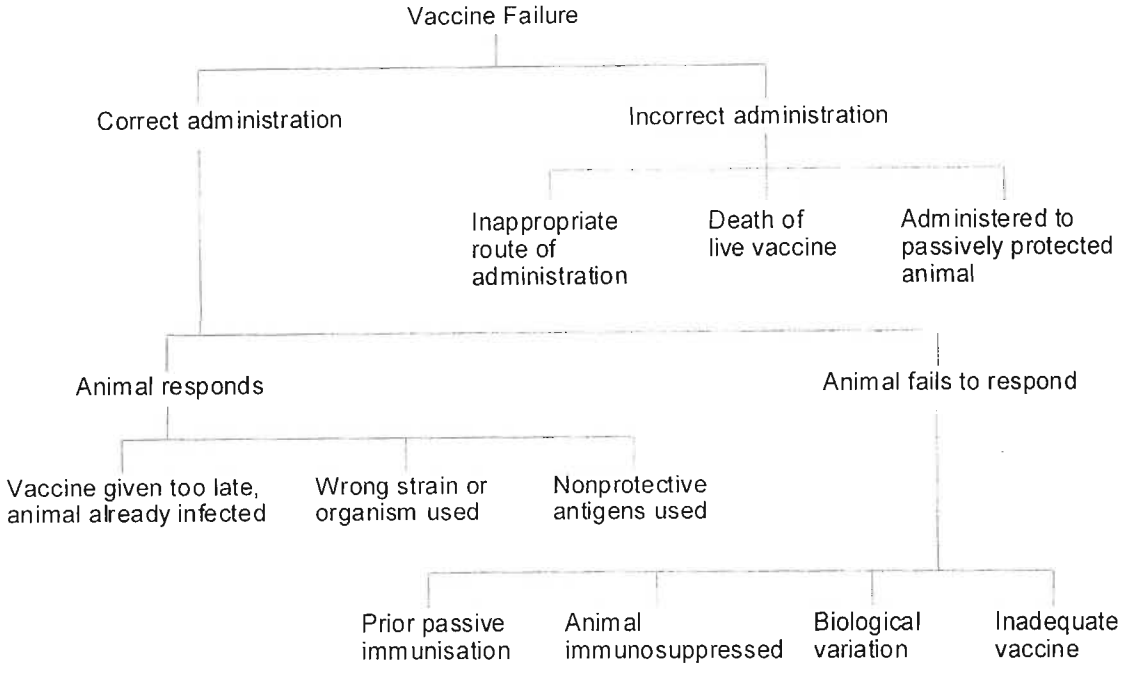


**Figure 1.11** Causes of vaccination failure (from Tizard, 2000)

The immune response development of the young animal is inhibited by the passive immunity acquired from the mother (Tizard, 2000). The presence of immunoglobulins from the mother prevents the successful vaccination of young animals by inhibiting immunoglobulin synthesis (Figure 1.12) (Tizard, 2000). The amount of antibody received from the mother and the half-life of the antibodies determine how long the inhibition of the immune system will last (Tizard, 2000). The amount of antibody transferred from the mother depends not only on her antibody level but also on how efficiently antibodies were transferred to the newborn (Tizard, 2000).

The general recommendation for a vaccination programme is to vaccinate the mare two to six weeks before foaling to maximise the colostral antibody concentration and then to

vaccinate the foals between six and nine months of age (Lunn & Townsend, 2000). However, although passively derived antibodies have similar decay rates, studies on the duration of maternal antibodies to different diseases indicate that the duration of the protection varies (Liu *et al.*, 1985). In addition, the decay rate of the antibodies is dependent on the amount of IgG absorbed by the foal (Liu *et al.*, 1985). The foal's antibody level is correlated to the antibody concentration in the mare's serum and the rate of decline is proportional to the initial antibody antibody titre (Alexander & Mason, 1941). If the mare is vaccinated or exposed to natural infection in the final two to six weeks of gestation, then the colostral antibodies will be at a maximum (Lunn & Townsend, 2000).

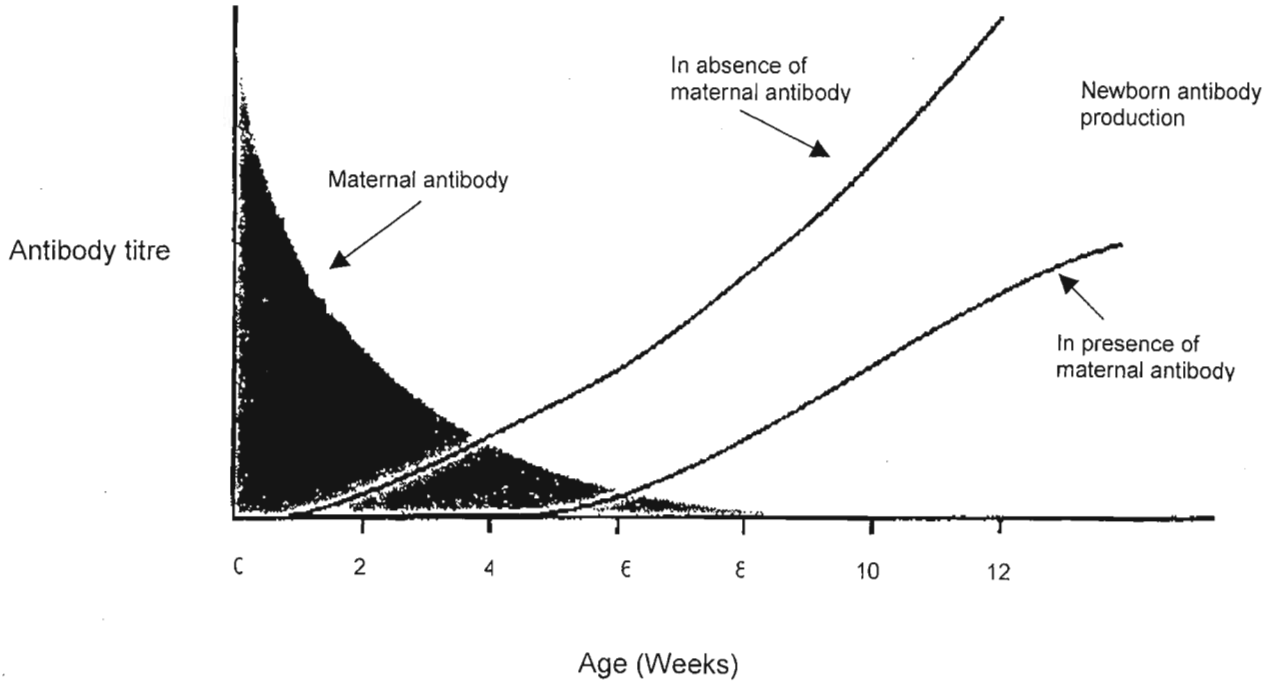


**Figure 1.12** The presence of maternal antibody in a newborn effectively delays the onset of immunoglobulin synthesis through a negative feedback process (Tizard, 2000).

It has been observed in the past few years that young foals, less than six months old, have died of AHS (Gerdes and Mullins, 2002, pers. comm.). This indicates that the recommended AHS vaccination programme needs to be revised. When looking at two of the nine AHS serotypes, Alexander and Mason (1941) found that colostral antibodies are demonstrable for up to six months and that foals are refractory to vaccination for even longer than this. These timings need to be reconsidered if foals are susceptible to AHS before six months of age, as concluded by Blackburn and Swanepoel (1988).

These researchers found that maternally derived antibodies (from regularly immunised mares) declined steadily over the five months the foals were tested from birth. Antibodies to some of the serotypes were undetectable within two to four months of birth. In addition, three foals vaccinated at three months and five foals vaccinated at four months of age showed only weak responses to the vaccine, but the vaccination did not adversely affect the existing immunity.

More research is needed to find the most effective time to vaccinate young foals against AHS for the first time. The vaccination should be given before the maternal immunity has faded but the maternal immunity needs to have faded sufficiently to allow an active response. It appears from the research already completed that this time may be in the period of three to five months of age.

## 1.8 Summary

The newborn foal is at risk from the development of infections as it adapts to its new environment. The risk is decreased by the passive transfer of immunity from the mare through colostrum. This immunity protects the foal until its own immune system is fully functional.

Tests are available which enable the transfer of passive immunity to the foal to be checked. The most effective tests are those that check the levels of immunoglobulin G in the foal's blood. Alternatively the quality of colostrum can be checked, but some foals ingest high quality colostrum and still experience failure of passive transfer (FPT) due to other factors such as the time between birth and the first drink, the amount of colostrum ingested and failure to absorb immunoglobulin, whilst others may not ingest the colostrum.

If the foals are tested and the results are received quickly, failure of transfer can be corrected by oral administration of colostrum or IgG. If the analyses take more than 24 hours, other treatment is necessary. Most commonly, this treatment consists of the intravenous administration of plasma or a concentrated IgG solution. The testing for and treatment of FPT results in fewer foals with infections and fewer deaths.

Passive immunity affects vaccination programmes, as the presence of maternal antibodies inhibits the active immune response. This means that passive immunity affects the timing of the first vaccination in two ways. The vaccination will only be effective when the passive immunity has faded sufficiently to allow an active response, but the vaccination should not be given after the passive immunity has declined to non-protective levels. A complication is that different antibodies have different half-life times

and so the most effective time for the first vaccination varies for different diseases. A disease such as AHS is even more complicated in that the antibodies to the different serotypes break down at different rates.

The objective of this literature review was to give the background of, and the link between the subject matter covered in the two experiments that are discussed in the next two chapters. The final chapter consists of the overall conclusions that can be drawn from the trial results. The first experiment looked at the different tests for determining whether the transfer of passive immunity from the mare to the foal has occurred, and which of these tests should be used preferentially. The second experiment looked at African horse sickness vaccination programmes focusing on when to vaccinate foals for the first time.

# CHAPTER 2

# TESTING FOR IMMUNOGLOBULIN IN NEONATAL FOALS

LJI Crow[1], DE Mullins[2] and GD Bradford[1]

## 2.1 Abstract

The results from four locally available tests for the transfer of passive immunity from the mare to the foal were compared to the results of the single radial immunodiffusion (SRID) test for immunoglobulinG (IgG). The SRID test yields quantitative estimates of IgG status (mg/dl serum) and is regarded as the benchmark or standard test in assessing transfer of immunity. Failure of passive transfer of immunity (FPT) is indicated at <400 mg/dl IgG, while partial failure (PFPT) may be indicated at IgG values <800mg/dl. Fifty foals born in 1999 on the Camargue stud in the KwaZulu-Natal Midlands were used in this trial. Samples of the colostrum, mare's blood and umbilical blood were taken at foaling. Repeat samples of the foal's blood were taken in the morning and afternoon following the birth of the foal and a final sample was taken the following morning. The colostrum was tested for specific density using a colostrometer and the blood samples were tested using the refractometer, zinc sulphate turbidity (ZST), glutaraldehyde coagulation (GC) and SRID tests for IgG.

There were significant correlations in the values for IgG status between the three inexpensive tests and the SRID test (colostrum, p=0.1; refractometer, GC and ZST, p=0.01). The ZST and GC tests express IgG status in categories, relative to the critical IgG levels of 400 and 800 mg IgG/dl serum. The refractometer yields a quantitative test result, allowing regression analysis to be used to give a prediction of the SRID result from refractometer readings. This analysis suggests that refractometer readings of 6.30 and 6.58 equate to SRID readings of 400 and 800 mg/dl, respectively. Chi-squared analysis of the GC, ZST and refractometer results showed there to be a significant relationship (p=0.1) between observed and expected IgG values for all three tests. Over- and under-estimation of IgG status (% samples, compared to the SRID test results) were found to be, respectively, 7.04 and 2.82 (GC), 11.97 and 4.93 (ZST) and 12.68 and 0.7 (refractometer).

[1] University of KwaZulu-Natal, Private Bag X01, Scottsville, 3209

[2] Mooi River Veterinary Clinic, P.O. Box 9, Mooi River, 3300

Although the SRID test gives the most accurate measure of IgG status in the foal, the other tests are inexpensive and provide a diagnosis within one hour (refractometer and ZST within 10 minutes, GC within 10 minutes if positive, longer if negative). This trial showed the results of three tests (refractometer, GC and ZST) to be strongly correlated with those of the SRID test. The choice of which test to use is therefore one based on cost and ease of use. The colostrum test is not a sufficiently reliable predictor of transfer of immunity from mare to foal.

The recommendation for the best time to take the sample from the foal remains at six hours of age or soon thereafter. The age of the mare, her parity and the gestation length did not have an effect on the transfer of passive immunity in this trial.

## 2.2 Introduction

Transfer of passive immunity is a very important factor in the health of the newborn foal. Tests are available to assess whether failure of passive transfer has occurred and treatments are available if failure is suspected. There is a need to determine which of the locally available foal-side (on the stud or in the veterinary clinic) tests - zinc sulphate turbidity, glutaraldehyde coagulation, protein refractometer and colostral specific density - provides the most accurate diagnosis of failure of passive transfer (FPT). If there is no difference in accuracy between methods, the simplest or cheapest test can be used. In addition, if the best time to take the foal's blood sample for testing can be determined then repeat samples and testing can be reduced and the foals that require treatment can be treated as quickly as possible.

The age of the mare, her parity (Clabough *et al.*, 1991) and the gestation length (Kruse-Elliot & Wagner, 1984, cited in LeBlanc *et al.*, 1992; Clabough *et al.*, 1991) have been reported to affect the transfer of immunity from the mare to foal. These three factors were also investigated in this trial.

The protein refractometer and colostral specific density test results are quantitative while the zinc sulphate turbidity and glutaraldehyde coagulation test results are categorical - either positive, partially positive or negative. The single radial immunodiffusion (SRID) test is assumed to be the most accurate means of evaluating the IgG status on a quantitative basis and is used as the benchmark test in this work.

## 2.3 Materials and methods

The trial was conducted on all the foals born to 50 mares during September and October 1999 on the Camargue stud farm in the KwaZulu-Natal Midlands.

The following samples were taken for each foal: mare's colostrum (100 ml into plastic tubes); mare's blood (10 ml into red-top (no additive) Vacutainer[3] test tubes (by jugular venipuncture with 20 G needles), within 30 minutes of the foal being born); foal's blood at birth (10 ml into red-top (no additive) Vacutainer test tubes (from umbilicus)); and three further samples from the foal in the following 36 hours (jugular venipuncture, 20 G needles, 10 ml into red-top (no additive) Vacutainer test tubes).

The blood samples were centrifuged (at room temperature, 4000 rpm) on arrival at the Mooi River Veterinary Laboratory (half-an-hour to several hours after sampling), for ten minutes or until the serum was liquid (some serum became gel-like during centrifuging). The serum was then poured into 5 ml plastic mailing tubes. The refractometer, ZST, and GC tests were performed immediately after the serum was separated, while the remaining serum was frozen (-5°C) in the mailing tubes, until the SRID test could be performed.

The colostrum was tested for specific density using a colostrometer (LeBlanc *et al.*, 1986). The blood samples from both mare and foal were tested for the presence of immunoglobulin G using four tests. The tests used were: protein refractometer (McBeath *et al.*, 1971); zinc sulphate turbidity (ZST; LeBlanc, 1990); glutaraldehyde coagulation (GC; Beetson *et al.*, 1985) and single radial immunodiffusion (SRID; Mancini *et al.*, 1965). The SRID test used was a commercially available kit from VMRD[4]. The SRID test is regarded as the standard or benchmark test for IgG (Ley *et al.*, 1990). The technique of SRID is the most widely used method for quantitative determination of classes of immunoglobulins and other serum and plasma proteins. This technique combines rapid and easy sample application with a high degree of accuracy and reproducibility. Antiserum specific for the protein to be measured is incorporated into agarose gel. The sample antigen diffuses into the gel containing the antibody, and a ring of precipitation forms that is proportional in size to the concentration of the antigen. A linear relationship exists between the diameter of the ring and the concentration of the antigen when plotted on semi-log graph paper. This method is time and temperature dependent (SRID information and instruction sheet from VMRD (Appendix 1) and Mancini *et al.*, 1965)

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[3] BD Vacutainer Systems, Preanalytical Systems, Bellliver Ind. Estate, Plymouth, PL6 7BP, UK.

[4] VMRD Inc, PO Box 502, Pullman, WA, 99163, USA

The refractometer[5] is calibrated using distilled water. Sufficient water to cover the screen over the prism face (Figure 2.1) is placed on the screen using a 1 ml syringe, the cover plate is lowered into place and the gauge is set to zero. The screen is then dried with tissue paper, a serum sample placed on it and the reading is taken.

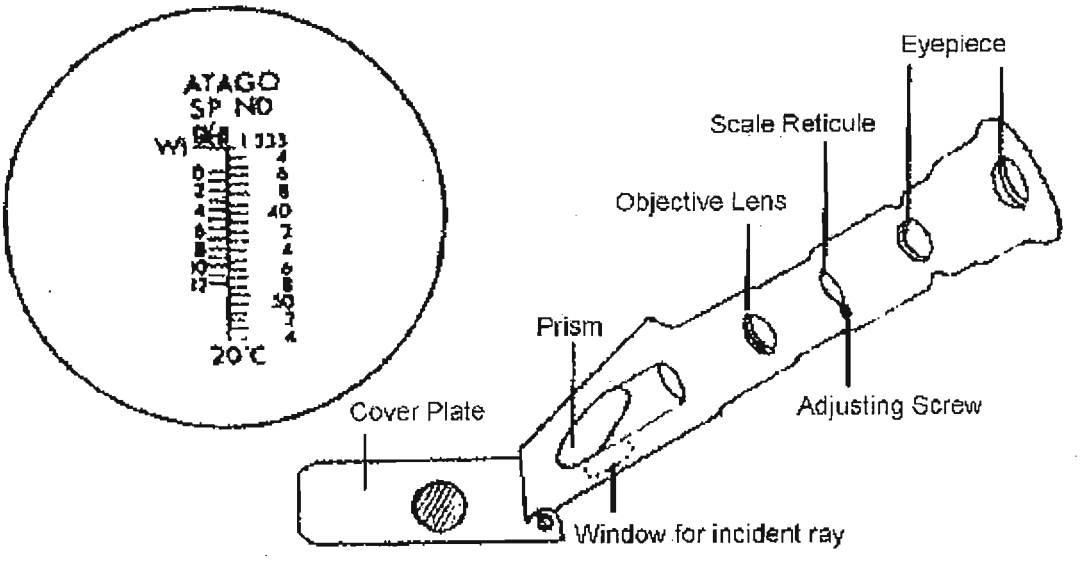


**Figure 2.1** The parts of a refractometer for measuring total serum protein. The inset shows the protein scale on the left (McBeath *et al.*, 1971).

The ZST test is performed using 0.1 ml of serum and 5 ml of zinc sulphate[6] solution (208 mg/l). The zinc sulphate is measured into test tubes and the serum is added. A precipitate forms. If newsprint can be read through the test tube, then the result is negative, i.e. failure of passive transfer of immunity from mare to foal has occurred and the foal has an IgG status of less than 400 mg/dl of serum. If newsprint cannot be read then the result is positive, i.e. passive transfer of immunity from mare to foal has occurred and the foal has an IgG status of more than 400 mg/dl.

For the glutaraldehyde coagulation test, 0.3 ml of serum is added to 0.01 ml of 10% glutaraldehyde. The test tube is checked after 10 minutes and again after an hour. If the resulting gel is solid within 10 minutes, the result is positive i.e. passive transfer of

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[5] Manufactured by Atago, Japan. Available from Polychem Supplies, P.O. Box 17254, Congella, 4013.

[6] Merck Chemicals (Pty) Ltd, Laboratory Supplies Division. P.O. Box 2805, Durban, 4000. Product code/Description: SAAR758286OEM, Zinc Sulphate unIVAR, Analysis Grade

immunity has occurred and the foal has an IgG status of more than 800 mg/dl. If the gel is solid between 10 minutes and an hour, the result is a weak positive i.e. partial failure of passive transfer has occurred and the foal has an IgG status of between 400 and 800 mg/dl. If there is no gel after an hour, the result is negative i.e. failure of passive transfer of immunity has occurred and the foal has an IgG status of less then 400 mg/dl.

The manufacturers instructions were followed for the SRID test (Appendix 1). Briefly, 3 µl of the standards (supplied) and the samples are added to the wells. The plates are covered and left at room temperature for 18 to 24 hours. The diameters of the standards are then read (in mm) and a standard curve developed. The diameters of the samples are then compared to the standard curve.

Minimums, maximums, means, standard errors and standard deviations were calculated for the SRID 12 hour, colostrums, SRID all sample, GC, ZST and refractometer results. Correlations were then calculated between the different testing methods. Linear regression was used to obtain a prediction of SRID values from the refractometer values. The chi-square analysis was used to test the association between the SRID test and each of the other tests. Finally analysis of variance was used to test whether the age of the mare, parity of the mare or gestation length had an effect on the IgG status of the foal at 12 hours of age.

## 2.4 Results

The full test results are shown in Appendix 2. The summarised, descriptive statistical results for each of the tests are shown in Table 2.1.

**Table 2.1**: Summary of descriptive statistics for tests for IgG status in newborn foals

| Test | No | Missing | Min | Max | Mean | SE | Std Deviation |
|---|---|---|---|---|---|---|---|
| SRID[a] 12 hours | 50 | 0 | 89 | 5442 | 2101 | 168.2 | 1190 |
| Colostrum | 47 | 3 | 1 | 1.1 | 1.07 | 0 | 0.024 |
| SRID (All samples) | 142 | 0 | 31 | 5969 | 2082 | 101.6 | 1210.8 |
| GC[b] | 142 | 0 | 1.0[d] | 3 | 2.8 | 0.04 | 0.48 |
| ZST[c] | 142 | 0 | 1.0[d] | 3 | 2.78 | 0.04 | 0.51 |
| Refractometer | 142 | 0 | 5.4 | 9.4 | 7.41 | 0.08 | 0.9 |

[a] SRID = Single radial immunodiffusion test (mg/dl) [b] GC= Glutaraldehyde coagulation test

[c] ZST= Zinc sulphate turbidity test [d] 1.0 = negative; 2 = weak positive; 3 = positive

Correlations were used as the first test to determine if there were differences in sensitivity and accuracy between the different testing methods. The ZST, GC and refractometer tests showed a positive, significant correlation with the SRID test at the 0.1% level of significance. The colostrum:SRID correlation was positively significant only at the 1% level. Given this difference and the large variation in the colostrum results it was decided not to proceed with further analysis of the colostrum results. Table 2.2 shows the correlation table for the three tests that warranted further analysis.

**Table 2.2**: Correlation values for the IgG tests (p = 0.01)

| Test | SRID | GC | ZST | Refractometer |
|---|---|---|---|---|
| SRID[a] | 1 | | | |
| GC[b] | 0.484 | 1 | | |
| ZST[c] | 0.432 | 0.57 | 1 | |
| Refractometer | 0.776 | 0.679 | 0.517 | 1 |

[a] SRID = Single radial immunodiffusion test [b] GC = Glutaraldehyde coagulation test
[c] ZST = Zinc sulphate turbidity test

Linear regression analysis was used to obtain a prediction of SRID values from the refractometer value, in order to determine refractometer values for the critical values of 400 and 800 mg/dl (the determining points for a positive, weak positive or negative IgG result) for a chi-squared analysis. For the ZST and GC tests, which are categorical in nature, results are stated in terms of these critical values, i.e. positive (>800 mg/dl), weak positive (400 - 800 mg/dl) and negative (<400 mg/dl), so no further manipulation of the data was necessary. The regression analysis was performed using Genstat 4.21 (2000). A small number of high leverage values were eliminated from the data set. The eliminated values were the last nine values when the data were sorted in ascending order on SRID value. The eliminated SRID values ranged from 4125 to 5970 mg/dl. This was done on the basis that the range of the refractometer is limited and that these nine SRID values were outside of this range. The eliminated values were all high SRID values. The resulting distribution graph is shown in figure 2.2. The resulting equation was:

$$IgG_{Refractometer} = 6.023 + 0.0006957 * IgG_{SRID} \ (r^2 \text{ value} = 60.5\%, SE = 0.540)$$

The critical refractometer values are therefore 6.30 and 6.58 for 400 and 800 mg/dl respectively. Practically, since the refractometer is measured in intervals of 0.2, this would equate to readings of less than 6.2 or 6.4 for an IgG level of less than 400 mg/dl (as 6.3 falls directly in the middle of these two values). The implications of choosing the

relevant cut off level (6.2 or 6.4) are covered in the Discussion below. A value of 6.6 or greater would be equal to 800 mg/dl IgG or greater. The values in between would be the values for a weak positive result (400 to 800 mg/dl).

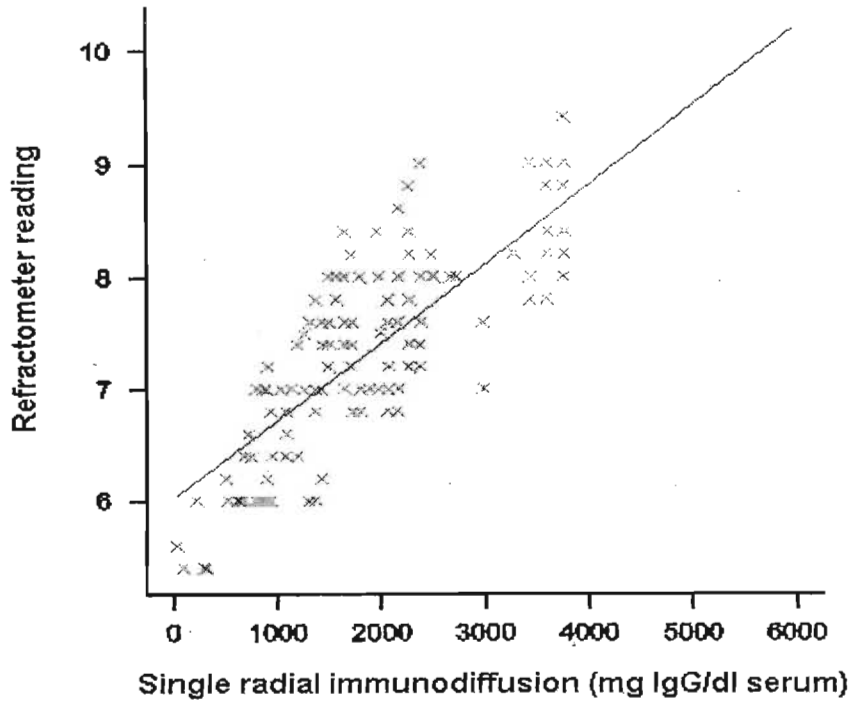


**Figure 2.2** Fitted and observed relationship between the refractometer readings and SRID IgG value.

The chi-square analysis, here, is a test of the association between the SRID test and the other test in question (ZST, refractometer or GC). A significant chi-square result means that the null hypothesis of no association between the readings is untrue. The chi-square analysis was used as a result of a problem with the experimental design. The blood samples were only tested once with each diagnostic test. This meant that, although there were multiple samples from each foal, there were no replications. The lack of replications meant that the regression for the prediction of a SRID result from the refractometer was not as accurate as it could have been. In addition, it limited the choice of statistical tests that could be used.

The chi-square analysis (n=142) (see Table 2.3 for calculations) showed a value of 130 for the GC test followed by 78 for the refractometer and 32 for the ZST tests (using refractometer readings of 6.4 and 6.6 for 400 and 800 mg/dl IgG, respectively). These

results were all significant (p=0.1), indicating a strong association between the results of the SRID test and each of the other tests.

The GC test over-estimated the successful transfer of immunity (7.04% false positives), while the ZST and refractometer tests produced 11.97 and 12.68% false positives, respectively (Figure 2.2). The number of false negatives recorded were 2.82, 4.93 and 0.7% for the GC, ZST and refractometer, respectively (Figure 2.3).

If 6.2 and 6.6 had been used as the refractometer readings for 400 and 800 mg/dl IgG, respectively, the false negatives would have been 11.97% and the false positives 1.40%. If less than 7 had been used as a negative result (Reid & Martinez, 1975), instead of 6.6, the false negatives would have been 18.8% and the false positives 0.70%.

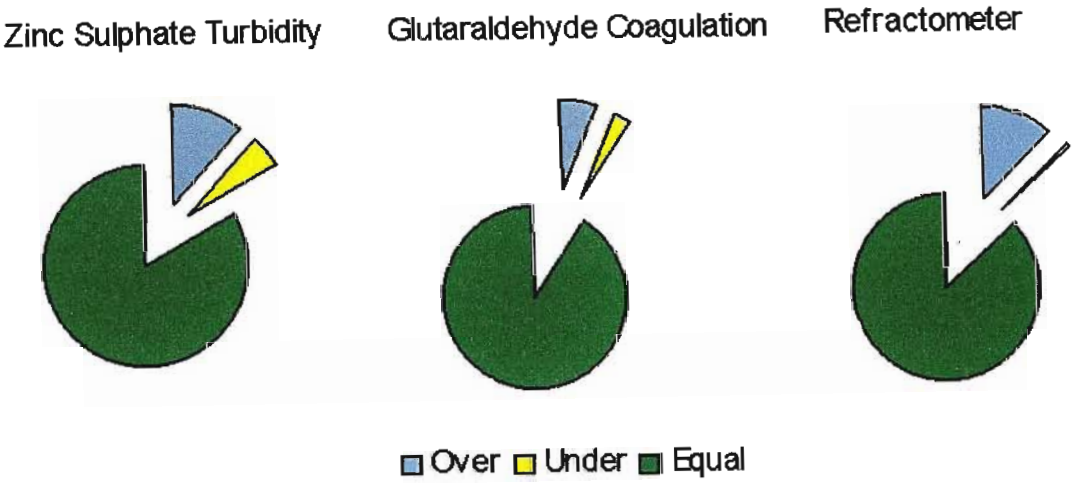


**Figure 2.3** Graphic representation of false positives and negatives when compared to SRID results.

Analysis of variance was used to determine whether the following three factors: age of the mare, parity of the mare and gestation length had an effect on the IgG status of the foal at 12 hours of age. The Genstat, 2000, generalised analysis of variance (for unbalanced treatment structure) was used as the factors were non-orthogonal to each other. None of the main effects (age of mare, parity of mare, gestation length) were significant at the 1% level of significance. Since the factors were non-orthogonal, it was not possible to test interactions between the factors. See Appendix 3 for the analysis of variance tables.

**Table 2.3**: Chi-square calculations between single radial immunodiffusion (SRID) and other tests for immunoglobulin G.

| | Glutaraldehyde coagulation | | | | Zinc sulphate turbidity | | | | Refractometer | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | **Observed** | | | | | | | |
| SRID | 1 | 2 | 3 | Totals | 1 | 2 | 3 | Totals | <6.4 | 6.4-6.6 | >6.6 | Totals |
| <400 | 4 | 1 | 0 | 5 | 1 | 2 | 2 | 5 | 6 | 0 | 0 | 6 |
| 400-800 | 0 | 8 | 3 | 11 | 2 | 6 | 3 | 11 | 6 | 3 | 1 | 10 |
| >800 | 1 | 9 | 116 | 126 | 3 | 12 | 111 | 126 | 8 | 4 | 114 | 126 |
| Totals | 5 | 18 | 119 | 142 | 6 | 20 | 116 | 142 | 20 | 7 | 115 | 142 |
| | | | | | **Expected** | | | | | | | |
| <400 | 0 | 1 | 4 | | 0 | 1 | 4 | | 1 | 0 | 5 | |
| 400-800 | 0 | 1 | 9 | | 0 | 2 | 9 | | 1 | 0 | 8 | |
| >800 | 4 | 16 | 106 | | 5 | 18 | 103 | | 18 | 6 | 102 | |
| | | | | | **(Obs - Exp)$^2$ /exp** | | | | | | | |
| <400 | 83 | 0 | 4 | | 3 | 2 | 1 | | 31 | 0 | 5 | |
| 400-800 | 0 | 31 | 4 | | 5 | 13 | 4 | | 15 | 13 | 6 | |
| >800 | 3 | 3 | 1 | | 1 | 2 | 1 | | 5 | 1 | 1 | |
| **Chi-Square** | | **130** | | | | **32** | | | | **78** | | |

Although there has been much research into the transfer of passive immunity in foals, there is very little data on the IgG levels of the mares. The VMRD information sheet for the SRID test cites values for Shetland ponies from the work of McGuire and Crawford in 1972. The mean IgG of 20 animals sampled in this work was 1334 ± 350 mg/dl serum. The mean value for the 50 Thoroughbred mares in the trial reported here was 2092 ± 660 mg/dl serum. The full summary statistics by age group are shown in Table 2.4.

**Table 2.4**: Summary statistics for Thoroughbred mares IgG levels (mg/dl)

| Age | Number of samples | Mean | Minimum | Maximum |
|---|---|---|---|---|
| 5 | 3 | 1870 | 1361 | 2369 |
| 6 | 5 | 2019 | 1425 | 2369 |
| 7 | 5 | 1707 | 1031 | 2160 |
| 8 | 1 | 1492 | 1492 | 1492 |
| 9 | 3 | 2082 | 1579 | 2507 |
| 10 | 4 | 1908 | 940 | 2369 |
| 11 | 10 | 2386 | 1361 | 3938 |
| 12 | 6 | 2042 | 1637 | 2262 |
| 13 | 3 | 1966 | 1773 | 2062 |
| 14 | 3 | 2497 | 1637 | 3591 |
| 15 | 5 | 2324 | 1299 | 3591 |
| 16 | 1 | 1563 | 1563 | 1563 |
| 20 | 1 | 2369 | 2369 | 2369 |

## 2.5 Discussion

The results from the trial reported here suggest that the foal should be tested between six and twelve hours of age. The earliest that there was a positive SRID result was for a sample taken 4.5 hours after birth. The other three tests all showed this sample as a negative (ZST and refractometer) or weak positive result (GC). A sample taken 6.25 hours after birth was the first that had a positive result from all four of the tests. There were several false positives after this time from the ZST, refractometer and GC tests.

Given the results above, the choice of which test to use for testing foals for the transfer of passive immunity will depend on the relative costs, availability and ease of use. The colostrum specific density is an indirect test of the foals IgG status. This tests the

colostral specific gravity and there are many factors other than the colostrum that affect the success or failure of the transfer of passive immunity. The colostrum test is not a sufficiently reliable predictor of transfer of immunity from mare to foal as no predictive point for failure of passive transfer could be determined from results in this trial. The other three tests are very similar to each other in cost, ease of use and the time taken to obtain a result. The refractometer gives a result that does not need interpretation and should be independent of the person performing the test, but it also gave the most false positive results, although it returned very few false negatives. The zinc sulphate turbidity test is open to different interpretation of results by different technicians, the number of false positive results was high and this test returned the most false negative results. The glutaraldehyde coagulation test result does not require any interpretation and returned the least number of false positive results.

A false positive result when testing the foal's IgG status is potentially more serious than a false negative. If the result is a false negative, the foal may be treated unnecessarily, while, if the result is a false positive, the foal requiring treatment will be missed and will not be treated.

Seventeen of the 50 foals in this trial were treated for failure of passive transfer of immunity having been diagnosed with a serum IgG level of less than 800 mg/dl at approximately 12 hours of age by any one of the three simple laboratory tests (ZST, GC or refractometer). An IgG of less than 800 mg/dl was assumed for refractometer readings of less than 7.0 (Reid & Martinez, 1975). Despite the results of the three simple tests, the SRID test showed that only six of these 17 foals had, in fact, suffered FPT[7] or PFPT[8]. Treatment was therefore unnecessarily administered to 11 foals. The treatment of choice on this stud was serum transfusions from the mare, on other farms colostrum or other more expensive treatments may be unnecessarily used in cases of false negatives.

Of the six foals that were diagnosed as FPT at 12 hours, only one attained a consistent level of IgG above 800 mg/dl after treatment, and this was reached at the test taken 56 hours after birth. This foal was accurately diagnosed as PFPT by the GC test at 12 hours, and inaccurately diagnosed as FPT by the ZST test and as positive by the refractometer test at 12 hours. At 56 hours, it was inaccurately diagnosed as PFPT by the ZST test.

One of the six foals diagnosed as FPT at 12 hours after birth never attained a blood IgG status of 400 mg/dl according to the SRID test even after treatment. The results for this

[7] FPT = Failure of passive transfer of immunity (<400 mg IgG/dl serum)

[8] PFPT = Partial failure of passive transfer of immunity (400 - 800 mg IgG/dl serum)

foal are shown in Table 2.5. The refractometer and GC tests correctly diagnosed this foal with FPT while the ZST test incorrectly identified a PFPT for the first sample and a successful transfer of immunity.

**Table 2.5**: Immunoglobulin G results for foal that remained below 400 mg/dl.

| Time (h) | Refractometer | ZST[c] | GC[b] (mg/dl) | SRID[a] (mg/dl) |
|---|---|---|---|---|
| 13.5 | 5.4 | Weak positive | < 400 | 89.99 |
| 36.5 | 5.4 | Positive | < 400 | 313.38 |
| 40.5 | 5.4 | Positive | < 400 | 285.72 |

[a] SRID = Single radial immunodiffusion test [b] GC = Glutaraldehyde coagulation test
[c] ZST = Zinc sulphate turbidity test

The remaining four foals diagnosed as FPT by the SRID test, all reached a blood IgG level of between 400 and 800 mg/dl before approximately 30 hours after birth (according to the SRID test) and after treatment. Of these foals, three were correctly diagnosed at 12 hours by all three tests as having PFPT and the last was correctly diagnosed by one test as FPT (ZST) and incorrectly by two tests as PFPT (refractometer and GC). The improvement in the blood serum IgG level of these foals was correctly identified by an increased refractometer reading. The GC test correctly identified an increase to between 400 and 800 mg/dl in two of the foals and incorrectly identified the other two foals as having an IgG level of >800mg/dl. The ZST test did not identify the improvement in one foal and incorrectly identified the other three results as > 800 mg/dl.

None of the foals requiring treatment were missed in this trial as the results of three tests were used and foals were treated if any one test showed less than full transfer of passive immunity. If only the refractometer had been used, five of the foals needing treatment would have been treated if a value of 7.0 was used as the 800 mg/dl reading. Only five of the foals would have been treated if either the GC or ZST tests had been used i.e. each test would have missed one foal (a different foal for each test!). Both the foals that were identified as having suffered FPT were identified by the refractometer as the readings were 5.4 and 6.0. Of the four foals identified PFPT, two would have been identified as FPT if either 6.2 or 6.4 had been used as the refractometer reading for FPT. One would have been identified as PFPT with 6.6 as the refractometer reading for PFPT and either 6.2 or 6.4 as the lower reading. The last foal had a refractometer reading of 7, so was not identified as PFPT using a cut- off level of less than 6.6 *or* 7.0.

All of these tests are easy to use. The costs involved would be that of the refractometer and colostrometer, and of the reactants for the other two tests. Other costs such as the test tubes and syringes are constant between the tests.

The results of this trial agree with the conclusions of Clabough *et al.* (1991) that it is better to test the blood directly, rather than testing the colostrum for IgG. This is because of the involvement of other factors affecting the absorption of the IgG. These factors include the time between birth and the first drink, the amount of colostrum ingested and failure to absorb immunoglobulin, whilst other foals may not ingest the colostrum. These factors mean that, although the colostrum is of a good quality and contains sufficient IgG, the foal may still suffer failure of passive transfer of immunity. Regarding the refractometer test, Reid and Martinez (1975) found that a refractometer reading of greater than 7.0 meant that calves and lambs had successful transfer of passive immunity. This is a higher reading than the values 6.4 or 6.6 reported in this trial. Rumbaugh *et al.* (1978) found a correlation of 0.58 between the SRID and the refractometer results, which is lower than the correlation of 0.776 found in this trial. These researchers (Rumbaugh *et al.*, 1978) found that the points were spread a long way from the regression line, indicating that false positive and false negative results were likely. Figure 2.2 (in results section) shows the distribution of the refractometer values from the trial reported here, while Figure 2.4 shows the data of Rumbaugh *et al.* (1978).

In a summary of the available tests for transfer of passive immunity, LeBlanc (1990) stated that the disadvantage of the zinc sulphate test is the number of false positive results. This problem was also evident in this trial with a high number of false positive results (17 foals, 11.97%). In addition, false negative results (7 foals, 4.93%) were also recorded in this trial.

The glutaraldehyde coagulation test was described by Beetson *et al.* (1985) as highly specific and sensitive at the critical IgG values of 400 and 800 mg/100 ml serum. This was confirmed in this trial as the GC test gave the lowest number of false positive results (10 foals, 7.04%) and second lowest number of false negative results (4 foals, 2.82).

There was no significant effect of age of the mare, parity of the mare and the gestation length on the IgG level that the foals attained, as tested using the SRID test on the sample taken as closely as possible to 12 hours of age. See ANOVA results (Appendix 3). These factors might have had an effect on the time that the foal achieved the 800 mg/dl level of immunity but this was not tested in this trial. Other researchers have found that the age of the mare (LeBlanc *et al.*, 1992), parity of the mare (Clabough *et al.*, 1991) and the gestation length (Clabough *et al.*, 1991 and Kruse-Elliot & Wagner, 1984 cited in LeBlanc *et al.*, 1992) have an affect on the transfer of passive immunity from the mare to the foal. FPT was most prevalent in mares greater than 15 years old (LeBlanc *et al.*, 1992). Mares which have had more foals were more likely to have foals with FPT (Clabough *et al.*, 1991), and primiparous were no more likely to have FPT than multiparous mares (Clabough *et al.*, 1991). The highest mean IgG concentration was in

foals born between 335 and 345 days of gestation and decreased either side of these values (LeBlanc *et al.*, 1992). No effect was found in this trial, but only one foal was born after a gestation period of less than 320 days and very few old mares were used in the trial.

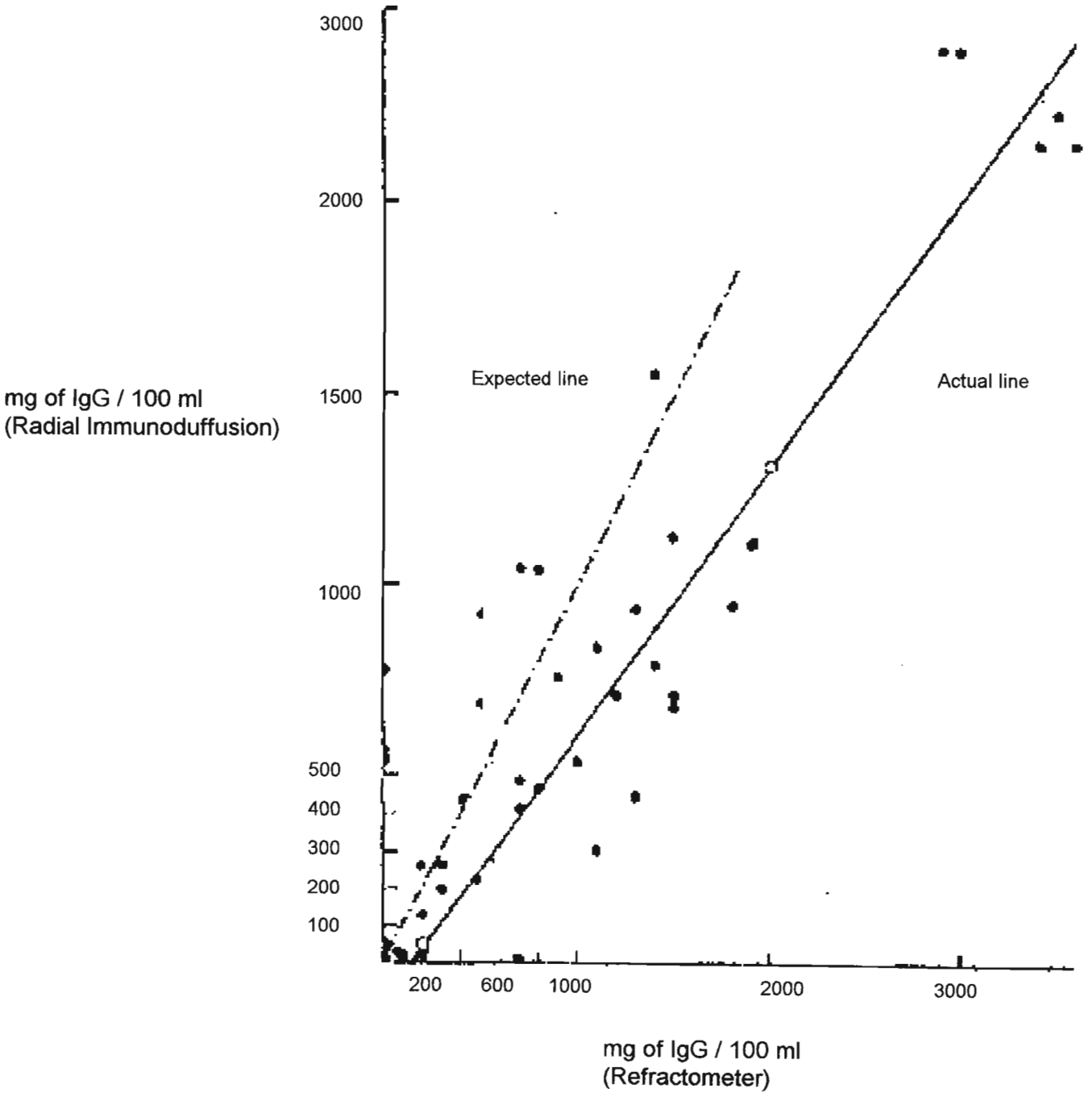


**Figure 2.4** Correlations of immunoglobulin concentrations for neonatal foals measured by radial immunodiffusion and estimated from total protein (refractometer readings) (Rumbaugh *et al.*, 1978)

The SRID test, which is a quantitative one, would be the ideal test to use if it were not for the length of the assay and the need for a prompt result. The foal-side test needs to be completed before the foal is 18 hours old in order that colostrum supplementation can be given before absorption of the IgG can no longer take place from the intestine. If other treatments are preferred, they still need to be administered as soon as possible for the foal's protection. The SRID test takes 18 hours to complete. The assay length for this test cannot be decreased since the accuracy of the test decreases as there is no longer sufficient time for the antibody-antigen complex to develop. This test can still be used as a confirmation test for use after a foal has been treated or for foals that continually test negative when the other tests are used. As an example, there was a foal in the trial reported here that did not have a positive result for any sample from any of the simple tests, but the SRID test results were positive for all of the samples. The SRID test should also be used occasionally to test the accuracy of the other tests under different laboratory conditions and with different technicians.

A more recently developed test than the ones covered in this trial is the SNAP Foal IgG test from IDEXX. Pusterla *et al.* (2002) compared this test with the SRID test and found it to be quick and easy to use. They found an overall agreement of 64% between the two tests, with the greatest agreement being for the high (>800 mg/dl; 97%) and low (<200 mg/dl; 93%) IgG levels. They concluded that the test would provide an adequate assessment of the passive immune status of the foal and allow treatment of any deficient foal early.

In conclusion, the specific density of colostrum was shown not to be a good predictor of the transfer of passive immunity. The colostrum specific density test should still be used to test the colostrum that is going to be stored for use in treating failure of passive transfer. The choice of the ZST, GC or refractometer tests as the foal-side test will have to be decided on by the stud manager and veterinarian, taking into account local conditions. The SNAP test may also be an option. The current recommendations of testing the foal six hours after birth and treating before 18 hours of age with colostrum remain. The age of the mare, parity of the mare and the gestation length did not appear to affect the transfer of immunity from the mare to the foal.

## 2.6 Acknowledgements

Many thanks to the following for all of their help in many different ways; The Equine Research Centre for funding, Mrs Kathy Devereaux for assistance in the laboratory, the staff at Camargue and Aldora studs, VMRD (Vice President, Robert Wildes) for SRID test kits and valuable advice, Sue Van Malsen for laboratory assistance and Dr Principal Ndlovu for assistance with the statistical analysis.

# CHAPTER 3

# OPTIMUM TIME FOR VACCINATION OF FOALS AGAINST AFRICAN HORSE SICKNESS

LJI Crow[9], DE Mullins[10] and T Gerdes[11]

## 3.1 Abstract

An investigation into the duration of the maternally derived antibodies for African horse sickness (AHS) in foals and the effect of the age of the foal at first vaccination on the reaction to the vaccination was undertaken.

Twenty-four foals, born on the Camargue stud in the KwaZulu-Natal Midlands in 2000 were used in this trial. The mares were all vaccinated on the same day, regardless of their expected foaling date. The foals were all vaccinated with the initial and booster vaccinations on two set days, regardless of the age of the foal when these vaccinations were given. Blood samples were taken from the mare at foaling; from the umbilical cord and from the foal at approximately twelve hours of age. Repeat samples were taken from the foals at monthly intervals until two months after the second set of vaccinations. These samples were tested with a virus neutralisation test to determine the antibody titres for each of the nine AHS serotypes.

The antibody titres for the nine serotypes of African horse sickness varied between the mares and within the mares. The immunity that the foal received from the mare (in terms of all nine serotypes) also varied between foals and within the foals. The maternal immunity faded to a negative antibody titre for all serotypes in all the foals by 18 weeks of age (mean = 4.44 ± 4.99 weeks; median = 4 weeks). In the majority of foals, the maternal immunity persisted for less than eight weeks. The number of serotypes that the mare carries positive antibody titres for appears to affect the maternal immunity that the foal receives from the mare. Although maternal immunity may interfere with the foal's response to the AHS vaccination, the data reported here provide worrying evidence that many foals are unprotected by either maternal immunity or vaccination for periods up to 16 weeks.

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[9] University of KwaZulu-Natal, Private Bag, X01, Scottsville, 3209

[10] Mooi River Veterinary Clinic, P.O. Box 9, Mooi River, 3300

[11] Virology Department, Onderstepoort Veterinary Institute, Private Bag X5, Onderstepoort, 0110

Results showed that successful reaction to the vaccine is affected by the age of the foal at the first vaccination, the anti-AHS titre of the foal on the day of vaccination, and the time between the mare vaccination and the birth of the foal (all of which are correlated). There was a wide variation in response to all nine serotypes between the foals and within the foals. Positive antibody titres were most numerous to Serotype 1. Foals born at either end of the foaling season enjoyed poorer protection against AHS than those born in the middle of the season. Early season foals received poor levels of maternal immunity but responded well to the first vaccination; whereas late season foals enjoyed more lasting maternal immunity which interfered with their response to the AHS vaccination and left them susceptible to the disease after vaccination. A case study of three foals is used to illustrate the differences in reaction in foals born at different times during the season (different lengths of time between mare vaccination and foal birth and different foal ages when vaccinated for the first time).

Fewer foals responded to the second vaccination than to the first but the response was faster indicating that the foals may have been building on a memory response from the first vaccination.

The pattern of response to vaccination observed in this trial is in large part due to the common management practice of vaccinating all mares on a farm on a single day, regardless of expected foaling date; and of vaccinating all foals on a single date, regardless of exact foal age. A recommendation is made that management practices need to change in order to adhere more closely to the recommended vaccination schedule. It is suggested that the existing vaccination schedule be revised as follows: the mare should be vaccinated at three and two months prior to her expected foaling date with AHS 1 and AHS 2 respectively; and the foal then vaccinated at four and five months of age with AHS 1 and AHS 2 and again at six (AHS 1) and seven months of age (AHS 2). This revised vaccination schedule seeks to address the lack of immunity present in many foals before the first vaccination, due to low levels of maternal immunity, and to improve the efficacy of the booster vaccination. The mares are vaccinated at the optimum time to improve the passive immunity given to the foals and the foals are vaccinated as early as possible, with respect to the disappearance of maternal immunity.

## 3.2 Introduction

Vaccination programmes are an integral part of animal husbandry, in order to minimise the risk of infection and the incidence of infectious diseases. Maternal immunity derives from immunoglobulins concentrated by the mammary gland from the blood and passed to the foal through the colostrum. Maternal immunity protects the foal but also affects vaccination programmes (Jeffcott, 1974).

Immunoglobulin synthesis in the young animal may be inhibited by the presence of antibodies from the mare, thus preventing a successful vaccination reaction if the foal is vaccinated at too young an age (Tizard, 2000). However, it is equally important that the foal is vaccinated as early as possible so that there is no period of time when the foal is not protected.

Liu *et al.* (1985) found African horse sickness virus (AHSV) maternal antibodies present until foals are approximately seven months old. However, Blackburn and Swanepoel (1998) found that antibodies to some serotypes disappear by two to four months after birth. Field observations seem to confirm that African horse sickness (AHS) antibodies reach very low levels in foals before six months of age (Gerdes and Mullins, 1999, pers. comm.).

African horse sickness is a severe, non-contagious, viral equine disease with mortality rates of up to 95% (Murphy *et al.*, 1999) but there is minimal available field data on the foal's immune response to the AHS vaccine. For an effective vaccination programme to be designed, data are needed on the duration of maternal immunity, and the variability in this duration. Data are also needed on the efficacy of repeated vaccinations, taking into account the age at which the animal is first vaccinated.

The African horse sickness vaccination[12] is a freeze dried, polyvalent vaccine containing live attenuated virus strains and is split into two vaccines. The animals are initially injected with the first vaccine (AHS 1) and then three weeks later with the second vaccine (AHS 2) of the combination. The first vaccine contains Serotypes 1, 3 and 4. The second vaccine contains Serotypes 2, 6, 7 and 8. Serotypes 5 and 9 are not included in the vaccine. There is some cross protection between the following serotype pairs: 1 and 2, 3 and 7, 5 and 8 and 6 and 9 (Coetzer & Erasmus, 1994). The vaccination package insert states that foals born to unvaccinated mares can be inoculated at any time but foals born to vaccinated mares should not be vaccinated until they are at least six to seven months of age. All animals should be vaccinated in early summer and mares should not be vaccinated in the first three months of pregnancy. Annual immunization is recommended. The insert further states that immunity starts to develop two to three weeks after complete inoculation, that protection to some serotypes is achieved after four weeks and that immunity cannot be guaranteed in all animals. The complete package insert is given in Appendix 4. The recommended vaccination programme is that foals born to susceptible mares are vaccinated at one month of age and foals from immune mares in high risk areas are vaccinated at five to six months of age and again in spring (August to September; approximately 12 months of age). The recommended vaccination programme for adult animals is annually, in spring, except for

[12] Onderstepoort Biological Products, Private Bag X07, Onderstepoort 0110, Registration No. G116 (Act 36 of 1947).

pregnant mares, which should be vaccinated six weeks before foaling (Onderstepoort Biological Products, 2005). This recommended programme is illustrated in Figure 3.1.

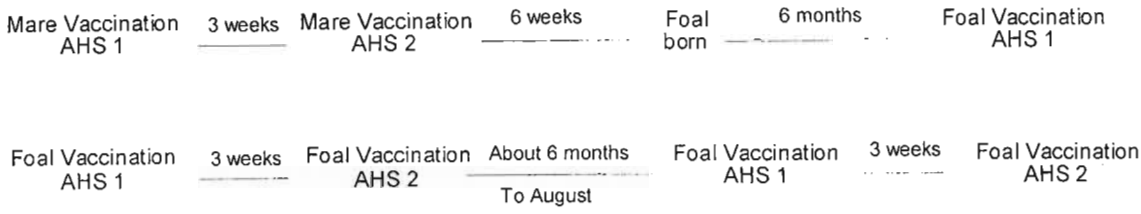


**Figure 3.1** Time line for the recommended vaccination schedule for pregnant mares and foals.

Different serotypes may be active during an outbreak but usually one serotype will dominate an outbreak, with a different dominant serotype the next season (Coetzer & Erasmus, 1994). It has never been reported that an animal has been infected by more than one serotype (Howell, 1968 cited in Coetzer & Erasmus, 1994). The infections may occasionally spread into provinces that are usually free of AHS, causing serious losses. The south-western Cape Province is the quarantine zone for export horses. Prior to 1990, the last outbreak in this area was in 1967 (Erasmus, 1990 cited in Coetzer & Erasmus, 1994).

The South African horse industry is a multimillion rand industry in a niche market. The figures from the latest outbreak of African horse sickness (January to April 2005) are as follows:

KwaZulu-Natal: 535 cases and 361 mortalities (including a yearling worth R550 000);
Mortalities in other provinces: Gauteng 42; Eastern Cape 33; Limpopo 2; North West: 3; Mpumalanga 2; Western Cape 2.
Total: 445 mortalities.

These figures represent reported cases and are likely to underestimate the severity of the outbreak, since many cases are not reported from the rural areas. The serotypes that were circulating in January to April 2005 outbreak were Serotype 1 (Gauteng, Limpopo), Serotype 2 (Eastern Cape), Serotype 5 (KwaZulu-Natal - mainly in the Natal Midlands, Gauteng and Western Cape - suspected introduction from KwaZulu-Natal), Serotype 6 (Gauteng) and Serotype 7 (KwaZulu-Natal - mainly in the Dundee district); (Gerstenberg, 2005, pers. comm.).

Results from samples submitted to the Onderstepoort Veterinary Institute for testing between 1981 and 2004 show that Serotypes 3, 5 and 9 are seldom found. Serotype 9

originates from North Africa and has only recently (1993) been isolated for the first time in South Africa (Gerdes, 2004, pers. comm.). Two isolates of AHS Serotype 9 were found in five years (1993 to 1998) and there have been fourteen isolates in the last six years (1999 to 2004). The prevalence of the nine serotypes is illustrated in Figure 3.2. The endemic zones of AHS are currently restricted sub-Saharan Africa and possibly Yemen (Mellor & Hamblin, 2004). But, the virus has a history of rapid expansion without warning into countries far beyond these endemic areas and climate changes have resulted in the major vector (*Culicoides imicola*) expanding into many areas of Europe previously considered AHS risk free (Mellor & Hamblin, 2004). The related bluetongue virus which is transmitted by the same *Culicoides* vector has already moved into this area suggesting that AHSV could do the same in the future (Mellor & Hamblin, 2004).

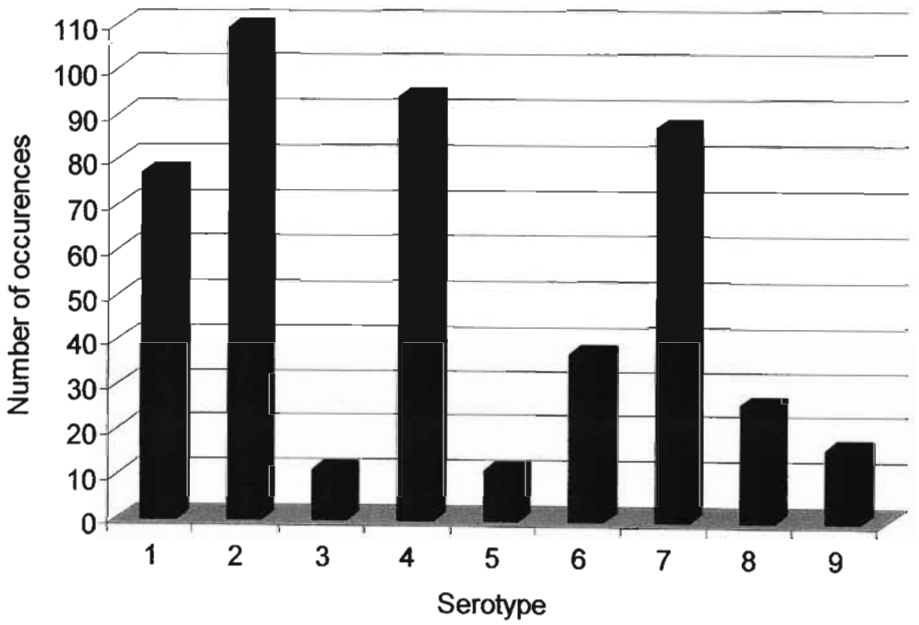


**Figure 3.2** Occurrences of different serotypes of African horse sickness in South Africa between 1981 and 2004 (Gerdes, 2005, pers. comm.)

The objectives of this trial were to determine when, after parturition, the maternal immunity to AHSV has decreased to a sufficiently low enough level to allow the first vaccinations for AHS to be given to the foals, and to determine the effect of repeated vaccinations on AHSV antibody levels in young horses, taking into account the age at which they were first vaccinated. Other factors which affect AHSV antibody levels in foals and which were studied in this work are the mare's antibody level on the day of foaling and the time period between the mare being vaccinated and the foal being born.

## 3.3 Materials and methods

All the foals born on the Camargue stud farm in the KwaZulu-Natal Midlands in 2000 were sampled at birth, 12 to 24 hours after birth and monthly thereafter until two months after their second set of AHS vaccinations, at approximately one year old. Each set of vaccinations consisted of both halves of the combination (AHS 1 and AHS 2). A sample of blood was taken aseptically by jugular venipuncture (20 G needles into 7 ml red-top (no additive) Vacutainer[13] test tubes) from the mare within 30 minutes of foaling. Blood was collected from the umbilicus at birth as the foal's zero-hour sample.

The blood samples were allowed to stand before being centrifuged. The serum was decanted into 5 ml plastic mailing tubes[14] and frozen until the analyses were performed. The 12 hr samples were tested for the transmission of passive immunity from the mare to foal by testing the immunoglobulinG (IgG) status using the glutaraldehyde coagulation (GC) (Beetson *et al.*, 1985) and zinc sulphate turbidity (ZST) (LeBlanc, 1990) tests before being frozen (Appendix 6).

Initially, 61 foals were sampled at birth, but a complete set of samples was collected on only 24 foals. Foals were excluded from the trial for the following reasons: a negative IgG status (according to either GC or ZST test); death (mainly due to physical injuries); missing samples (foals not brought in from the field, too small a sample taken or other management reasons) or because the mare and foal were moved to another stud farm during the trial period. Two foals (numbers 20 and 21) that tested negative for IgG status were included in the trial in error. Only 24 of the remaining foals were tested with the virus neutralisation test due to time and funding constraints. The foals were all born between August and November 2000 and all were vaccinated on the same day, so their ages ranged from three to six months at the time of the first vaccination and from 12 to 15 months at completion of sampling. This vaccination schedule does not comply with the Onderstepoort Biological Products (OBP) recommendations (Figure 3.1, above) in so much that the mares were vaccinated from 4.5 weeks to 19.5 weeks before foaling and the foals were vaccinated for the first time from nine weeks to 24 weeks of age. The foals were then vaccinated six months later as per the OBP recommendations. This type of schedule represents standard practice on stud farms in the KwaZulu-Natal Midlands (Mullins, 2005, pers. comm.).

The 24 complete serum sample sets were tested in the Onderstepoort Veterinary Institute virology laboratories using the virus neutralisation test (Appendix 5) for the presence of sero-specific antibodies for each of the nine AHS serotypes.

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[13] BD Vacutainer Systems, Preanalytical Systems, Bellliver Ind. Estate, Plymouth, PL6 7BP, UK.

[14] Mailing tubes with polyethylene screw-on caps, AEC Amersham, (Pty) Ltd, P.O. Box 1596, Kelvin 2054.

The virus neutralisation test is performed using a constant amount of virus against dilutions of serum and the criterion for positive antibody by microscopic reading is the complete neutralisation of the virus input, i.e. the absence of viral cytopathic effect (CPE) (Anderson & Rowe, 1982). Thus, the virus neutralisation test is a quantitative assay for the determination of antibody titre in, for example, serum, based on the ability of the serum to neutralise a known amount of a standard virus antigen (Aleff Group, 2000). Neutralisation tests estimate the ability of antibody to neutralise the biological activity of antigen, when mixed with it *in vitro*. Viruses may be prevented from infecting cells after a specific antibody has combined with and blocked their critical attachment sites. This reaction is the basis of neutralisation tests that can be used for the identification of unknown viruses or the measurement of specific antiviral antibody (Tizard, 2000). Neutralisation tests are highly specific and sensitive (Tizard, 2000). The African horse sickness virus neutralisation test is a sero-specific test that measures the amount of IgG and IgM antibody specific to each serotype in the serum. The test uses decreasing concentrations of the serum against a set amount of virus to determine how much neutralising antibody is present in the serum. The test result is read in antibody titres doubling in strength from 1:2 to 1:4 to 1:8, increasing to 1:256. An antibody titre of 1:40 is considered to indicate a positive result (Onderstepoort Veterinary Institute, 1995). The layout of the analysis in this work meant that the results measured were either side of 1:40 (1:32 and 1:64); 1:32 will be considered the lowest positive reading for the rest of this discussion.

## 3.4 Results and discussion

For the purposes of this discussion, Vaccination 1 and 2 refer to when the first and second sets of the vaccine were administered, not the individual vaccines (AHS 1 and AHS 2) within the combination. When an individual vaccine within the combination is referred to, AHS 1 and 2 will be the terminology used. AHS 1 refers to the combination of Serotypes 1, 3 and 4; AHS 2 refers to the combination of Serotypes 2, 6, 7 and 8.

### 3.4.1 Duration of maternal immunity

Figure 3.3 shows the mares' antibody titres for each serotype on the day of foaling. It can be seen that there is a large variation in antibody titres both between mares and within mares for the different serotypes.

Only one mare had a negative titre for Serotype 6 and two mares had a negative antibody titre for Serotype 1, while 8 had negative antibody titres for Serotype 3 and 14 had negative antibody titres for Serotype 2. The antibody titres for Serotypes 5 and 9 would result from the cross reactions with Serotypes 8 and 6 respectively, or from natural exposure. The antibodies from vaccine reactions and those from natural exposure are not differentiated in the test.

Mare 10 had antibody titres at each of the measured levels (1:2 to 1:256). Serotypes 1, 2, 3, 6 and 9 had negative antibody titres and the rest were positive. In contrast the range of antibody titres for Mare 2 was all positive from 1:32 to 1:256.

Figure 3.4 shows the variation in the immunity that the foal received from the mare as determined when the foal was approximately twelve hours old. The variation between foals and within one foal between serotypes is clearly illustrated. There are fewer foals shown in this figure than mares shown in Figure 3.3 because there was not sufficient serum for all the foals to test for all the serotypes for this set of samples.

Factors which may affect the maternal immunity that the foal receives are the age of the mare and the number of serotypes that she is positive for. Figure 3.5 shows the mare age against the number of serotypes that the foal had positive results for at four to six weeks of age. There is no clear pattern of response but, if the number of serotypes that the mare had a positive test result for on the day of foaling is compared to the number of serotypes that the foal is positive for at four to six weeks of age (Figure 3.6), then a clearer pattern is visible. The more serotypes that the mare is positive for, the more serotypes the foal is positive for.

Table 3.1 shows the duration of the maternal immunity (weeks) for each of the serotypes. The mean and median values show that the majority of the foals have maternal immunity that lasts less than eight weeks. The overall mean (all foals, all serotypes) is 4.44 ± 4.99 weeks. The median is 4 weeks. Only 11.1% of the foals had a maternal immunity (all serotypes) that lasted for eight weeks or longer. Figure 3.7 illustrates the variation in the duration of the passive immunity received from the mare and that the immunity is not long lasting. Across all serotypes, maternal immunity did not extend beyond 18 weeks after foaling. These data (Figure 3.7) include four foals that still had a positive antibody titre at the time of the vaccination. Therefore, in these cases, the maternal immunity may have lasted until after vaccination and possibly beyond 18 weeks, although this is unlikely.

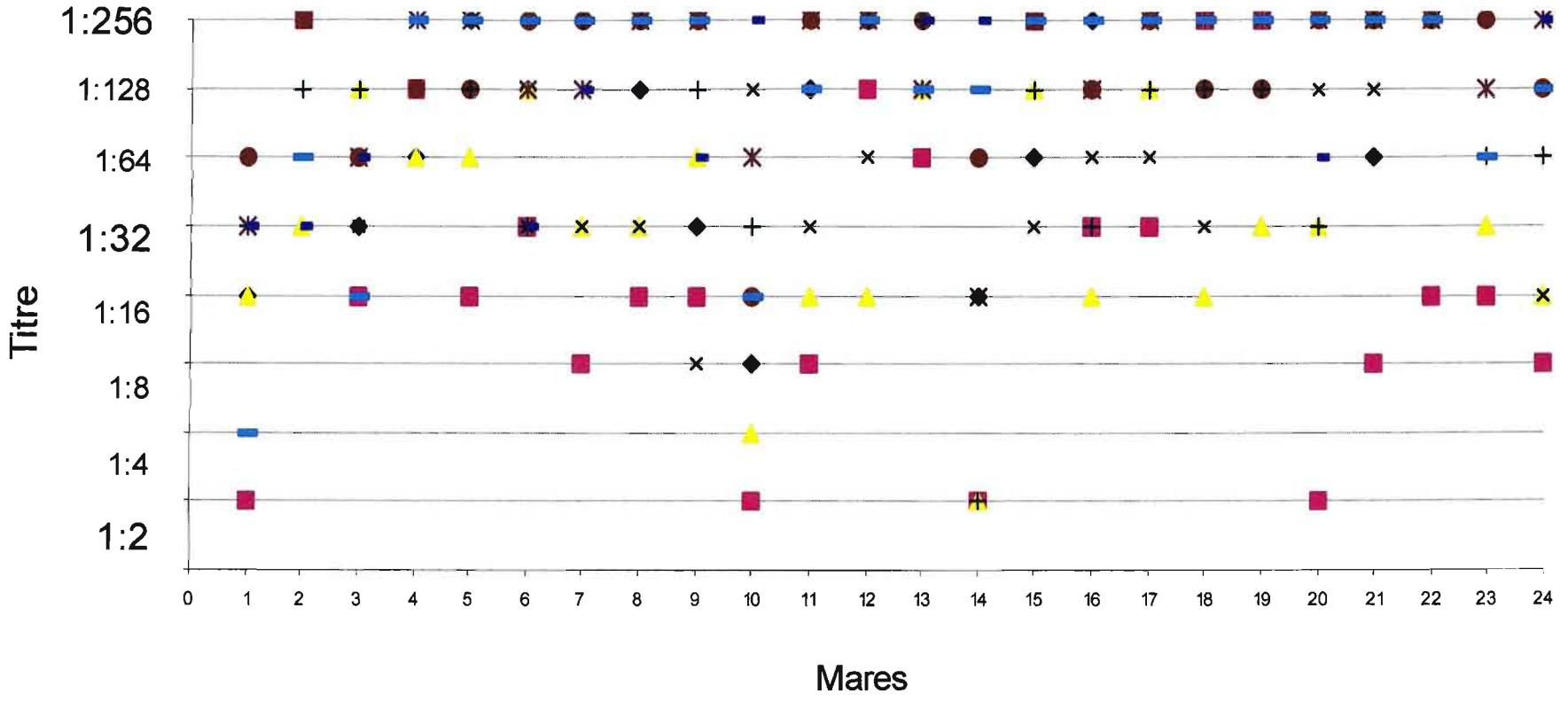


**Figure 3.3** Mare antibody titres for AHS serotypes (1 to 9) on the day of foaling (Serotype: 1 ◆, 2 ■, 3 ▲, 4 ×, 5 ∗, 6 ●, 7 +, 8 –, 9 – )

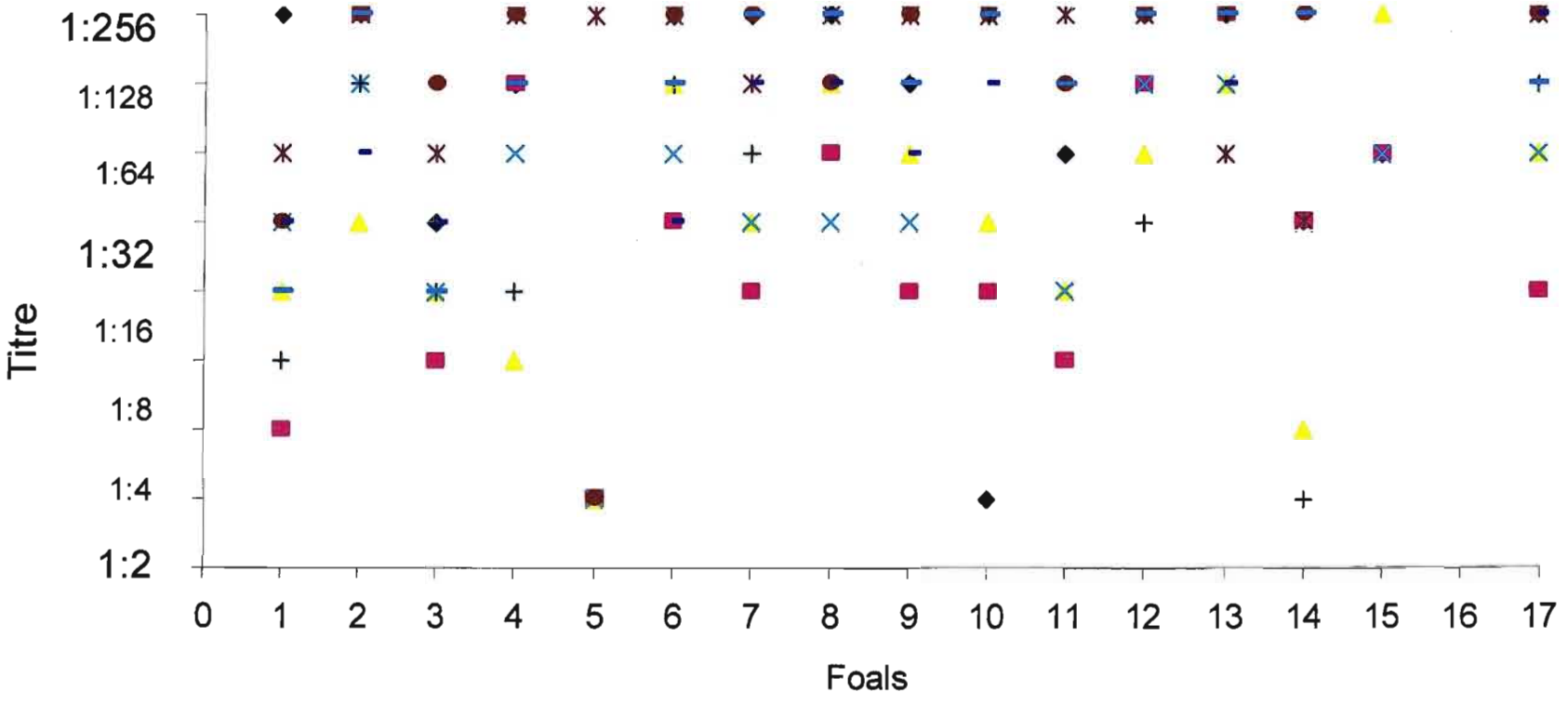


**Figure 3.4** Antibody titres for foals at twelve hours of age for each of the nine African horse sickness serotypes (Serotypes: 1 ◆, 2 ■, 3 ▲, 4 ×, 5 ∗, 6 ●, 7 +, 8 –, 9 –)

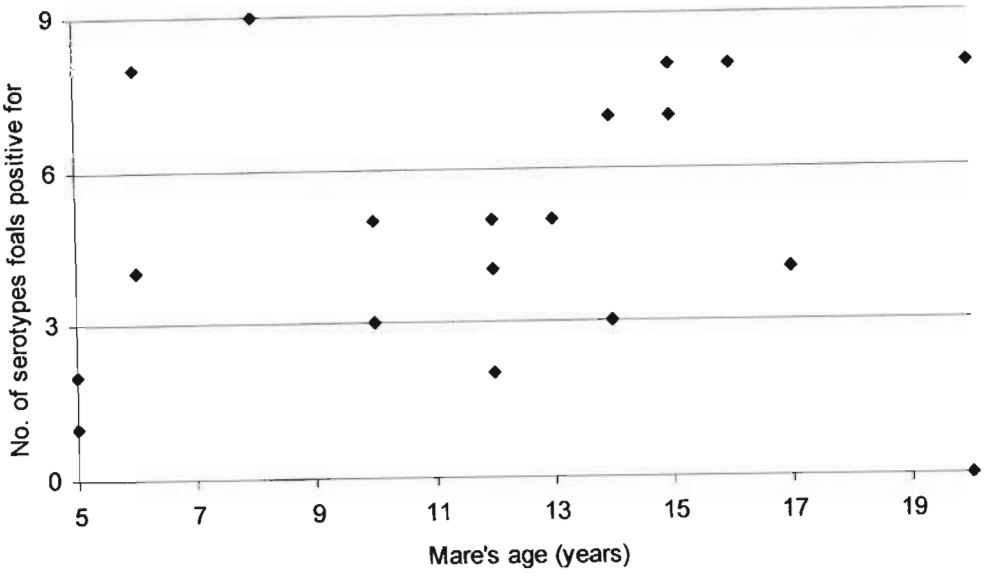


**Figure 3.5** The effect of the mare's age on the number of serotypes that the foal is positive for at four to six weeks of age.

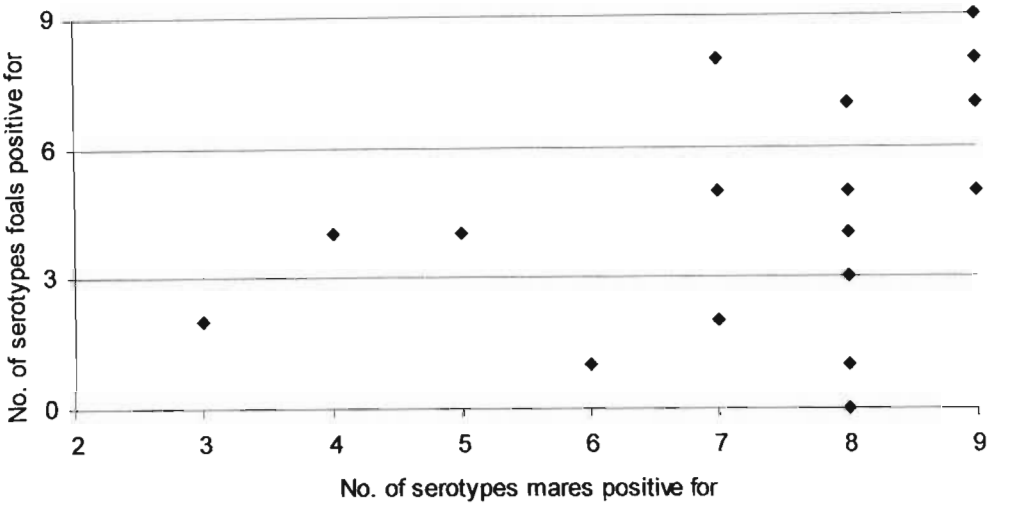


**Figure 3.6** The effect of the number of serotypes that the mare is positive for at foaling on the number of serotypes that the foal is positive for at four to six weeks of age.

The difference between the maternal antibodies and those produced by the foal in response to vaccination cannot be determined in these four foals. If these foals are removed from the data set (Table 3.1, highlighted in green) then the pattern of the degradation of maternal immunity remains the same (Table 3.2, Figure 3.8). Of the four foals that still had a positive antibody titre on the day that they were vaccinated for the

first time, two of these foals had a positive antibody titre for more than one serotype (Serotypes 1, 4 and 6; and Serotypes 1 and 6). These antibody titres were recorded on the day that the foals were vaccinated with the vaccine combination (AHS 1 or AHS 2) containing the relevant serotype. The serotypes highlighted in purple for Foal 5 are those for which this foal had negative antibody titres from birth, with sudden, unexpected positive antibody titres on the day of the vaccination. The duration of maternal immunity for these serotypes, in this foal, has been taken as zero weeks as it is not known whether the antibody titres on vaccination day are a testing error or genuine antibody titres resulting from natural exposure to these serotypes.

**Table 3.1**: Duration of maternal immunity (weeks)

| Foal | S 1 | S 2 | S 3 | S 4 | S 5 | S 6 | S 7 | S 8 | S 9 |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 8‡ | 0 | 0 | 4 | 4 | 12 | 0 | 0 | 0 |
| 2 | 10 | 10 | 0 | 10 | 10 | 12 | 4 | 4 | 10 |
| 3 | 4 | 0 | 8 | 4 | 8 | 8 | 8 | 4 | 4 |
| 4 | 8 | 4 | 0 | 4 | 8 | 10 | 8 | 10 | 8 |
| 5 | 0¿ | 0 | 0 | 0 | 12 | 0 | 0 | 0 | 0 |
| 6 | 10 | 10 | 10 | 4 | 10 | 12 | 10 | 4 | 12 |
| 7 | 10 | 0 | 0 | 6 | 8 | 12 | 0 | 10 | 8 |
| 8 | 6 | 0 | 0 | 0 | 8 | 8 | 0 | 8 | 16 |
| 9 | 0 | 0 | 4 | 0 | 8 | 12 | 8 | 0 | 4 |
| 10 | 0 | 0 | 0 | 14 | 4 | 0 | 0 | 8 | 8 |
| 11 | 4 | 0 | 0 | 0 | 8 | 0 | 0 | 0 | 0 |
| 12 | 14 | 4 | 0 | 4 | 14 | 4 | 0 | 12 | 4 |
| 13 | 14 | 4 | 4 | 4 | 0 | 8 | 8 | 8 | 8 |
| 14 | 0 | 0 | 0 | 0 | 0 | 4 | 0 | 12 | 0 |
| 15 | 6 | 0 | 6 | 0 | 12 | 18 | 6 | 16 | 18 |
| 16 | 6 | 0 | 0 | 0 | 6 | 12 | 0 | 0 | 4 |
| 17 | 0 | 16 | 0 | 0 | 16 | 8 | 6 | 8 | 6 |
| 18 | 6 | 0 | 0 | 0 | 12 | 6 | 0 | 16 | 6 |
| 19 | 12 | 4 | 0 | 12 | 12 | 4 | 4 | 12 | 18 |
| 20 | 6 | 0 | 0 | 0 | 6 | 6 | 0 | 0 | 0 |
| 21 | 0 | 0 | 0 | 6 | 0 | 0 | 0 | 0 | 0 |
| 22 | 0 | 0 | 0 | 0 | 0 | 6 | 0 | 6 | 6 |
| 23 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 24 | 0 | 0 | 0 | 0 | 0 | 12 | 0 | 0 | 0 |
| Mean | 5.17 | 2.17 | 1.33 | 3.00 | 6.92 | 7.25 | 2.58 | 5.75 | 5.83 |
| Std dev | 4.82 | 4.21 | 2.87 | 4.13 | 4.97 | 5.03 | 3.61 | 5.48 | 5.75 |
| Median | 6.00 | 0.00 | 0.00 | 0.00 | 8.00 | 8.00 | 0.00 | 5.00 | 5.00 |

‡ Positive at vaccination; ¿Odd results (see text)

**Table 3.2**: Mean and median duration of maternal immunity when foals that had positive antibody titres on the day of vaccination are removed from the data set.

| | Serotype 1 | Serotype 4 | Serotype 6 |
|---|---|---|---|
| Mean | 4.38 | 2.18 | 6.82 |
| se | 4.54 | 3.14 | 5.04 |
| Median | 4.00 | 0.00 | 7 |

Table 3.3 shows the anti-AHS antibody titre of each foal, for each of the seven serotypes included in the vaccinations, on the day that the foal was vaccinated for the first time (light blue cells represent the positive antibody titres). As can be seen from the limited colour in Table 3.3, very few of the foals still had a positive level of maternal immunity when vaccinated. The oldest foal with a positive antibody titre when vaccinated was 108 days old (14 weeks). These results do not agree with the findings of Alexander and Mason (1941) who found that passive immunity to African horse sickness lasts for approximately 6 months, but confirm the findings of Blackburn and Swanepoel (1998) and field observations (Gerdes and Mullins, 2000, pers. comm.) that maternal immunity fades before the foals are six months old. In addition, it implies that the foals could be vaccinated earlier than six months of age without maternal immunity compromising the reaction to the vaccine. If the foals were vaccinated at 120 days (four months) for the first time, none would have had a positive antibody titre for any of the serotypes on the day of vaccination.

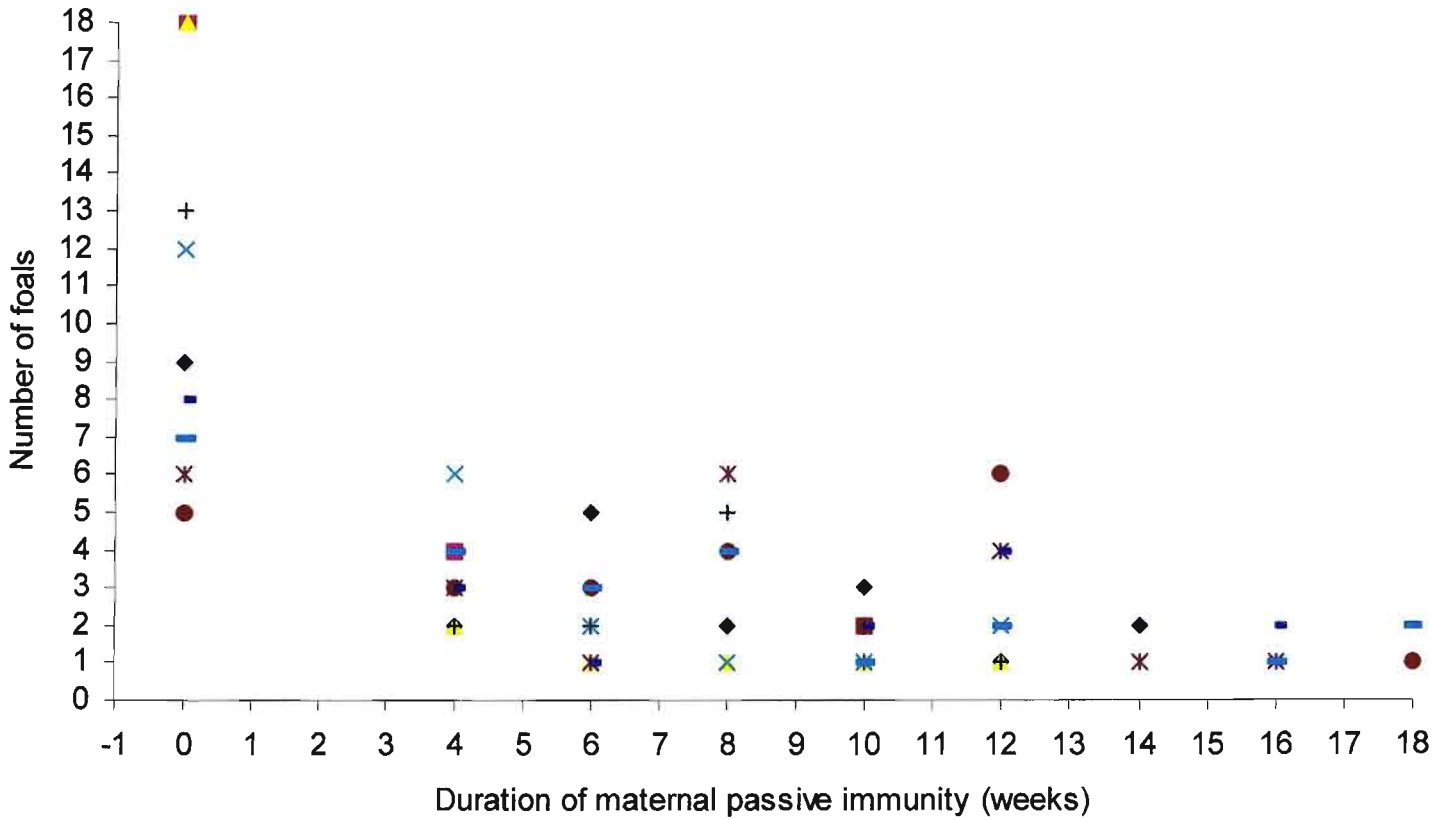


**Figure 3.7** Duration of maternal passive immunity in foals for each of the nine African horse sickness serotypes (all 24 foals) as measured by positive virus neutralisation antibody titres (Serotypes: 1 ◆, 2 ■, 3 ▲, 4 ×, 5 ∗, 6 ●, 7 +, 8 –, 9 – )

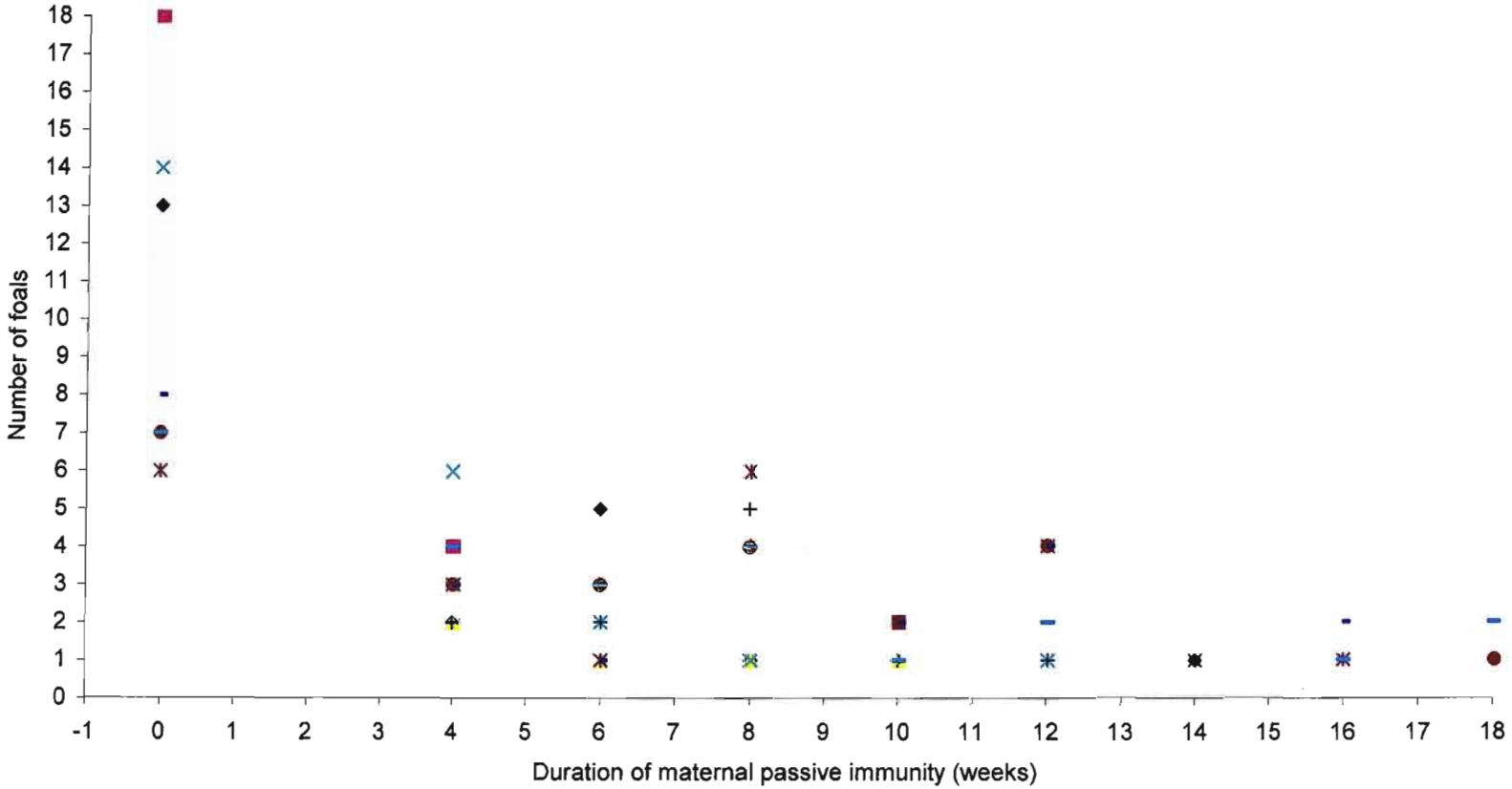


**Figure 3.8** Duration of maternal passive immunity in foals for each of the nine African horse sickness serotypes (four foals positive at vaccination removed) as measured by positive virus neutralisation antibody titres
(Serotypes: 1 ◆, 2 ■, 3 ▲, 4 ×, 5 ✳, 6 ●, 7 +, 8 –, 9 –)

**Table 3.3**: Foal antibody titres for the African horse sickness virus serotypes in the vaccine on the day of the first vaccination (Vaccination 1) with the relevant vaccine combination (AHS 1 or AHS 2)

| Foal No. | Age at 1st | Serotype | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 6 | 7 | 8 |
| 1 | 65 | 1:32[§] | 1:2 | 1:2 | 1:4 | 1:64 | 1:2 | 1:2 |
| 2 | 69 | 1:256 | 1:16 | 1:8 | 1:64 | 1:256 | 1:8 | 1:4 |
| 3 | 74 | 1:4 | 1:4 | 1:4 | 1:2 | 1:8 | 1:8 | 1:4 |
| 4 | 83 | 1:16 | 1:4 | 1:2 | 1:8 | 1:2 | 1:2 | 1:8 |
| 5 | 86 | 1:256 | 1:2 | 1:256 | 1:128 | 1:2 | 1:2 | 1:2 |
| 6 | 91 | 1:8 | 1:8 | 1:8 | 1:4 | 1:16 | 1:16 | 1:2 |
| 7 | 96 | 1:16 | 1:2 | 1:2 | 1:2 | 1:8 | 1:2 | 1:8 |
| 8 | 99 | 1:8 | 1:4 | 1:4 | 1:4 | 1:8 | 1:2 | 1:4 |
| 9 | 105 | 1:4 | 1:2 | 1:2 | 1:2 | 1:16 | 1:2 | 1:2 |
| 10 | 106 | 1:2 | 1:2 | 1:2 | 1:32 | 1:2 | 1:2 | 1:8 |
| 11 | 108 | 1:2 | 1:2 | 1:2 | 1:2 | 1:4 | 1:2 | 1:2 |
| 12 | 108 | 1:256 | 1:2 | 1:2 | 1:4 | 1:2 | 1:2 | 1:8 |
| 13 | 122 | 1:16 | 1:2 | 1:4 | 1:4 | 1:8 | 1:2 | 1:2 |
| 14 | 128 | 1:2 | 1:4 | 1:2 | 1:2 | 1:2 | 1:2 | 1:8 |
| 15 | 130 | 1:8 | 1:4 | 1:2 | 1:2 | 1:16 | 1:2 | 1:4 |
| 16 | 131 | 1:8 | 1:2 | 1:2 | 1:2 | 1:16 | 1:2 | 1:4 |
| 17 | 134 | 1:4 | 1:2 | 1:2 | 1:4 | 1:4 | 1:4 | 1:4 |
| 18 | 139 | 1:4 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 |
| 19 | 143 | 1:8 | 1:2 | 1:2 | 1:4 | 1:4 | 1:2 | 1:8 |
| 20 | 160 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 |
| 21 | 161 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 |
| 22 | 163 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 |
| 23 | 168 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 |
| 24 | 170 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 |

[§]Positive Antibody titres

### 3.4.2 Response of foals to the first African horse sickness vaccination

There was wide variation in the response of each foal to each of the seven serotypes in the vaccinations and in the antibody titre levels of Serotypes 5 and 9. The reaction to Serotype 1 was the most positive and the main part of the discussion will focus on this

result and possible methods of achieving the same level of reaction for the other serotypes. The variation in the response of the mares and foals to the seven serotypes in the vaccinations is illustrated in Figure 3.9. The response of the foals is shown at four different points in time: 12 hours of age, four or six weeks of age, eight weeks after the first vaccination and eight weeks after the second vaccination. The mares have the most negative antibody titres to Serotype 2 and no negative antibody titres to Serotype 8. The foals exhibited a similar pattern with few positive antibody titres to Serotype 2, and many positive antibody titres to Serotypes 5, 6 and 8 at the 12 hours sampling. This is expected, since the foal's immunity at this age is a reflection of the antibodies they received in the colostrum, as their own immune systems are not yet fully functional. Although Serotype 5 is not in the vaccine, the mares did show positive antibody titres for this serotype (either through cross protection from Serotype 8 or natural exposure).

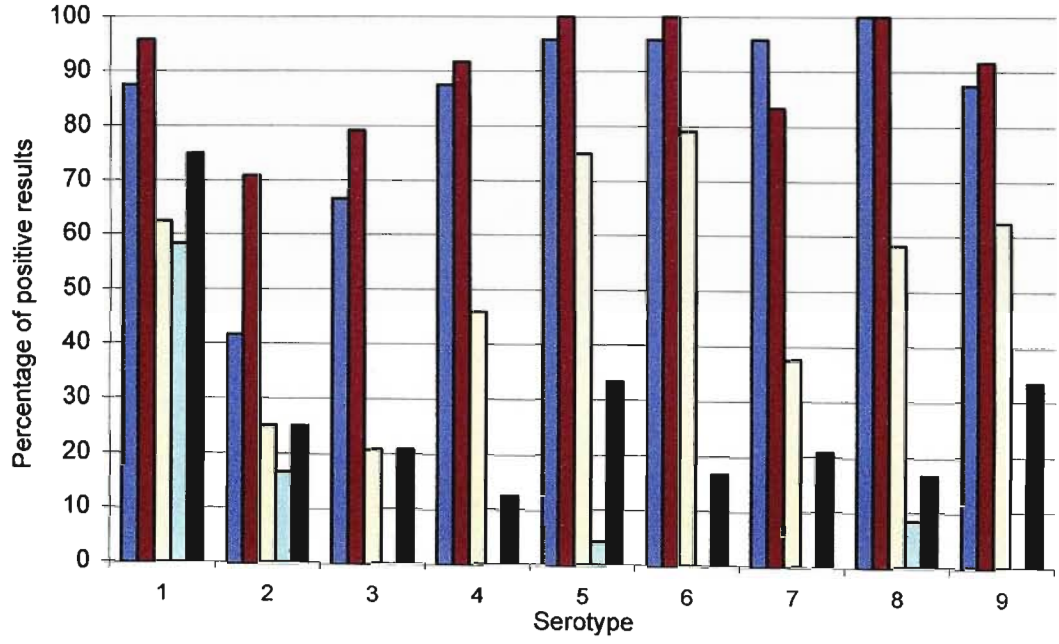


**Figure 3.9** Differences in antibody titres as shown by the percentage of positive results for the mares (within 30 mins of birth) and at four points in time for the foals; ■ Mares, ■ Foals 12 hours, ■ Foals 4/6 weeks old, ■ Foals 8 weeks after Vaccination 1, ■ Foals 8 weeks after Vaccination 2

By the time the foals were four to six weeks old, the proportion of negative antibody titres had increased for all serotypes. Serotype 3 had the greatest proportion of negative antibody titres at this stage while Serotype 6 had the lowest proportion of negative antibody titres. In this trial, the foals showed no positive antibody titres to Serotype 4 eight weeks after Vaccination 1, and only three positive antibody titres (12.5 % of foals) eight weeks after Vaccination 2. There were also no positive antibody titres for

antibodies against Serotypes 3, 6, 7 and 9 eight weeks after Vaccination 1. Vaccinations 1 and 2 resulted in 58 % and 75 % positive antibody titres to Serotype 1, respectively. Perhaps surprisingly, Vaccination 2 was followed by 33 % positive antibody titres for Serotypes 5 and 9 (there were 16.67 % positive antibody titres for Serotypes 6 and 8, the cross protective serotypes for Serotypes 9 and 5, respectively) since they are not included in the vaccination. Serotypes 5 and 9 will not be discussed further for this reason.

Table 3.4 shows the virus neutralisation results for Serotype 1, with the first born (older) foals on the right hand side of the table. Each column represents one foal, with the sequential samples running down the column. The blue-coloured blocks indicate positive antibody titres and the yellow blocks represent negative antibody titres (an antibody titre of less than 1:32). The white squares show where no samples were taken. The fourth and fifth lines of Table 3.4 show the number of days between the mare's vaccination and the birth date and the age at first vaccination for each of the 24 foals respectively. Of particular importance, as will be discussed later, is the fact that all the mares were vaccinated on the same day, irrespective of the anticipated foaling date, and all the foals were vaccinated on the same day, irrespective of age. This means that there is a perfect negative correlation (-1.0) between the age of the foal at first vaccination and the length of time between the mare's vaccination and the birth of the foal. Therefore, the foals that were born early in the season are associated with a short gap between the mare being vaccinated and their birth, whilst they are the oldest foals when vaccinated themselves. The rows labelled Vaccination 1 and Vaccination 2 illustrate when the foals were vaccinated with the vaccine combination containing the relevant serotype (AHS 1 or AHS 2). Serotype 1 is shown in Table 3.4 while the other eight serotypes are presented in Appendix 6. The other half of the vaccine combination (AHS 1 or AHS 2) would have been given three weeks later for Serotypes 1, 3 and 4, and three weeks earlier for Serotypes 2, 6, 7 and 8.

There are two clear areas of negative results (yellow blocks). The first occurs after the foals which were the youngest when first vaccinated (i.e. the left of the table) have been vaccinated for the first time. This group of foals (1 to 7) was born at least three and a half months after the mares had been vaccinated. The second area of negative results is on the right hand side of the table, where the first born (older) foals appear to lack maternal immunity. This group of foals (21 to 24) was born within six weeks of the mare being vaccinated. Although not as clear (there are more negative results), the pattern is repeated for the other eight serotypes (Appendix 6). The poor level of immunity in these two groups thus appears to be related to the time between the mare being vaccinated and the foal birth and/or the age at which the foal was vaccinated for the first time. Foal 24 does not fit the pattern as the only positive antibody titres this foal had were at one and two months of age for Serotype 6 and at 13 months of age for Serotype 5.

**Table 3.4**: Virus neutralisation results for AHS Serotype 1

| ZST result | + | + | + | Weak + | Weak + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | - | - | + | + | Weak + |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| GC result | + | + | + | Weak + | + | + | + | + | + | + | Weak + | + | + | + | + | + | + | Weak + | + | + | | | | |
| **Foal number** | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** | **13** | **14** | **15** | **16** | **17** | **18** | **19** | **20** | **21** | **22** | **23** | **24** |
| Days: mare vac to foaling | 138 | 134 | 129 | 120 | 117 | 112 | 107 | 104 | 98 | 97 | 95 | 95 | 81 | 75 | 73 | 72 | 69 | 64 | 60 | 43 | 42 | 40 | 35 | 33 |
| Foal age at vac 1 (days) | 65 | 69 | 74 | 83 | 86 | 91 | 96 | 99 | 105 | 106 | 108 | 108 | 122 | 128 | 130 | 131 | 134 | 139 | 143 | 160 | 161 | 163 | 168 | 170 |
| Mare | 1:16 | 1:256 | 1:32 | 1:64 | 1:256 | 1:128 | 1:256 | 1:128 | 1:32 | 1:8 | 1:128 | 1:256 | 1:256 | 1:16 | 1:64 | 1:256 | 1:256 | 1:128 | 1:256 | 1:256 | 1:64 | 1:256 | 1:256 | 1:128 |
| Day 0 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 |
| Day 1 | 1:256 | 1:256 | 1:32 | 1:128 | 1:2 | 1:256 | 1:256 | 1:256 | 1:128 | 1:2 | 1:64 | 1:256 | 1:256 | 1:32 | 1:64 | | 1:256 | | | | | | | |
| vac 1 - 3.5 months | | | | | | | | | | | | | | | | | | | 1:128 | 1:32 | | | 1:8 | |
| vac 1 - 3 months | | | | | | | | | | | | | 1:256 | 1:8 | 1:32 | 1:64 | 1:8 | 1:64 | | | | | | 1:2 |
| vac 1 - 2.5 months | | | | | | | | | 1:16 | 1:2 | 1:32 | 1:256 | | | 1:8 | | 1:8 | 1:16 | 1:64 | | 1:2 | | 1:2 | |
| vac 1 - 2 months | | | | | 1:16 | 1:256 | | 1:32 | | | | | 1:128 | | | 1:8 | | | | 1:16 | | 1:4 | | |
| vac 1 - 1.5 months | | 1:256 | 1:128 | 1:128 | 1:2 | | 1:64 | 1:8 | 1:4 | 1:8 | 1:4 | 1:256 | | 1:4 | 1:8 | 1:4 | 1:8 | 1:4 | 1:32 | | 1:2 | 1:4 | 1:2 | 1:2 |
| vac 1 - 1 month | 1:64 | 1:256 | | | | | 1:32 | | | | | | | 1:2 | | | | | | 1:2 | | | | |
| vac 1 - 0.5 months | 1:32 | | 1:16 | 1:32 | | 1:64 | 1:32 | 1:8 | 1:8 | 1:8 | 1:2 | 1:256 | 1:64 | 1:2 | 1:4 | 1:8 | 1:256 | 1:4 | 1:4 | 1:2 | 1:2 | 1:4 | 1:2 | 1:2 |
| Vaccination 1 | **1:32** | **1:256** | **1:4** | **1:16** | **1:256** | **1:8** | **1:16** | **1:8** | **1:4** | **1:2** | **1:2** | **1:256** | **1:16** | **1:2** | **1:8** | **1:8** | **1:4** | **1:4** | **1:8** | **1:2** | **1:2** | **1:2** | **1:2** | **1:2** |
| vac 1 + 0.5 months | | 1:128 | 1:4 | | 1:2 | | | 1:16 | | | | | | | 1:8 | 1:2 | 1:2 | | | | 1:2 | 1:2 | | |
| vac 1 + 1 month | 1:4 | | | 1:16 | | 1:32 | 1:16 | | 1:2 | 1:4 | 1:8 | 1:256 | 1:32 | 1:256 | | | | 1:32 | 1:16 | 1:16 | | | 1:2 | 1:2 |
| vac 1 + 1.5 months | | | | | | | | | | | | | | | | | | | | | | | | |
| vac 1 + 2 months | 1:2 | 1:16 | 1:2 | | 1:2 | 1:2 | 1:2 | 1:32 | 1:2 | 1:256 | 1:32 | 1:256 | 1:8 | 1:256 | 1:256 | 1:256 | 1:64 | 1:256 | 1:128 | | 1:256 | 1:64 | 1:256 | 1:2 |
| vac 1 + 2.5 months | | 1:8 | | 1:16 | | | | 1:256 | | | | | | | 1:256 | | | | | 1:256 | | | | |
| vac 1 + 3 months | 1:2 | | 1:2 | 1:8 | 1:2 | 1:4 | 1:2 | | 1:256 | 1:256 | 1:64 | 1:256 | 1:8 | 56 | | 1:128 | 1:256 | 1:256 | 1:256 | 1:256 | 1:256 | 1:256 | 1:64 | 1:2 |
| vac 1 + 3.5 months | | | | | | | | | | | | | | | | | | | | | | | | |
| vac 1 + 4 months | | 1:16 | 1:2 | | 1:2 | | | 1:64 | 1:8 | | | | | | 1:256 | 1:256 | 1:256 | | | | 1:256 | 1:256 | | |
| vac 1 + 4.5 months | 1:2 | | | 1:2 | | 1:2 | 1:4 | | | 1:256 | 1:256 | 1:256 | 1:8 | 1:256 | | | | 1:256 | 1:256 | 1:256 | | | 1:256 | 1:2 |
| vac 1 + 5 months | | 1:8 | 1:2 | | 1:8 | | | 1:128 | 1:16 | | | | | | 1:256 | 1:64 | 1:256 | | | | 1:256 | 1:256 | | |
| vac 1 + 5.5 months | 1:2 | | | 1:2 | | 1:4 | 1:2 | | | 1:256 | 1:256 | 1:256 | 1:2 | 1:256 | | | | 1:256 | 1:256 | 1:256 | | | 1:64 | 1:2 |
| vac 1 + 6 months | | 1:2 | 1:2 | | 1:2 | 1:2 | | 1:256 | 1:32 | 1:256 | 1:256 | 1:256 | 1:2 | | 1:256 | 1:32 | 1:256 | 1:256 | | | 1:256 | 1:256 | 1:32 | 1:2 |
| vac 1 + 6.5 months | 1:2 | | | 1:2 | | | 1:2 | | | | | | | 1:128 | | | | | 1:256 | 1:256 | | | | |
| | | | | | | | | | | | | | | | | | | | | | | | | |
| Vaccination 2 | **1:2** | **1:2** | **1:2** | **1:2** | **1:2** | **1:2** | **1:2** | **1:256** | **1:32** | **1:256** | **1:256** | **1:256** | **1:2** | **1:128** | **1:256** | **1:32** | **1:256** | **1:256** | **1:256** | **1:256** | **1:256** | **1:256** | **1:32** | **1:2** |
| vac 2 + 0.5 month | 1:2 | 1:4 | | 1:4 | 1:4 | | 1:8 | 1:64 | | | | | | 1:256 | 1:256 | 1:256 | 1:256 | | 1:256 | 1:128 | 1:256 | 1:256 | | |
| vac 2 + 1 month | | | 1:2 | | | 1:16 | | | 1:256 | 1:256 | 1:256 | 1:256 | 1:256 | | | | | 1:256 | | | | | 1:64 | 1:2 |
| vac 2 + 1.5 months | 1:2 | 1:2 | 1:2 | 1:256 | 1:8 | | 1:4 | 1:64 | | | | | | 1:256 | 1:256 | 1:128 | 1:256 | | 1:256 | 1:128 | 1:128 | 1:256 | | |
| vac 2 + 2 months | | | | | | 1:8 | | | 1:64 | 1:256 | 1:128 | 1:256 | 1:256 | | | | | 1:256 | | | | | 1:128 | 1:2 |
| vac 2 + 2.5 months | 1:256 | 1:2 | 1:2 | 1:256 | 1:2 | 1:4 | 1:4 | 1:128 | 1:16 | 1:256 | 1:32 | 1:256 | 1:256 | 1:256 | 1:256 | 1:256 | 1:256 | 1:256 | 1:256 | 1:128 | 1:128 | 1:256 | 1:128 | |
| vac 2 + 3 months | | | | | | | | | | | | | | | | | | | | | | | | 1:2 |
| vac 2 + 3.5 months | 1:256 | 1:2 | 1:2 | | 1:2 | | 1:4 | | | | | | | 1:128 | 1:256 | | 1:256 | | 1:256 | | 1:64 | 1:256 | | |
| vac 2 + 4 months | | | | 1:256 | | 1:2 | | 1:128 | 1:32 | 1:256 | 1:32 | 1:256 | 1:64 | | | 1:128 | | 1:256 | | 1:32 | | | 1:32 | 1:2 |

The combined effects of the time between the mare being vaccinated and the foal's birth and that of the mare's antibody titre on the foal's antibody titre at four to six weeks of age are shown in Figure 3.10a and Figure 3.10b. Two figures are used to illustrate this point, as the three dimensional aspect may not be easily interpreted. The superimposed plane in Figure 3.10a illustrates that as the mare's antibody titre decreases and the interval between the mare's vaccination and foal birth decreases, so the foal's antibody titre decreases when tested between four and six weeks of age. This is also shown by the contours in Figure 3.10b. Those foals (21 - 24) born within six weeks of the mare being vaccinated, do not appear to have received any maternal antibodies to the AHSV. Interestingly though, the ZST test was positive for Foals 22 and 23, and a weak positive for Foal 24, indicating that these foals had achieved a successful transfer of passive immunity. Foal 21 had a negative result for the ZST test but the pattern of response to the vaccinations is the same as for Foals 22 to 24. This negative result for antibodies for AHSV may be because the antibodies are not yet present in the colostrum.

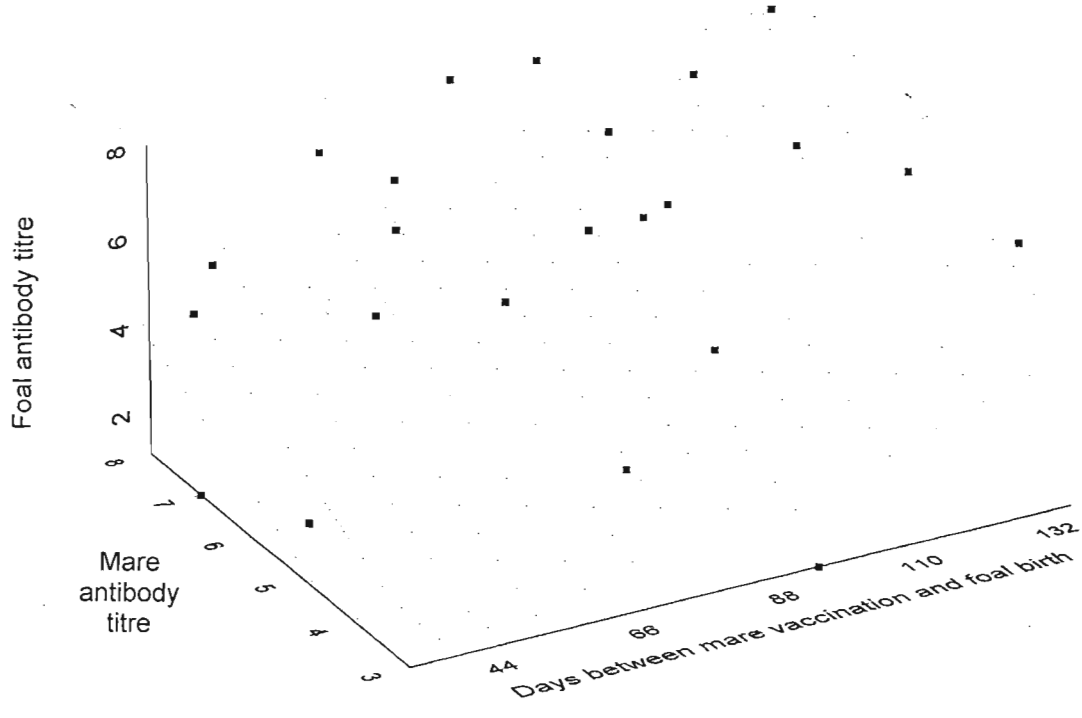


**Figure 3.10a** The effect of i) the mare's anti-African horse sickness Serotype 1 antibody titre and ii) the days between the mare's vaccination and the foal's birth on the foal's African horse sickness Serotype 1 antibody titre at four to six weeks of age; 1= 1:2, 2=1:4, 3=1:8, 4=1:16, 5=1:32, 6=1:64, 7=1:128, 8=1:256.

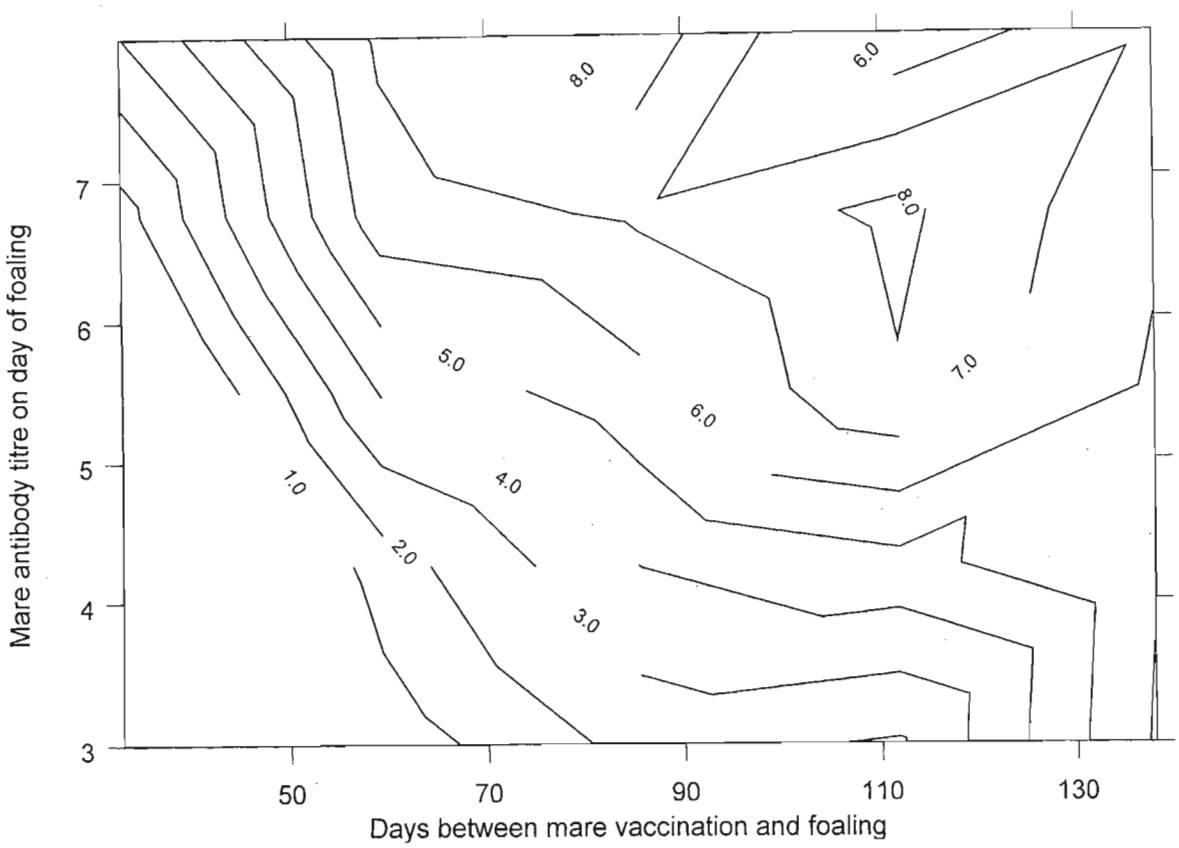


**Figure 3.10b** The effect of i) the mare's anti-African horse sickness Serotype 1 antibody titre and ii) the days between the mare's vaccination and the foal's birth on the foal's African horse sickness Serotype 1 antibody titre at four to six weeks of age; 1= 1:2, 2=1:4, 3=1:8, 4=1:16, 5=1:32, 6=1:64, 7=1:128, 8=1:256.

Figure 3.11 illustrates the foal antibody titre on the day of vaccination with the age of the foal at first vaccination. The lower antibody levels are found in the older foals on the day of the vaccination. Whether the low antibody titres in the older foals are due to the age of the foal *per se*, or the short interval between the mare's vaccination and the birth of the foal cannot be answered conclusively from this trial (except for Foals 21 to 24, which did not have a positive antibody titre at one month of age), since all the mares, and subsequently the foals, were vaccinated on one day by the stud farms involved. Nevertheless, the lack of maternal immunity in the slightly older group of foals (15 to 20), for periods of up to sixteen weeks (from fading of maternal immunity to positive antibody titre after vaccination) (Table 3.5) leaves this group vulnerable to African horse sickness and raises questions about the practice of blanket vaccination on a single day.

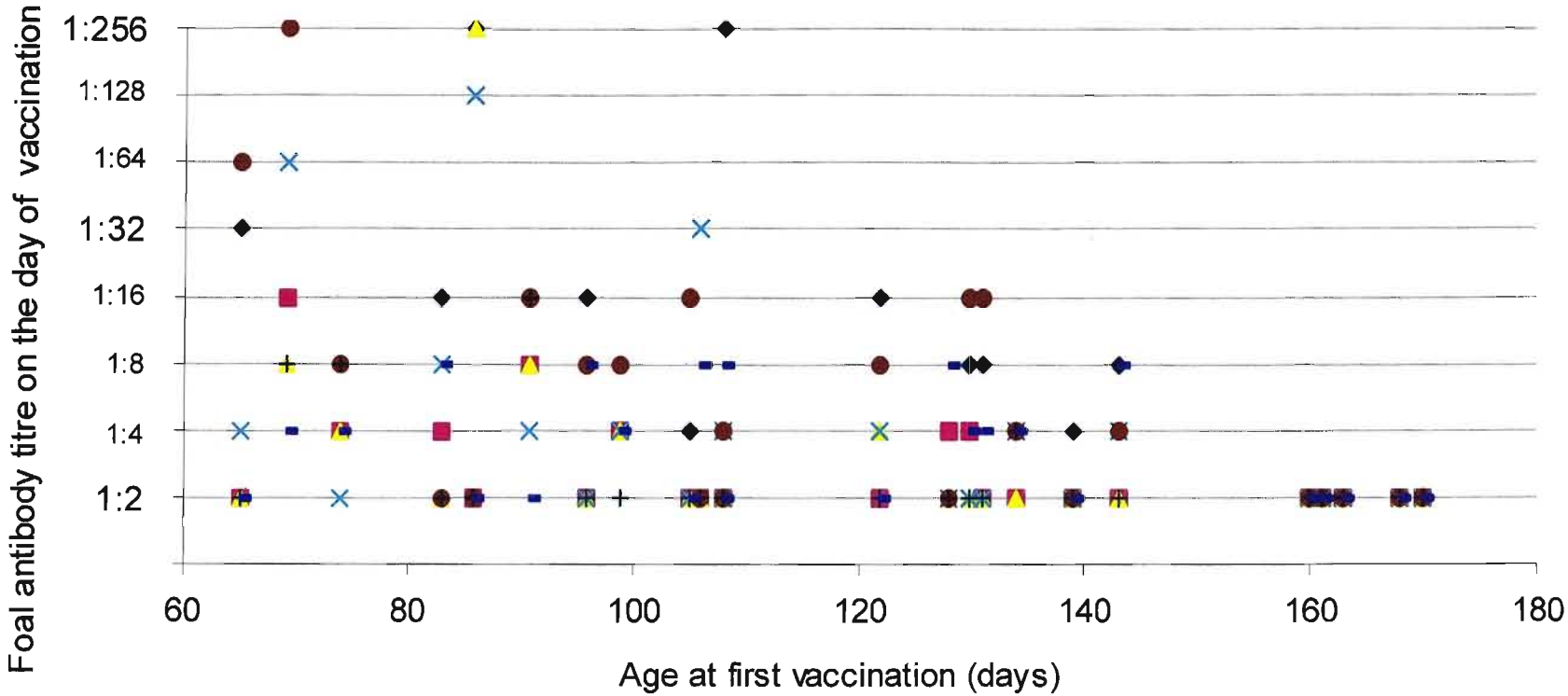


**Figure 3.11** The effect of foal age (days) at first vaccination on the antibody titre on the day of the first vaccination (Serotypes: 1 ◆, 2 ■, 3 ▲, 4 ×, 6 ●, 7 +, 8 –).

**Table 3.5:** Virus neutralisation antibody titres for selected foals (15 – 20) to illustrate the relationship between the time between the maternal immunity fading and a positive antibody titre after the vaccination

| Foal | 15 | 16 | 17 | 18 | 19 | 20 |
|---|---|---|---|---|---|---|
| Age (Weeks) | | | | | | |
| 4 | | | | | 1:128 | |
| 6 | 1:32[§] | 1:64 | 1:8 | 1:64 | | 1:32 |
| 8 | 1:8 | | 1:8 | 1:16 | 1:64 | |
| 10 | | 1:8 | | | | |
| 12 | 1:8 | 1:4 | 1:8 | 1:4 | 1:32 | 1:16 |
| 14 | | | | | | |
| 16 | 1:4 | 1:8 | 1:256 | 1:4 | 1:4 | 1:2 |
| 18 | **1:8[£]** | **1:8** | **1:4** | **1:4** | **1:8** | 1:2 |
| 20 | 1:8 | 1:2 | 1:2 | | | **1:2** |
| 22 | | | | 1:32 | 1:16 | |
| 24 | | | | | | 1:16 |
| 26 | 1:256 | 1:256 | 1:64 | 1:256 | 1:128 | |
| 28 | 1:256 | | | | | |
| 30 | | 1:128 | 1:256 | 1:256 | 1:256 | 1:256 |
| Weeks with negative antibody titres | 16 | 16 | 24? | 12 | 8 | 16 |

**[§]Positive Antibody titres; [£]Day of vaccination**

The antibody status of the foal on the day of the first vaccination is another factor that affects the reaction to the vaccine (Figure 3.12). Only one foal had a positive antibody titre on the day of the vaccination and still had an unchanged positive antibody titre two months later. This foal (12) did not have any negative antibody titres for Serotype 1. Two foals had unchanged negative antibody titres at both samplings, one of these foals (Foal 24) had an antibody titre of only 1:2, throughout the trial period. The majority of the foals (13; 54.2 %) had an increase in antibody titre at the sample taken two months after the vaccination and all of these foals had a negative antibody titre on the day of the vaccination and a positive antibody titre two months later. Of the remaining eight foals, three (12.5 %) had a positive antibody titre on the day of the vaccination and a negative two months later and the last five (20.8 %) had negative antibody titres for both samples, with a decrease in antibody titres between the samples. This indicates that the foal has a greater chance of reacting to the vaccination if the antibody titre has dropped to a negative antibody titre before the vaccination is administered. This pattern changes from the majority of the foals ending with a positive antibody titre to the majority of the foals having an unchanged antibody titre when all the serotypes are included in the analysis (Figure 3.13). The pattern for Serotype 1 represents the pattern of response of an effective vaccination.

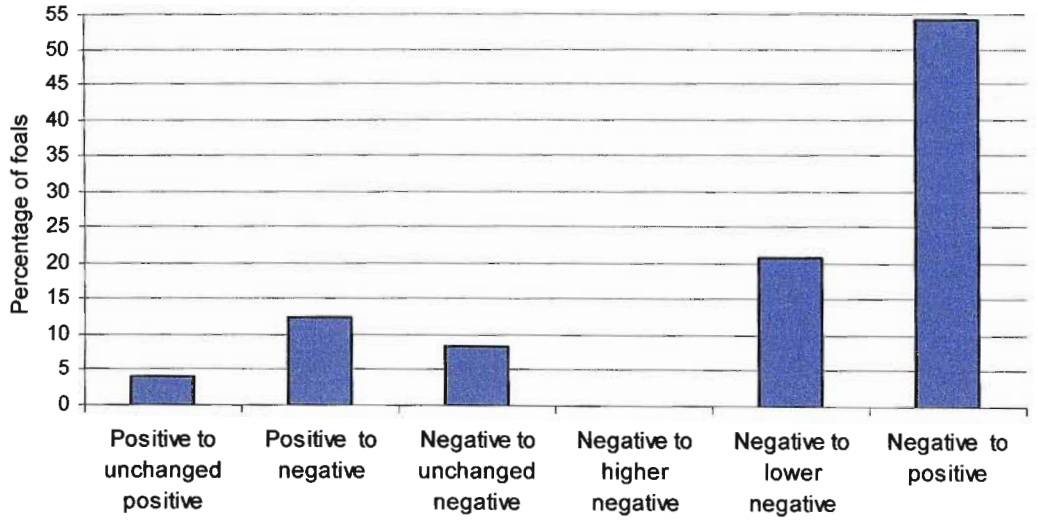


**Figure 3.12** The effect of the foal's antibody titre for Serotype 1, on the day of Vaccination 1, on the subsequent reaction to the vaccination, as shown by the change in antibody titre two months from vaccination day.

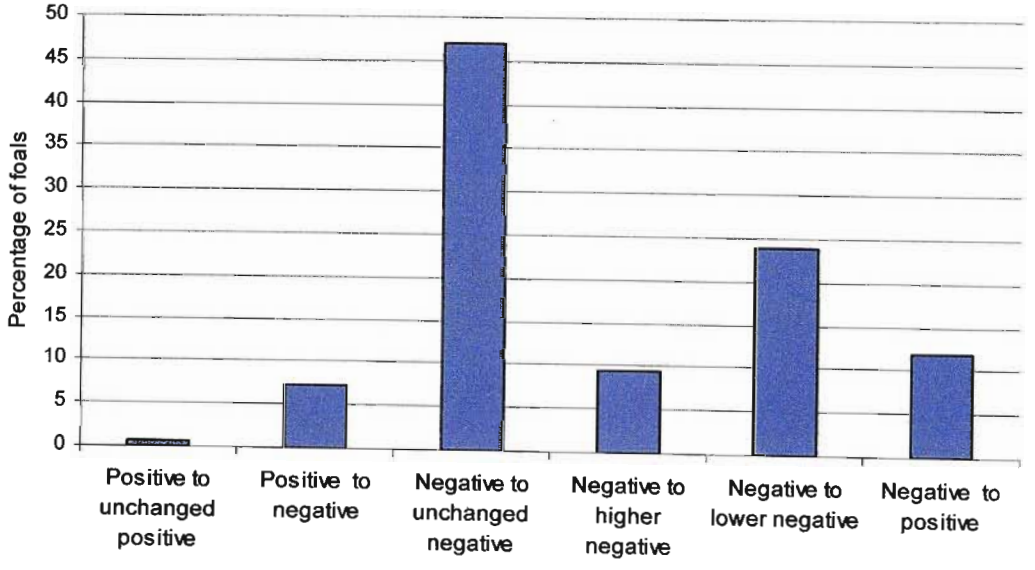


**Figure 3.13** The effect of the foal's antibody titre (all serotypes) on the day of Vaccination 1 on the subsequent reaction to the vaccination, as shown by the change in antibody titre two months from the day of vaccination.

The greater chance of a positive reaction to the vaccination if the antibody titre is a negative before the vaccination is also illustrated when a group of seven foals is tracked[15] (for Serotype 1, Figure 3.14) from before vaccination to 12 weeks after the vaccination. It can be seen that the anti-AHSV antibody titres of the two foals with a positive antibody titre on the day of vaccination dropped after vaccination, while the antibody titres of the other three foals increased. However, for a few of the foals (four for Serotype 1) the antibody titres did not rise after the vaccination. The decrease in antibody titre after vaccination indicates that there is a reaction between the existing passive immunity in the foal and the live virus in the vaccine. The virus and the maternal antibodies possibly react and no new antibodies are produced due to the negative feedback effect of the presence of the passively transferred antibodies (Tizard, 2000).

Immune responses are in part regulated by a negative feedback system, the presence of IgG antibodies depresses the production of IgM and IgG, while the presence of IgM antibodies depresses the further synthesis of IgM (Tizard, 2000). A specific antibody will tend to suppress a specific immune response better than non-specific immunoglobulin. Antibodies from maternal immunity have the same effect (Tizard, 2000), hence the poor reaction to the vaccination in these foals.

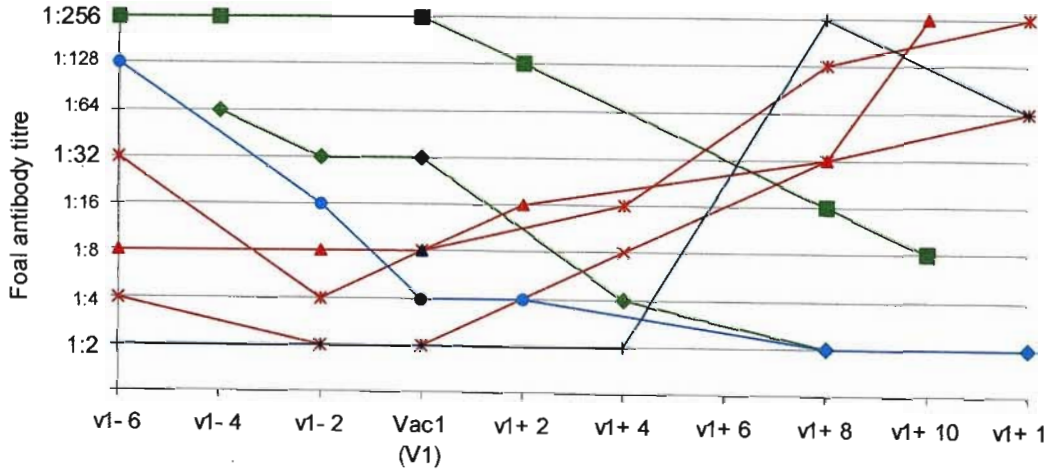


**Figure 3.14** Foal antibody titres contrasting the response of foals to the vaccination with positive and negative antibody titres at vaccination;
♦ Foal 1; ▪ Foal 2; ▲ Foal 8; ● Foal 9; x Foal 11; + Foal 14; * Foal 19.

[15] These seven foals were chosen to illustrate each of the reaction patterns for Serotype 1.

A possible theoretical explanation for the pattern of the foals' reactions to the vaccination is that the virus from the vaccination given to the mare is still alive in the mare and is transmitted at birth to the foals born early in the season (i.e. foals born within 3 months of mare vaccination; Foals 12 to 24) (Weber, 2004, pers. Comm.), or that remnants of the virus are transmitted to the foal and act as an antigen. This would mean that the antigen and the maternal antibodies would react in the foal, and the very first foals of the season (those born within 2 months of mare vaccination; Foals 19 to 24) would appear to receive no immunity from the mares. The next group of foals; born two to three months after the mare vaccination (Foals 12 to 19), would still retain some of the maternal immunity and the last foals born (Foals 1 to 11) would not be affected by the antigen. This infection at birth, in foals born to mares vaccinated late in pregnancy, would be the foal's initial exposure to the antigen and the foal's own immune system will register the antigen, thereby improving the subsequent reaction to the vaccination. The vaccination will therefore effectively be the "booster" rather than the initial exposure to the virus. If the foals are being exposed to the antigen at birth then, although the first foals (Foals 19 to 24) appear to be unprotected in the *post partum* period, the reaction to further exposure to the virus will result in a prompt immune reaction as demonstrated by the reaction to the vaccination. However, this is still not an ideal situation as the reaction may not be prompt enough for complete protection and some foals may still be lost. The foals born slightly later, two to three months after mare vaccination (Foals 12 to 19), have the best protection as they retain some of the maternal immunity and react to the first vaccination.

For the foals born at the end of the season (Foals 1 to 11), the vaccination will represent the initial exposure to the live virus. These foals can be further split into two groups. The first is the foals born more than three months after the mare was vaccinated and older than three months at first vaccination (Foals 6 to 11). The second is the foals born more than 3 months after the mare was vaccinated and less than three months old at first vaccination (Foals 1 to 5).

The first group of foals (Foals 6 to 11) will react to the vaccine as the maternal immunity will have faded before they are vaccinated and they will therefore have some protection after the first vaccination. The final group of foals (Foals 1 to 5) will have retained the antibodies from the mare. These antibodies will react with the virus in the vaccine causing a decrease in the antibody titre rather than the expected increase. These foals are thus protected from birth until the first vaccination but are subsequently unprotected.

### 3.4.3 Response of foals to the second African horse sickness vaccination

Only three of the nine foals with no reaction to the first vaccination reacted to the second vaccination for Serotype 1 (Table 3.4). There are two possibilities for the lack of reaction to the second vaccination in foals less than three months at first vaccination. As there is little known about the immune status of equines (Mullins, pers. comm., 2006) either possibility or a combination could be the reason. The first is that the first vaccination was given too early and the passive immunity received from the mare possibly interfered with the reaction to the vaccine, and this effect continued to the second vaccination. The second possibility is that the second set of vaccines is given too long after the first. The second vaccination is thus not acting as a booster but rather as the primary vaccination again (Weber, 2004, pers. comm.). Newborn animals are capable of an immune response at birth. However, the primary response is limited with a long lag period and low antibody concentrations (Tizard, 2000). The response to the booster or second exposure is faster and greater due to the presence of already formed memory cells (Tizard, 2000). Immunoglobulin M and IgG are the two most important immunoglobulins in the development of immunity (reaction to vaccination) (Tizard, 2000). Some IgM is present in the serum of the foal when it is born (Tizard, 2000). Immunoglobulin G and IgM are antibodies that are formed during active immunity, and are part of the B cell response to an antigen. When the body is exposed to an antigen for the first time, B cells are activated and divide into three types of cells - IgM producing B cells (majority), some IgG producing B cells and memory B cells (Figure 3.15). When the same antigen is encountered again, the memory B cells are activated producing mainly IgG, some IgM and more memory B cells. This secondary response is much greater than the primary response and the lag period is shorter as more antibodies are produced and the antigen is detected sooner (Tizard, 2000). After each vaccination, the base of memory cells from the previous vaccination will be built on. Demonstrable antibodies to all the serotypes may only be present after three or four vaccinations (Gerdes, pers. comm., 2005). The more effective the vaccination programme, the quicker this will occur. The tests measure the circulating or humoral antibodies, but there is also an unmeasured cellular response providing protection against AHS (Gerdes, pers. comm., 2005).

The poor response to the second vaccination could be because the time period between the two sets of vaccinations is too great and so the second set of vaccinations may be acting as an initial exposure to the virus again, rather than as a booster exposure. The multiple serotypes complicate the vaccination programme as there is not an equal reaction to all of the serotypes. The best reaction will be to the most immunogenic strain in the vaccine (this appears to be Serotype 1 in AHS 1 and Serotype 2 in AHS 2 for the work reported here as is shown by the number of foals with a positive antibody titre at the end of the trial period, Table 3.6) and, as the attractiveness of a serotype to the immune system decreases, so will the reaction to that serotype (Tizard, 2000).

Stressors will also reduce the ability of the immune system to react to the vaccine (Tizard, 2000) and the foals are generally under some stress when they are vaccinated as they are not fully used to being handled and, in general, other vaccinations and management practices (e.g. hoof trimming) are completed on the same day.

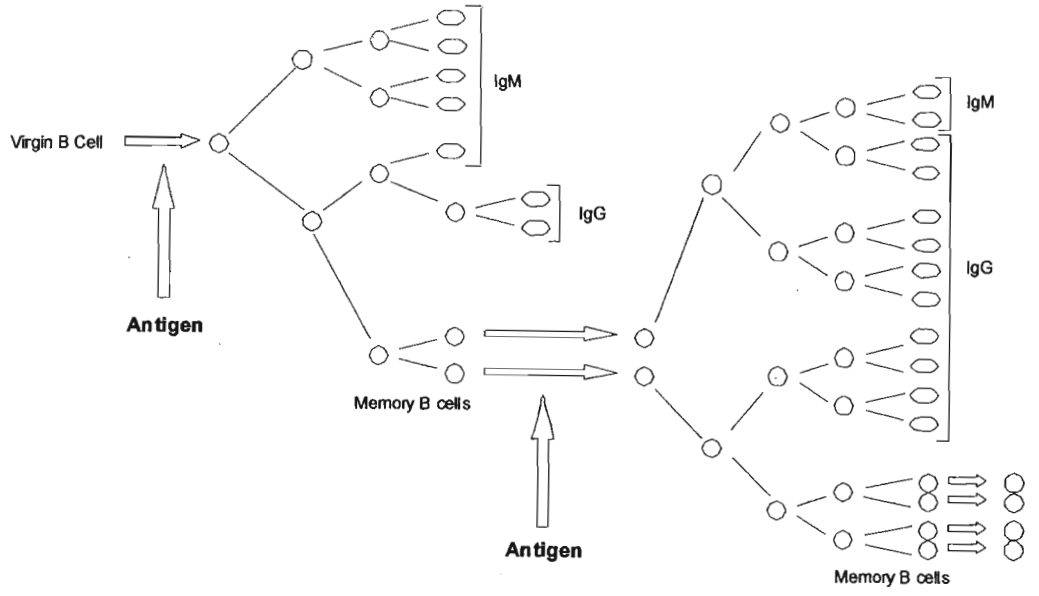


**Figure 3.15** The time course of a B-cell response and the cellular events that accompany it. Note how some IgG is made in the primary immune response while a little IgM is made in the secondary immune response (Tizard, 2000)

**Table 3.6** The number of positive and negative antibody titres at the end of the trial period for each of the nine AHS serotypes.

| Serotype | Positive antibody titres | Negative antibody titres |
|---|---|---|
| 1 | 18 | 6 |
| 2 | 7 | 17 |
| 3 | 3 | 21 |
| 4 | 1 | 23 |
| 5 | 5 | 19 |
| 6 | 3 | 21 |
| 7 | 5 | 19 |
| 8 | 5 | 19 |
| 9 | 7 | 17 |

The package insert for the vaccinations (Appendix 4) states that immunity starts to develop two to three weeks after inoculation and protection against some of the virus serotypes is achieved after four weeks. Figures 3.16 and 3.17 show the large variation

in the time taken to develop a positive antibody titre after Vaccinations 1 and 2, respectively. Some of the foals only achieved a positive antibody titre after the immunity of other foals had already started to wane. The variation in the number of positive reactions between serotypes, and the time taken to develop a positive antibody titre are also evident. Vaccination 2 resulted in a positive antibody titre in a shorter period of time, although fewer foals reacted to this vaccination.

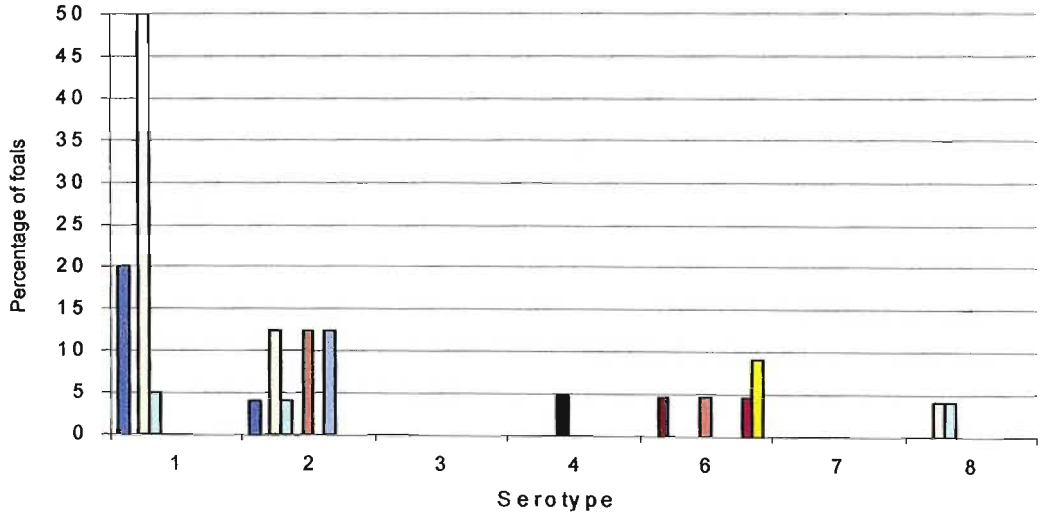


**Figure 3.16** Time from first vaccination to positive antibody titre as shown by the percentage of new positive antibody titres in foals previously testing negative (note: no positive antibody titres for Serotypes 3 and 7);
4 w, 6 w, 8 w, 10 w, 12 w, 14 w, 16 w, 18 w, 20 w, 22 w, 24 w

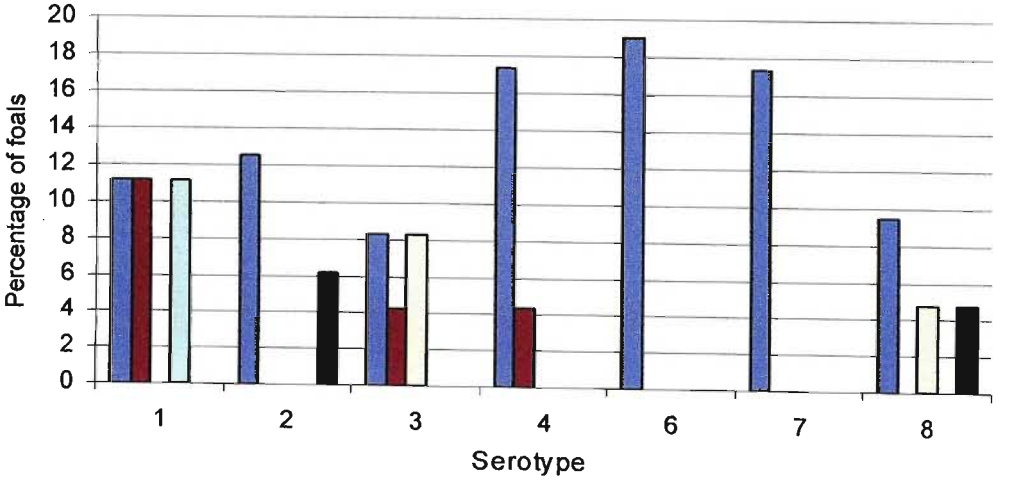


**Figure 3.17** Time from second vaccination to positive antibody titre as shown by the percentage of new positive antibody titres in foals previously testing negative (note: no positive antibody titres for Serotypes 3 and 7);
4 w, 6 w, 8 w, 10 w, 12 w

### 3.4.4 Overall reactions and patterns

Figure 3.18 shows the different time lines for vaccination and antibody development for three foals from mare vaccination until the last sample taken; for the second foal from either end of the foaling season (i.e. second oldest and second youngest foal on the day of the first vaccination) and for a foal from the middle of the season (born 73 days after the mare vaccination and 130 days old when vaccinated for the first time. Foals 2 and 23 were chosen instead of 1 and 24 because Foal 24 does not fit the pattern (as discussed previously); Foal 15 was chosen because it has the best protection overall.

Foal 2 is representative of the foals born late in the season, with some level of maternal immunity, a positive antibody titre when first vaccinated and no response to either of the vaccinations.

The time line, with respect to maternal and foal vaccinations, experienced by the foal 15 resulted in the best protection for the foal against Serotype 1 when compared to the other foals. This foal also had the best protection (when compared to the other foals) against Serotypes 3, 6 and 7. It had a good level of protection against Serotypes 2 and 9, but no positive results for Serotypes 4, 5 and 8. The antibody titres at each sampling for this foal from six weeks of age are shown in Figures 3.19, 3.20 and 3.21. Figure 3.19 shows Serotypes 1, 3, 6 and 7; Figure 3.20 shows Serotypes 2 and 9 and Figure 3.21 shows Serotypes 4, 5 and 8.

Although foal 15 had no positive antibody titres for Serotypes 4, 5 and 8, two foals within the same group outlined in the discussion about Table 3.4 had the best pattern of protection. The foal with the best pattern of protection for Serotypes 4 and 5 was Foal 14 and for Serotype 8 was Foal 13. These foals are within the same group outlined in the discussion about Table 3.4; i.e. those foals born two to three months after the mare vaccination and about four months of age when vaccinated for the first time. This group of foals (Foals 12 to 19) demonstrate that positive results from the vaccine are possible, but the recommendations for the vaccination programme may need revising and any recommended programme must be adhered to. There are positive antibody titres outside of this group of foals for some of the serotypes, but at least one foal in this group had a positive antibody titre at the end of the trial for each serotype, and the majority of the foals with positive antibody titres at the end of the sampling period.

Foal 23 is representative of the first foals of the season, with no passive immunity; a minimal response to the first vaccination and no further response to the second vaccination.

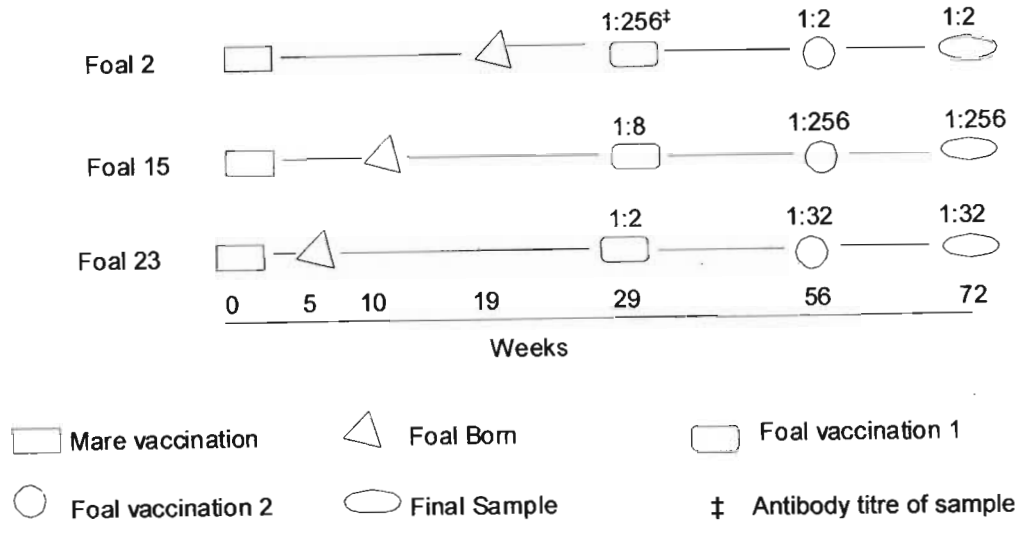


**Figure 3.18** Time frames and response of three selected foals (Serotype 1).

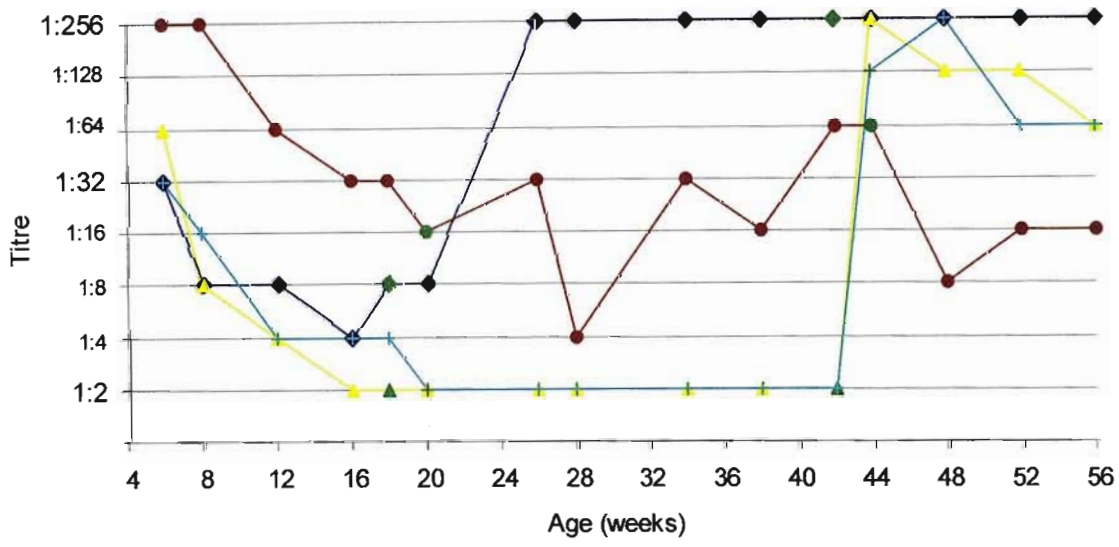


**Figure 3.19** Anti-AHSV antibody titres for foal 15 from six weeks of age to end of the trial period for Serotypes 1, 3, 6 and 7 (green points are when the foal was vaccinated for each serotype) (Serotypes: 1 ◆, 3 ▲, 6 ●, 7 +)

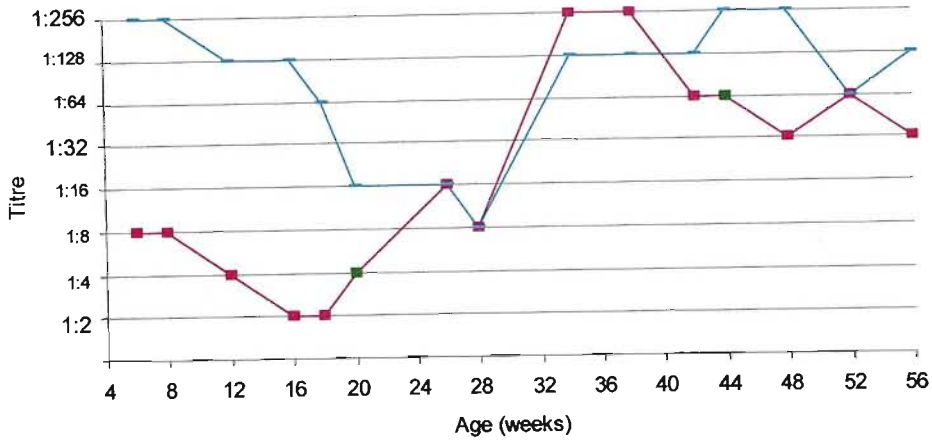


**Figure 3.20** Anti-AHSV antibody titres for foal 15 from six weeks of age to end of the trial period for Serotypes 2 and 9 (green points are when the foal was vaccinated) (Serotypes: 2 ■, 9 –)

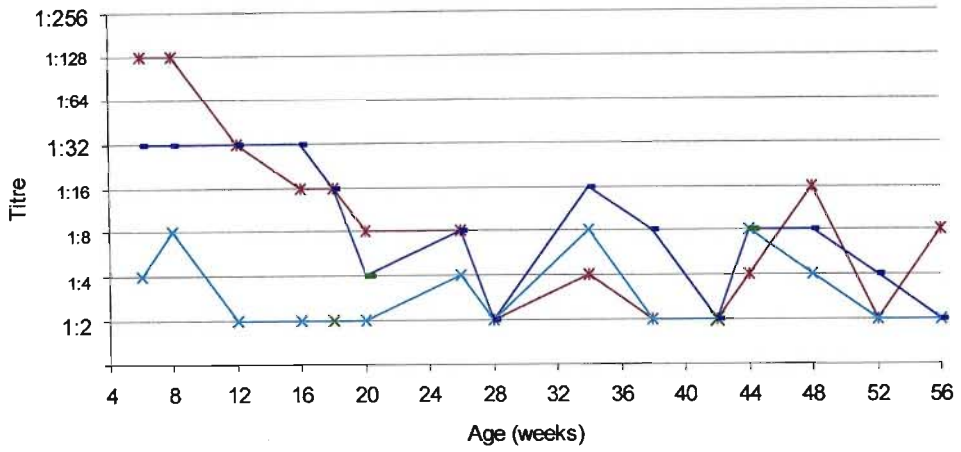


**Figure 3.21** Anti-AHSV antibody titres for foal 15 from six weeks of age to end of the trial period for Serotypes 4, 5 and 8 (green points are when the foal was vaccinated for each serotype) (Serotypes: 4 ×, 5 *, 8 –)

The effect of the time gap between the mare's vaccination and the foal being born and the foal's age at first vaccination on the foal's response to the vaccinations have already been covered in this discussion. The recommendation for a vaccination program needs to take these factors (the timing of the vaccinations including the mare's vaccination date and the foal's age at first vaccination) into account to try to achieve the ideal pattern of response for all of the foals. Vaccinating all the mares and, subsequently, all the foals

on a single day will result in the greatest possible range of reactions and may result in more animals than necessary being unprotected. The principle of herd immunity is that the individual animal is at the greatest risk when the proportion of susceptible animals in the herd is high, while the susceptible individual is indirectly protected when the proportion of susceptible animals is low. The principle of herd immunity works well when the majority of the animals are protected (Tizard, 2000) but a vaccination program should strengthen the herd immunity rather than relying on the herd immunity for protection of the animals within the herd.

On the basis of the work reported here, a revised vaccination schedule is recommended in which the mare would be vaccinated annually at three months and two months prior to her expected foaling date, with African horse sickness AHS 1 and AHS 2, respectively. The foals would then be vaccinated at four and five months of age with African horse sickness AHS 1 and AHS 2, and then again at six and seven months of age. This should give the majority of the foals the same pattern of reaction as discussed for Foal 15, in which they are protected from birth until the first vaccination by the maternal immunity but the maternal immunity would have decreased sufficiently to allow a reaction to take place to the first vaccination. The second vaccination will then be at the correct time to act as a booster vaccination and to give the foals a longer lasting immunity. The second vaccination needs to be close enough to the first vaccination to act as a booster, but still sufficiently spaced so that the negative feedback effect of the presence of antibodies to the virus will not reduce the response to the vaccine. This revised vaccination schedule is based on the results of the trial reported here, in which the maternal immunity had decreased to a sufficiently low level to have allowed the first vaccination by four months of age. The time between the vaccinations has been decreased, as in the reported trial the second dose did not appear to be acting as a "booster" vaccination should, but rather as a primary vaccination.

Any revised vaccination schedule should be based on the expected foaling date for each mare and the age of each foal. It could be based on the dates for a group of mares and the ages of a group of foals (dates and ages within a month of each other). Vaccinating all the animals on a farm on the same day may make management easy but this means that there is a large reliance on herd immunity protecting the individual, since this system results in wide deviations from the recommended vaccination schedule. The case study used in this discussion and many of the other principles discussed have shown that confidence in the herd immunity will be unjustified if the majority of the animals are in fact, unprotected.

## 3.5 Acknowledgements

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# CHAPTER 4

# GENERAL CONCLUSION

The literature review looked at the passive transfer of immunity from the mare to the foal. The newborn foal is at risk from the development of infections as it adapts to its new environment. The risk is decreased by the passive transfer of immunity from the mare through colostrum. This immunity protects the foal until its own immune system is fully functional. Factors affecting the transfer of passive immunity and the consequences of failure of passive transfer of immunity were discussed. The importance of testing for the transfer of passive immunity and different methods of testing whether the transfer of passive immunity has occurred were reviewed. Each test method and its associated advantages and disadvantages were described. Passive immunity affects vaccination programmes, as the presence of maternal antibodies inhibits the active immune response. This means that passive immunity affects the timing of the first vaccination in two ways. Firstly, the vaccination will only be effective when the passive immunity has faded sufficiently to allow an active response but, secondly, the vaccination should not be given after the passive immunity has declined to non-protective levels. The review concluded with a discussion of vaccination programmes using African horse sickness as an example.

Trial One looked at several different, locally available, methods of testing for the transfer of passive immunity. The tests investigated were the measurement of the colostral specific gravity, the measurement of total serum protein by the refractometer and the measurement of serum immunoglobulin G by the zinc sulphate turbidity (ZST) and glutaraldehyde coagulation (GC) tests. The results of these methods were compared to the results of the measurement of immunoglobulin G by the single radial immunodiffusion test (SRID).

The single radial immunodiffusion test is the benchmark test for immunoglobulin testing, and represents the ideal test to use as it gives a quantitative result and is not subjective. However, the test takes too long to complete for routine use in newborn foals, since the gastrointestinal becomes impermeable to immunoglobulins within approximately 18 to 24 hours of birth. This means that treatments for failure of transfer of passive immunity need to be given very soon after birth. The specific density of colostrum was shown not to be a good predictor of the transfer of passive immunity. However, the colostrum specific density test should still be used to test the colostrum that is going to be stored for use in treating failure of passive transfer. There were significant correlations in the values for IgG status between the three inexpensive tests and the SRID test (colostrum, $p=0.1$; refractometer, GC and ZST, $p=0.01$). The ZST and GC tests express IgG status

in categories, relative to the critical IgG levels of 400 and 800 mg IgG/dl serum. The refractometer yields a quantitative test result, allowing regression analysis to be used to give a prediction of the SRID result from refractometer readings. This analysis suggests that refractometer readings of 6.30 and 6.58 equate to SRID readings of 400 and 800 mg/dl, respectively. Chi-squared analysis of the GC, ZST and refractometer results showed there to be a significant relationship ($p=0.1$) between observed and expected IgG values for all three tests.

Over- and under-estimation of IgG status (% samples, compared to the SRID test results) were found to be, respectively, 7.04 and 2.82 (GC), 11.97 and 4.93 (ZST) and 12.68 and 0.7 (refractometer). The final choice of the ZST, GC or refractometer tests as the foal-side test will have to be decided on by the stud manager and veterinarian, taking into account local conditions but the recommendation of the author would be to use the GC test. The current recommendation to test the foal six hours after birth and to treat before 18 hours of age with colostrum remain valid. The age of the mare, parity of the mare and the gestation length did not appear to affect the transfer of immunity from the mare to the foal.

Trial Two looked at the African Horse Sickness vaccination programme, focusing on when to vaccinate foals for the first time and what factors affect the response of the foals to the vaccine. A recommendation was made that management practices need to change in order to adhere more closely to the vaccination schedule recommended by the manufacturer. Suggested revisions to the existing vaccination schedule were also presented. The revised vaccination schedule is as follows: the mare should be vaccinated at three and two months prior to her expected foaling date with AHS 1 and AHS 2 respectively. The foal would then be vaccinated at four and five months of age with AHS 1 and AHS 2 and then again at six and seven months of age (AHS 1 and AHS 2).

The results of this work have raised many questions and there is little doubt that further research is needed into the response of animals in the field to available vaccines. Adjusting the current vaccination programme is only a start in the battle to protect South African horses as fully as possible against AHS.

Specific questions that further research should aim to answer are:

1) The effectiveness of any revised or proposed vaccination programmes (from foals to mature animals).

2) Should the vaccine be split further into monovalent vaccines or polyvalent vaccines with fewer serotypes?

3) How long before the expected foaling date should the mare be vaccinated in order for antibodies to be present in the colostrum?

4) How long does it take to transfer AHSV antibodies into the colostrum and does it differ for the different serotypes?

5) When should the third set of vaccinations take place?

6) Is the virus from the vaccine being transferred to the foal at birth?

This thesis makes available valuable field data on the effectiveness of the African horse sickness vaccination under practical conditions in the KwaZulu-Natal Midlands. It is hoped that, beyond the recommendations made above, other researchers will be able to make use of the data presented in the Appendices attached to this work to help design more efficient means of controlling African horse sickness in the Southern African region.

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## PERSONAL COMMUNICATIONS

Gerdes, G.H., Virology Department, Onderstepoort Veterinary Institute, Private Bag X5, Onderstepoort, 0110

Gerstenberg, C., Directorate Animal Health, Sub Directorate Disease Control, Private Bag 138, Pretoria, 0001

Mullins, D.E., Mooi River Veterinary Clinic, P.O. Box 9, Mooi River, 3300

Weber, G., 2004, Disease Control Africa, Postnet Suite 490, Private Bag X1, Die Wilgers, 0041, jc.weber@mweb.co.za

# APPENDIX 1

# SINGLE RADIAL IMMUNODIFFUSION PROTOCOL

This is the package insert that comes with the Single Radial Immunodiffusion test kits.

## A1.1 Kit contents

1) Ready to use single radial immunodiffusion (SRID) plates containing monospecific antisera in buffered agarose.
2) Reference standards (0.09% Sodium Azide).
3) Instruction sheet with graph paper.
4) Disposable micropippettes with reusable plungers.

## A1.2 Materials needed

1. Test tubes if dilutions are necessary.
2. Samples to be tested. Specimens should be collected by approved veterinary techniques. Allow the blood to clot, and centrifuge to collect serum. Use serum within 72 hours, or freeze until ready to assay. Avoid repeated freezing and thawing.

## A1.3 Precautions

Store plates in an inverted position at 4-8°C. DO NOT FREEZE. Keep the plate tightly closed at all times. Between uses, plates may be stored inverted in their zip lock bags. The expiration date is listed on the outside package label.

**For Veterinary Research Use Only**

## A1.4 Procedure

Add reference standards by carefully pipetting 3 microliters of each into the first wells. To use the micropipetting system, insert the reusable plunger into the end of the disposable micropipette marked by the yellow band. Depress the plunger to the other end of the micropipette. Draw the standard up to the black line marked on the micropipette. Insert the pipette into the well to be filled and depress the plunger. Be sure to lift the pipette off the well bottom once the filling process has begun. The pipette may then be discarded.

Pipette 3 microliters of the specimen(s) to be tested into the remaining wells.

Replace the cover firmly on the plate and leave undisturbed, right side up at room temperature for 18-24 hours outside of its zip lock bag.

After 18-24 hours, the diameters of the rings may be read and a standard curve established. Measure each diameter either directly off the plate or by using an inexpensive comparator. Read each diameter in terms of mm. If all wells are not used, replace the lid securely and store at 4-8° C inverted in the zip lock bag provided.

Plot the diameters of the reference standards versus the concentrations of the reference standards as indicated on the supplied graph paper. Draw a standard curve. Alternatively, a calculator with the capability of establishing a regression line may be used to determine the concentrations of the unknowns. Specimens with diameters beyond the range of the standard curve may be retested either by redilution if the rings are too large or by concentration if the rings are too small.

**Do not freeze!**
**Store inverted at 4-8°C**

## A1.5 Interpretation

Information sheets regarding immuno-deficiencies and immunoproliferative disorders of horses, cows and dogs are available through **VMRD, Inc.** (Section A1.8).

## A1.6 Principles

The technique of single radial immunodiffusion (SRID) is the most widely used method for quantitative determination of classes of immunoglobulins and other serum and plasma proteins. This technique combines rapid and easy sample application with a high degree of accuracy and reproducibility.

The method is derived primarily from the works of Fahey, and of Mancini. Antiserum specific for the protein to be measured is incorporated into agarose gel. The sample antigen diffuses into the gel containing the antibody, and a ring of precipitation forms that is proportional in size to the concentration of the antigen (Figure A1.1). A linear relationship exists between the diameter of the ring and the concentration of the antigen when plotted on semi-log graph paper. This method is time and temperature dependant.

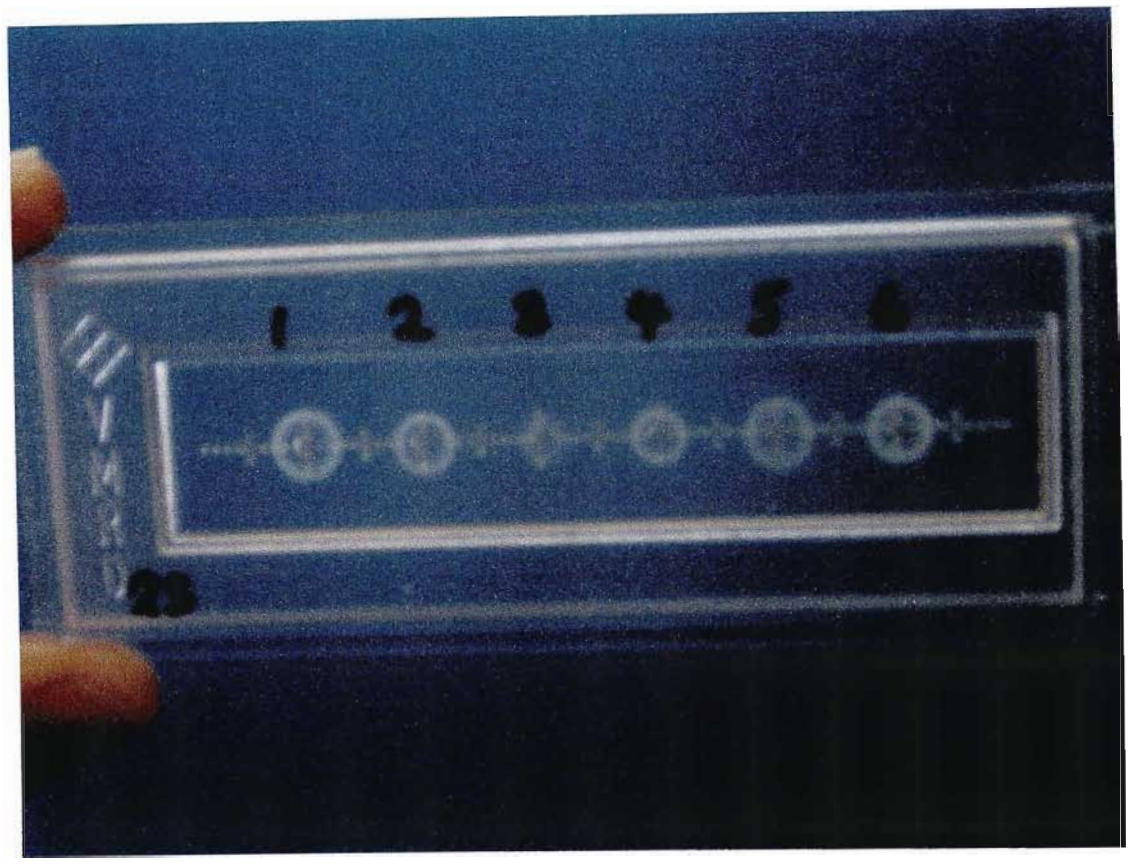


**Figure A1.1** Single radial immunodiffusion test plate

## A1.7 Limitations

Results are limited by the assay range of the kit, although dilution of the specimen to be tested will extend the range. When diameters greater than the highest reference standard occur, dilute the sample and rerun the assay. If the diameter of the ring is too small, the sample may be concentrated.

## A1.8 Equine information sheet

VMRD, Inc's radial immunodiffusion plates are ready-to-use with standards calibrated to determine samples within the normal range. Custom plates can be prepared to meet your specific needs.

Prevalence of Immunodeficiency Disorders

| | |
|---|---|
| FPT and partial FPT: | as high as 20% of all foals, the percentage varies depending on neonatal foal management |
| CID: | 2.3% of all Arabian foals |
| Selective IgM Deficiency: | unknown; 19 of 2,092 submissions |

| | |
|---|---|
| Agammaglobulinemia; | unknown; 3 of 2,092 submissions |
| Transient hypogammaglobulinemia: | unknown; 2 of 2,092 submissions |
| Selective IgA Deficiency: | unknown |
| Lymphosarcoma with paraproteins: | unknown |

**Normal Equine Immunoglobulin Values**

| Age | Breed | No. of Animals | Ref. No. | IgA | IgG | IgG (T) | IgM |
|---|---|---|---|---|---|---|---|
| **Serum (mg/dl +/- S.D.)** | | | | | | | |
| 1 – 20 days | Arabian | 28 | 1 | 21+/-13 | 814+/-583 | 143+/-88 | 28+/-11 |
| 1 – 13 days | Arabian | 37 | 2 | 38+/-14 | 944+/-523 | 171+/-94 | 31+/-10 |
| 1 – 14 days | TB | 66 | 3 | ND | 1335+/-652* | ND | ND |
| 21 – 40 days | Arabian | 22 | 1 | 17+/-13 | 480+/-293 | 126+/-41 | 30+/-10 |
| 41 – 60 days | Arabian | 19 | 1 | 20+/-6 | 264+/-193 | 96+/-33 | 42+/-12 |
| 61 – 80 days | Arabian | 9 | 1 | 25+/-10 | 252+/-128 | 75+/-17 | 36+/-12 |
| 81 -140 days | Arabian | 15 | 1 | 64+/-28 | 248+/-92 | 162+/-126 | 39+/-15 |
| 3 - 5 months | Mixed | 27 | 4 | 38+/-14 | 380+/-188 | 211+/-148 | 61+/-22** |
| 6 -12 months | TB/Mixed | 95 | NA | ND | 863+/-298 | ND | 50+/-16 |
| Adults | Shetland | 20 | 6 | 153+/-86 | 1334+/-350 | 821+/-310 | 120+/-31 |
| **Tears (mg/dl)** | | | | | | | |
| Adults | Shetland | 4 | 6 | 135 | 15 | 3 | 0 - 5 |
| **Milk (mg/dl)** | | | | | | | |
| | Horse | NA | 10 | 50 - 100 | 20 - 50 | 5 - 20 | 5 - 10 |
| **Postpartum Presuckle Colostrum** | | | | | | | |
| | TB | 32 | 4 | IgG: mean = 2354; range = 1080 - 8960 | | | |

* This IgG value is higher than other data for similarly aged foals because all foals with less than 400 mg/dl were diagnosed as failures and removed from the normal data.

** Since most selective IgM deficiencies are diagnosed between 3-5 months, an IgM value of 17 mg/dl (61 minus 2 standard deviations) has been used as a cutoff point in this age group.

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## APPENDIX 2

RESULTS TABLES OF TESTS FOR IMMUNOGLOBULIN G (IgG)

**Table A2.1**: Details of the mares

| Mare No. | Colostrum | Refractometer | ZST | GC (mg/dl) | SRID (mg/dl) | Age (years) | Parity (No. of foals) | Gestation length (days) | Foal 12 hr SRID (mg/dl) |
|---|---|---|---|---|---|---|---|---|---|
| 1 | | 9 | + | >800 | 1299 | 15 | 10 | 334 | 1969 |
| 2 | | 10 | + | >800 | 1969 | 7 | 3 | 344 | 3591 |
| 3 | | 8 | + | >800 | 2160 | 6 | 1 | 350 | 2160 |
| 4 | 1.06 -1.09 | 9 | + | >800 | 1579 | 9 | 4 | 351 | 2507 |
| 5 | >>1.09 | 9.5 | + | >800 | 1773 | 13 | 7 | 355 | 1253 |
| 6 | 1.08 | 9 | + | >800 | 2507 | 9 | 3 | 342 | 790 |
| 7 | >>1.10 | 9 | + | >800 | 2369 | 11 | 8 | 343 | 1492 |
| 8 | >1.09 | 8.8 | + | >800 | 3761 | 11 | 4 | 351 | 4738 |
| 9 | 1.08 | 9.2 | + | >800 | 2369 | 10 | 4 | 327 | 2659 |
| 10 | 1.08 | 9.2 | + | >800 | 2369 | 11 | 6 | 352 | 5443 |
| 11 | 1.06 | 8.8 | + | >800 | 2160 | 7 | 1 | 339 | 1361 |
| 12 | 1.06 | 8.6 | + | >800 | 1492 | 8 | 4 | 353 | 1080 |
| 13 | 1.07 | 10 | + | >800 | 1637 | 14 | 6 | 334 | 3761 |
| 14 | 1.08 | 8.2 | + | >800 | 2369 | 5 | 1 | 355 | 1492 |
| 15 | 1.10 | 9 | + | >800 | 1880 | 5 | 1 | 334 | 1425 |
| 16 | >1.10 | 9 | + | >800 | 2262 | 6 | 1 | 338 | 1425 |
| 17 | 1.10 | 8 | + | >800 | 1492 | 7 | 3 | 341 | 1299 |
| 18 | 1.09 | 9 | + | >800 | 1880 | 7 | 1 | 348 | 3429 |
| 19 | 1.08 | 9 | + | >800 | 2062 | 13 | 7 | 347 | 3591 |
| 20 | 1.07 | 8 | + | >800 | 3591 | 15 | 8 | 338 | 3429 |

**Table A2.1 (continued)**: Details of the mares

| Mare No. | Colostrum | Refractometer | ZST | GC (mg/dl) | SRID (mg/dl) | Age (years) | Parity (No. of foals) | Gestation length (days) | Foal 12 hr SRID (mg/dl) |
|---|---|---|---|---|---|---|---|---|---|
| 21 | 1.04 | 9.6 | + | >800 | 1563 | 11 | 6 | 353 | 1563 |
| 22 | 1.09 | 9.6 | + | >800 | 3938 | 11 | 3 | 333 | 3761 |
| 23 | >1.10 | 8.8 | + | >800 | 940 | 10 | 3 | 347 | 2262 |
| 24 | <1.00 | 9.4 | + | >800 | 1714 | 15 | 6 | 334 | 620 |
| 25 | 1.03 | 9 | + | >800 | 1425 | 6 | 1 | 318 | 898 |
| 26 | 1.08 | 9.6 | + | >800 | 2262 | 10 | 5 | 344 | 1425 |
| 27 | 1.06 | 10 | + | >800 | 2062 | 12 | 6 | 343 | 2481 |
| 28 | 1.07 | 9.8 | + | >800 | 1637 | 12 | 5 | 344 | 2262 |
| 29 | 1.10 | 9.8 | + | >800 | 1880 | 11 | 4 | 353 | 1492 |
| 30 | 1.04 | 10.6 | + | >800 | 2262 | 14 | 6 | 341 | 790 |
| 31 | >1.10 | 10 | + | >800 | 2369 | 20 | 8 | 349 | 2062 |
| 32 | 1.09 | 9.4 | + | >800 | 2160 | 9 | 3 | 343 | 3591 |
| 33 | 1.08 | 10.6 | + | >800 | 3591 | 14 | 5 | 338 | 4320 |
| 34 | 1.06 | 9.4 | + | >800 | 2369 | 11 | 6 | 346 | 3591 |
| 35 | 1.05 | 9.8 | + | >800 | 2160 | 12 | 5 | 346 | 940 |
| 36 | 1.09 | 9.8 | + | >800 | 1563 | 16 | 7 | 338 | 1637 |
| 37 | 1.06 | 8.4 | + | >800 | 2062 | 13 | 2 | 346 | 1131 |
| 38 | 1.05 | 9.2 | + | >800 | 2369 | 11 | 6 | 338 | 90 |
| 39 | 1.07 | 10 | + | >800 | 1969 | 12 | 7 | 343 | 2062 |
| 40 | 1.05 | 10.6 | + | >800 | 3591 | 15 | 6 | 341 | 1969 |

**Table A2.1 (continued)**: Details of the mares

| Mare No. | Colostrum | Refractometer | ZST | GC (mg/dl) | SRID (mg/dl) | Age (years) | Parity (No. of foals) | Gestation length (days) | Foal 12 hr SRID (mg/dl) |
|---|---|---|---|---|---|---|---|---|---|
| 41 | 1.07 | 9.2 | + | >800 | 2369 | 6 | 1 | 349 | 1361 |
| 42 | 1.07 | 10.2 | + | >800 | 2160 | 12 | 7 | 332 | 1080 |
| 43 | >>1.10 | 9 | + | >800 | 2062 | 10 | 2 | 338 | 2369 |
| 44 | >1.10 | 9.4 | + | >800 | 2262 | 12 | 6 | 339 | 3591 |
| 45 | 1.08 | 8 | + | >800 | 1880 | 6 | 1 | 358 | 1637 |
| 46 | 1.00 | 8.2 | + | >800 | 1425 | 15 | 7 | 342 | 497 |
| 47 | 1.06 | 9 | + | >800 | 1361 | 5 | 2 | 329 | 857 |
| 48 | 1.08 | 9 | + | >800 | 1361 | 11 | 6 | 345 | 2062 |
| 49 | 1.09 | 8.6 | + | >800 | 1031 | 7 | 3 | 341 | 1714 |
| 50 | 1.07 | 9.2 | + | >800 | 1880 | 11 | 4 | 338 | 2062 |

**Table A2.2**: Foal details

| Foal and sample no. | Age (hours) at sampling | Refractometer | ZST | GC (mg/dl) | SRID (mg/dl) |
|---|---|---|---|---|---|
| 1.1 | 8 | 7 | + | > 800 | 1131 |
| 1.2 | 16 | 8 | + | > 800 | 1969 |
| 1.3 | 32 | 8 | + | > 800 | 2160 |
| 2.1 | 6.5 | 9 | + | > 800 | 2369 |
| 2.2 | 14.5 | 9 | + | > 800 | 3591 |
| 2.3 | 30.5 | 9 | + | > 800 | 3429 |
| 3.1 | 7 | 7 | + | > 800 | 1425 |
| 3.2 | 15 | 7 | + | > 800 | 2160 |
| 3.3 | 31 | 7 | + | > 800 | 1880 |
| 4.1 | 12 | 8 | + | > 800 | 2507 |
| 4.2 | 24 | 7.5 | + | > 800 | 1990 |
| 5.1 | 22 | 7.5 | + | > 800 | 1254 |
| 6.1 | 12 | 6 | + | 400-800 | 790 |
| 6.2 | 20 | 6 | weak + | 400-800 | 790 |
| 6.3 | 36 | 6 | weak + | 400-800 | 665 |
| 7.1 | 11.75 | 8 | + | > 800 | 1492 |
| 7.2 | 19.75 | 8 | + | > 800 | 1492 |
| 7.3 | 35.75 | 7.4 | + | > 800 | 1492 |
| 8.1 | 0 | 5.6 | weak + | < 400 | 31 |
| 8.2 | 9.5 | 8.4 | + | > 800 | 3761 |
| 8.3 | 16.5 | 8.8 | + | > 800 | 4738 |
| 8.4 | 33.5 | 8.8 | + | > 800 | 3761 |
| 9.1 | 11 | 8 | + | > 800 | 2659 |
| 9.2 | 19 | 7.8 | + | > 800 | 3591 |
| 9.3 | 35.5 | 8 | + | > 800 | 2369 |
| 10.1 | 8 | 8.2 | + | > 800 | 2481 |
| 10.2 | 16 | 9 | + | > 800 | 5443 |
| 10.3 | 32 | 9 | + | > 800 | 5700 |
| 11.1 | 8 | 6.8 | + | > 800 | 1361 |
| 11.2 | 28.5 | 6.4 | + | > 800 | 1185 |
| 12.1 | 7.5 | 6.4 | + | > 800 | 1080 |
| 12.2 | 31.5 | 6.6 | weak + | > 800 | 1080 |
| 13.1 | 8 | 9 | + | > 800 | 4125 |
| 13.2 | 16 | 9 | + | > 800 | 3761 |
| 13.3 | 35.5 | 8.8 | + | > 800 | 3591 |
| 14.1 | 9 | 7.6 | + | > 800 | 1493 |
| 14.2 | 33 | 7 | + | > 800 | 2160 |
| 15.1 | 5.5 | 6 | weak + | 400-800 | 898 |
| 15.2 | 9 | 7 | weak + | 400-800 | 1425 |
| 15.3 | 29.5 | 7 | + | > 800 | 2985 |
| 16.1 | 7.5 | 6.2 | weak + | 400-800 | 1425 |
| 16.2 | 28 | 8.2 | + | > 800 | 4320 |

**Table A2.2 (continued)**: Foal details

| Foal and sample no. | Age (hours) at sampling | Refractometer | ZST | GC (mg/dl) | SRID (mg/dl) |
|---|---|---|---|---|---|
| 17.1 | 7.5 | 6.8 | + | > 800 | 1714 |
| 17.2 | 15.5 | 7.6 | + | > 800 | 1299 |
| 17.3 | 35.5 | 7.6 | + | > 800 | 1425 |
| 18.1 | 8.75 | 7.2 | + | > 800 | 898 |
| 18.2 | 12.75 | 8 | + | > 800 | 3429 |
| 18.3 | 28.75 | 7.8 | + | > 800 | 3429 |
| 19.1 | 5 | 7 | weak + | > 800 | 1031 |
| 19.2 | 25.75 | 8.4 | + | > 800 | 3591 |
| 19.3 | 49.75 | 8.2 | + | > 800 | 3761 |
| 20.1 | 23 | 7.8 | + | > 800 | 3429 |
| 20.2 | 47 | 7.2 | - | > 800 | 2369 |
| 21.1 | 7 | 8 | + | > 800 | 1563 |
| 21.2 | 31 | 7.2 | + | > 800 | 1714 |
| 21.3 | 87 | 7.2 | + | > 800 | 2062 |
| 22.1 | 6 | 6.4 | weak + | 400-800 | 940 |
| 22.2 | 14 | 9.4 | + | > 800 | 3761 |
| 22.3 | 30 | 9 | + | > 800 | 5970 |
| 23.1 | 9 | 8.4 | + | > 800 | 1969 |
| 23.2 | 17 | 8.4 | + | > 800 | 2262 |
| 23.3 | 33 | 8.8 | + | > 800 | 2262 |
| 24.1 | 8.5 | 6.4 | weak + | > 800 | 680 |
| 24.2 | 16.5 | 6 | weak + | 400-800 | 620 |
| 24.3 | 32.5 | 6.6 | + | > 800 | 712 |
| 25.1 | 7.5 | 6 | weak + | 400-800 | 516 |
| 25.2 | 15.5 | 6.2 | + | 400-800 | 898 |
| 25.3 | 31.5 | 6.4 | + | > 800 | 746 |
| 26.1 | 6.25 | 7.4 | + | > 800 | 1425 |
| 26.2 | 30.5 | 8.6 | + | > 800 | 2160 |
| 26.3 | 38.5 | 8.2 | + | > 800 | 2262 |
| 27.1 | 5.75 | 8.2 | + | > 800 | 3274 |
| 27.2 | 13.75 | 8 | + | > 800 | 2481 |
| 27.3 | 29.75 | 8 | + | > 800 | 2160 |
| 28.1 | 12.75 | 7.8 | + | > 800 | 2262 |
| 28.2 | 20.75 | 7.6 | + | > 800 | 2062 |
| 29.1 | 9.5 | 7.4 | + | > 800 | 1637 |
| 29.2 | 17.5 | 7.2 | + | > 800 | 1492 |
| 29.3 | 33.5 | 7.4 | + | > 800 | 1425 |
| 30.1 | 16.5 | 7 | - | 400-800 | 790 |
| 30.2 | 56.5 | 7 | weak + | > 800 | 866 |
| 30.3 | 81.5 | 7 | weak + | > 800 | 1254 |
| 31.1 | 10 | 7.6 | + | > 800 | 1714 |
| 31.2 | 18 | 7 | + | > 800 | 2062 |
| 31.3 | 34 | 7 | + | > 800 | 1637 |

**Table A2.2 (continued)**: Foal details

| Foal and sample no. | Age (hours) at sampling | Refractometer | ZST | GC (mg/dl) | SRID (mg/dl) |
|---|---|---|---|---|---|
| 32.1 | 7 | 8 | + | > 800 | 2721 |
| 32.2 | 15 | 8.2 | + | > 800 | 3591 |
| 32.3 | 31 | 8.2 | + | > 800 | 3591 |
| 33.1 | 7 | 8.2 | + | > 800 | 3591 |
| 33.2 | 10.5 | 8.4 | + | > 800 | 4320 |
| 33.3 | 26 | 8 | + | > 800 | 4738 |
| 34.1 | 10.5 | 8.2 | + | > 800 | 3591 |
| 34.2 | 18.5 | 8 | + | > 800 | 3761 |
| 34.3 | 34.5 | 8 | + | > 800 | 3761 |
| 35.1 | 10.5 | 6.8 | weak + | > 800 | 940 |
| 35.2 | 18.5 | 7 | weak + | > 800 | 898 |
| 35.3 | 34.5 | 6.8 | + | > 800 | 2160 |
| 36.1 | 20.5 | 8 | + | > 800 | 1637 |
| 36.2 | 28.5 | 7.8 | + | > 800 | 1563 |
| 36.3 | 44.5 | 7.6 | + | > 800 | 1637 |
| 37.1 | 7 | 7.2 | + | > 800 | 898 |
| 37.2 | 15 | 7 | + | > 800 | 1131 |
| 37.3 | 31 | 7.4 | + | > 800 | 1185 |
| 38.1 | 13.5 | 5.4 | weak + | < 400 | 90 |
| 38.2 | 36.5 | 5.4 | + | < 400 | 313 |
| 38.3 | 40.5 | 5.4 | + | < 400 | 286 |
| 39.1 | 15 | 7 | + | > 800 | 2062 |
| 39.2 | 19 | 7 | + | > 800 | 1969 |
| 39.3 | 35.5 | 7 | + | > 800 | 2160 |
| 40.1 | 13.5 | 8 | + | > 800 | 1969 |
| 40.2 | 17.5 | 7.6 | + | > 800 | 2160 |
| 40.3 | 34 | 7.8 | + | > 800 | 5443 |
| 41.1 | 12.5 | 7 | + | > 800 | 1361 |
| 41.2 | 20 | 7.4 | + | > 800 | 1637 |
| 41.3 | 40 | 6.8 | + | > 800 | 1795 |
| 42.1 | 4.5 | 6 | - | 400-800 | 1361 |
| 42.2 | 11.75 | 6.8 | weak + | > 800 | 1080 |
| 42.3 | 31.75 | 6 | + | 400-800 | 1299 |
| 43.1 | 14.5 | 7.2 | + | > 800 | 2369 |
| 43.2 | 18.5 | 7.4 | + | > 800 | 2262 |
| 43.3 | 34.5 | 7.6 | + | > 800 | 2369 |
| 44.1 | 13 | 7.8 | + | > 800 | 3591 |
| 44.2 | 17 | 8 | + | > 800 | 2721 |
| 44.3 | 33 | 7.6 | + | > 800 | 2985 |
| 45.1 | 10.5 | 7.8 | + | > 800 | 1361 |
| 45.2 | 14.5 | 8.4 | + | > 800 | 1637 |
| 45.3 | 30.5 | 8 | + | > 800 | 1795 |
| 46.1 | 6 | 6 | - | 400-800 | 217 |
| 46.2 | 13 | 6.2 | - | 400-800 | 497 |
| 46.3 | 29 | 6 | weak + | 400-800 | 627 |

**Table A2.2 (continued)**: Foal details

| Foal and sample no. | Age (hours) at sampling | Refractometer | ZST | GC (mg/dl) | SRID (mg/dl) |
|---|---|---|---|---|---|
| 47.1 | 11.5 | 6 | - | < 400 | 857 |
| 47.2 | 15.5 | 6 | weak + | 400-800 | 940 |
| 47.3 | 31.25 | 6 | + | 400-800 | 818 |
| 48.1 | 9.5 | 7.4 | + | > 800 | 2369 |
| 48.2 | 17.5 | 6.8 | + | > 800 | 2062 |
| 48.3 | 33.5 | 7.2 | + | > 800 | 2262 |
| 49.1 | 7.5 | 8.2 | + | > 800 | 1714 |
| 49.2 | 15.5 | 7.4 | + | > 800 | 1714 |
| 49.3 | 31.5 | 7.8 | + | > 800 | 2062 |
| 50.1 | 13 | 7.2 | + | > 800 | 2062 |
| 50.2 | 37 | 7 | + | > 800 | 1795 |
| 50.3 | 43 | 7 | + | > 800 | 1880 |

# APPENDIX 3

## ANALYSIS OF VARIANCE (ANOVA) TABLES FOR AGE, PARITY AND GESTATION LENGTH OF MARES.

**Table A3.1**: ANOVA: age

Variate: Foal 12 hour SRID

| Source of variation | d.f. | s.s. | m.s. | v.r. | F pr |
|---|---|---|---|---|---|
| Age | 12 | 11363472 | 946956 | 0.6 | 0.825 |
| Residual | 37 | 58023455 | 1568201 | | |
| Total | 49 | 69386928 | | | |

Not significant at the 1% level of significance

**Table A3.2**: ANOVA: parity

Variate: Foal 12 hour SRID

| Source of variation | d.f. | s.s. | m.s. | v.r. | F pr |
|---|---|---|---|---|---|
| Parity | 8 | 6192704 | 774088 | 0.5 | 0.847 |
| Residual | 41 | 63194224 | 1541323 | | |
| Total | 49 | 69386928 | | | |

Not significant at the 1% level of significance

**Table A3.3**: ANOVA: Gestation length

Variate: Foal 12 hour SRID

| Source of variation | d.f. | s.s. | m.s. | v.r. | F pr |
|---|---|---|---|---|---|
| Gestation length | 22 | 36417135 | 1655324 | 1.36 | 0.224 |
| Residual | 27 | 32969792 | 1221103 | | |
| Total | 49 | 69386928 | | | |

Not significant at the 1% level of significance

# APPENDIX 4

## AFRICAN HORSE SICKNESS VACCINE PACKAGE INSERT

FOR ANIMAL USE ONLY

BIO ONDERSTEPOORT PROD

P 2063

### HORSESICKNESS VACCINE

Reg.No.G 0116 (Act 36/1947)

Namibia:NSR 0586

Freeze-dried, polyvalent, live attenuated horsesickness virus strains for the prophylactic immunisation of horses, mules and donkeys against horsesickness.

**The vaccine is presented as two separate injections with different horsesickness virus types. First administer combination I and at least three weeks later combination II.**

**Store the vaccine in a refrigerator at a temperature of 4 °C to 8 °C.**
**Do not use the vaccine after the expiry date printed on the bottle.**

### RECOMMENDATIONS FOR USE

Foals born of unvaccinated dams can be inoculated at any age but foals of immune dams should not be vaccinated until they are at least six to seven months old. Animals should preferably be immunised during early summer. Immunisation of mares should be avoided during the first three months of pregnancy.

Annual immunisation is recommended.

It takes up to 2 – 3 vaccinations for horses to become immune to all the serotypes in the vaccine. It is therefore important to combine vaccination with the control of the *Culicoides* midges which transmit the disease. Horses can be protected from midge bites by stabling them from dusk to dawn, using insect repellents and keeping animals away from low-lying vlei areas or other surface water during the day.

**WARNINGS**

**Do not slaughter animals for human consumption within 7 days of vaccination.**

**Vaccinate healthy animals only.**

**Keep out of reach of children, uninformed persons and animals.**

**The vaccine will not necessarily stimulate a complete immunity in all animals and additional measures must necessarily be taken to ensure protection of horses against horsesickness during the time of the year when the risk of transmission of infection by biting insects is greatest.**

**Although this product has been extensively tested under a large variety of conditions, failure thereof may ensue as a result of a wide range of reasons. If this is suspected, seek veterinary advice and notify the registration holder.**

**Do not vaccinate horses more than once a year.**

**DIRECTIONS FOR USE**

**Use only as directed.**

The active ingredient of the vaccine is in the form of a powder or pellet in a small bottle. Connect one of the enclosed needles to one of the syringes with diluent and transfer the contents to bottle no.1. Mix thoroughly until the powder is dissolved and withdraw the contents into the syringe. The vaccine is now ready for use and must be injected without delay. Avoid exposure to high temperatures and direct sunlight during inoculation. Keep the remainder of the package at 4°C – 8°C for use at least 3 weeks later.

**DOSAGE** : 2 ml subcutaneously

**EFFECTS OF THE VACCINE**

Animals inoculated for the first time may react slightly between the seventh and fourteenth day following inoculation. During this period and for a further week these animals should not be worked excessively. Immunity starts to develop two to three weeks after complete inoculation and protection against some of the virus types are achieved after four weeks. Immunity cannot be guaranteed in all animals.

**PACKING**

Available in series of 2 bottles sufficient for 1 dose.

Registration holder: Onderstepoort Biological Products (Ltd.), Private Bag X07, ONDERSTEPOORT 0110

Tel. (012) 522 -1500 Co Reg no.2000/022686/06

# APPENDIX 5
# VIRUS NEUTRALISATION TEST PROTOCOL

## A5.1. Introduction

The virus neutralisation test is a quantitative assay for the determination of antibody titre in, for example, serum, based on the ability of the serum to neutralise a known amount of a standard virus antigen (Aleff Group, 2000). The African horse sickness virus neutralisation test is a sero-specific test that measures the amount of IgG antibody specific to each serotype in the serum.

Full procedural details of the method are given in Section A5.2 but, in summary, the Biobest (2005) laboratory in the U.K. describes the test as follows: "The test uses decreasing concentrations of the serum against a set amount of virus to check how much neutralising antibody is present in the serum. Serial dilutions of the heat inactivated serum are prepared in a 96 well plate and are incubated with a known amount of virus ($100TCID_{50}$). Virus susceptible cells are added to the virus/serum mix.

After incubation, each well of the plate is examined for evidence of the viral cytopathic effect (CPE), i.e. how many of the cells have been killed by the virus. Sera containing antibodies to the test virus are able to neutralise the virus and prevent infection of the cells when they are added to the plate. Where the serum sample contains high concentrations of antibodies, neutralisation of the antibodies will occur at even high serum dilutions. Conversely, where the sample contains little or no antibodies, it is unable to neutralise the virus even at the lowest dilution used in the test. The result of the test is the point at which the serum sample has been diluted so that it is unable to neutralise all the virus in the test. This dilution is reported as the antibody titre of the test sample."

Figure A5.1 is a diagrammatic representation of the test procedure. In this trial, the results were measured in antibody titres doubling in strength from 1:2 to 1:4 to 1:8, increasing to 1:256. The virology department at Onderstepoort Veterinary Institute considers an antibody titre of 1:40 to be positive (Gerdes, 2004, pers. comm.). A positive antibody titre in the work reported here was 1:32 due to the layout of the test plates.

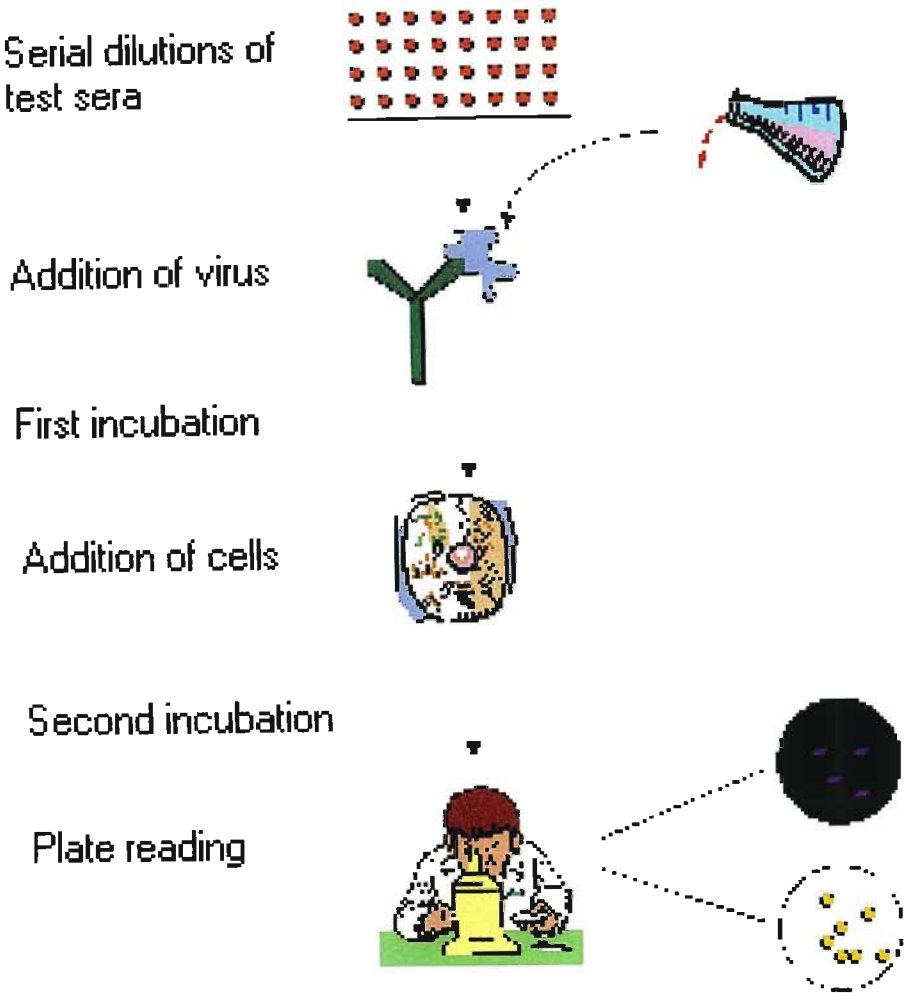


**Figure A5.1**Diagrammatic representation of the virus neutralisation test (Biobest, 2005)

## A5.2. Procedure

All work is conducted under Class II biohazard hoods and staff wear laboratory coats and latex gloves. Sterile conditions are employed for all procedures

**Step 1**: Numbered plates[16] are marked with sample numbers. Each sample is tested in duplicate for each serotype and all the samples for one mare and her foal are tested together (i.e. at the same time) in a series of plates. See the example of Plate 1 in Figure A5.2.

[16] (NUNC, Microwell 96 well plate, flat bottom, AEC Amersham, item no. 167008)

| Dilution | 1 | 1 | 1.1 | 1.1 | 1.2 | 1.2 | 1.3 | 1.3 | 1.4 | 1.4 | 1.5 | 1.5 | Sample numbers |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1:2 | | | | | | | | | | | | | Row A |
| 1:4 | | | | | | | | | | | | | Row B |
| 1:8 | | | | | | | | | | | | | Row C |
| 1:16 | | | | | | | | | | | | | Row D |
| 1:32 | | | | | | | | | | | | | Row E |
| 1:64 | | | | | | | | | | | | | Row F |
| 1:128 | | | | | | | | | | | | | Row G |
| 1:256 | | | | | | | | | | | | | Row H |

**Figure A5.2**: Example of plate layout and labelling (Plate 1)

Plates 2 and 3 are labelled as per Plate1, but with sample numbers from 1.6 to 1.11 and 1.12 to end, respectively. Any remaining columns on Plate 3 are used as cell control wells (at least two columns). Cell control wells (cells and medium only) are used to check cell growth. A set of three plates as described are labelled for each of the nine serotypes and numbered as below.

- Plates 1 - 3: AHS 1
- Plates 4 - 6: AHS 2
- Plates 7 - 9: AHS 3
- Plates 10 - 12: AHS 4
- Plates 13 - 15:AHS 5
- Plates 16 - 18: AHS 6
- Plates 19 - 21:AHS 7
- Plates 22 - 24: AHS 8
- Plates 25 - 27: AHS 9

**Step 2**: Twenty five (25) µl of the cell growth medium is aliquoted into Rows B to H of each plate. This medium consists of EMEM (Minimum Essential Medium Eagle) [17], with a penicillin/ streptomycin/fungizone mix (PSF)[18], non-essential amino acids (NEAA)[19],

[17] Bio-Whittaker, Cat. No. 12-136 F

[18] Bio-Whittaker, Cat. No. 17-745 E

[19] Bio-Whittaker, Cat. No. 13-114 E

and L-glutamine[20] mix added and supplies the necessary nutrients for the cell growth as well as being the diluent for the serum.

**Step 3**: Fifty (50) µl of undiluted serum (inactivated at 56°C for 30 min) is aliquoted into Row A of each plate.

**Step 4**: Two-fold dilutions of each plate are made by mixing serum and medium. Twenty five (25) µl of serum is taken from Row A. This is mixed in Row B by evacuating and refilling the micro-pipette ten times. Twenty five (25) µl is taken from Row B and mixed in Row C. This procedure is repeated to Row H, where the remaining 25µl is discarded into a biocidal solution.

**Step 5**: Twenty five (25) µl of $100TCID_{50}$ virus is added to all wells except the cell control wells (see Section A5.3 for description and calculation of $100TCID_{50}$)

**Step 6**: Plates 28 and 29 are constructed as back titration plates (virus and vero cells (see Step 9) only). These are used to check when the plates are ready to be read. Examples are shown in Figure A5.3. Dilutions are 10-fold from $100TCID_{50}$ (see Figure A5.3, Plate 28, AHS 1 for an example of the dilutions). $100TCID_{50}$ is the working dilution, see Section A5.3 for the calculation and explanation of the working dilution.

**Step 7**: All plates (1 to 29) are placed on a rocker (Stuart Scientific Platform Shaker STR 6) for 10 minutes to mix the virus and serum.

**Step 8**: All plates are then placed in an incubator (Forma Scientific Water Jacketed $CO_2$ Inc, Model: 3164 S/N 36558/2762) for half an hour to an hour to allow the antibody-virus binding to take place.

**Step 9**: Vero cell preparation:
The cells are grown in tissue culture flasks (roller, roux or 75 cm vero bottles[21]) containing EMEM with non-essential amino acids, L-glutamine and PSF.

a) Pour the medium out of the flask.

b) Rinse the flask with Hanks Balanced Salt Solution[22] (HBSS) and place on the rocker for 10 minutes.

c) Pour the Hanks solution out and replace with trypsin[23] to release the cells from the surface of the flask.

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[20] Bio-Whittaker, Cat. No. 17-605 E

[21] Adcock Ingram

[22] with phenol red; Bio-Whittaker, Cat. No. 10-508 F

[23] Bio-Whittaker

d) Place on a rocker until all the cells are loose.

e) Pour 500 µl fetal calf serum[24] (FCS) into the flask to stop the action of the trypsin.

f) Spray any left-over cells off the surface of the flask with a syringe and needle. Pour into a 20 ml glass bottle and centrifuge for 10 minutes at 4°C to form a cell pellet.

g) Pour off supernatant and replace with EMEM.

h) Disperse the cells with a syringe and needle.

i) Mix the cells with the required amounts of EMEM and FCS. (See calculations in Section A5.3).

j) Use in the test wells or pour into a new flask to grow again.

Plate 28

| AHS 1 | | | | AHS 2 | | | | AHS 3[a] | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| $100TCID_{50}$ | | $10^{-2.5}$ | | | | | | $4^+$ | $4^+$ | $4^+$ | $4^+$ |
| 10 x Dil | | $10^{-3.5}$ | | | | | | $4^+$ | $4^+$ | $4^+$ | $4^+$ |
| 10 x Dil | | $10^{-4.5}$ | | | | | | $3^+$ | $^+$ | $2^+$ | $^+$ |
| 10 x Dil | | $10^{-5.5}$ | | | | | | Nothing | | | |
| | | | | | | | | | | | |
| | | | | | | | | | | | |
| | | | | | | | | | | | |
| | | | | | | | | | | | |
| AHS 4 | | | | AHS 5 | | | | AHS 6 | | | |

Plate 29

| AHS 7 | | | | AHS 8 | | | | AHS 9 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | | |
| | | | | | | | | | | | |
| | | | | | | | | | | | |
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| | | | | | | | | | | | |
| | | | | | | | | | | | |
| | | | | | | | | | | | |
| Cell Control Wells | | | | | | | | | | | |

[a] Degree of cytopathic effect (CPE) : $^+$ = 0 - 25 % CPE or 0 - 25 % cells dead
$2^+$ = 25 - 50 % CPE
$3^+$ = 50 - 75 % CPE
$4^+$ = 75 - 100 % CPE.

**Figure A5.3**: Example of Plate 28 and 29, back titration plates, including dilution and result examples.

[24] Delta Bioproducts, Cat. No. 14-501AI

**Step 10**: Add 100 µl cells to every well in each of the plates 1 - 29.

**Step 11**: Incubate the plates in the $CO_2$ (5%) incubator at 37°C.

Day 1 and 2 - Check the cell control wells: the cell growth should be fully confluent (100% coverage) in the wells within 24 to 48 hours.

Day 3 onward - Check the back titrations on Plates 28 and 29: the plates are checked by looking at each well under a microscope to determine the degree of the CPE. The CPE is visible in the number of cells that the virus has killed. The serotype is ready when the back titration shows the pattern of CPE illustrated in Figure A5.3, Plate 28, AHS 3. When ready, fix all the plates for that serotype with 70% alcohol and stain plates with 1% basic fuchsin[25]. When the plates for the final serotype are ready, fix those plates and the back titration plates.

The cells adhere to the base of the plate so when fixing the plates, the liquid is emptied out, the plates are rinsed under gently running cold tap water and filled with 70% alcohol for at least 1 minute. The alcohol is then emptied and then the plates are ready for staining. Twenty five (25) µl of basic fuchsin is aliquoted into each well by means of a 12-channel pipette, allowed to stand for at least one minute and then rinsed with tap water and dried in the incubator. The living cells are stained pink by the basic fuchsin. If there are no cells in the wells (destroyed due to CPE), the stain is washed away by the water and the well appears clear.

**Step 12**: The plates are read either under a microscope or over a light box. The degree of CPE is determined (as for the back titration plates) but on the basis of colour rather than on the cells. As the living cells have been stained pink, if the well is pink then there was no virus action (antibodies present). If there is no pink colouration then the cells have all been killed by the virus. The CPE for each well is recorded into a table representing the plate. The final antibody titre is taken as the dilution where there is less than 25% CPE (i.e. the first row where the reading is a "+" or no CPE present. An antibody titre of 1:40 is regarded as positive and indicative of protection against clinical infection (Gerdes, 2004, pers. comm.). Figure A5.4 shows an example of the final plate and Figure A5.5 shows an example of the results for a plate.

[25] Saarchem, made up as a 1% solution in 70% ethanol

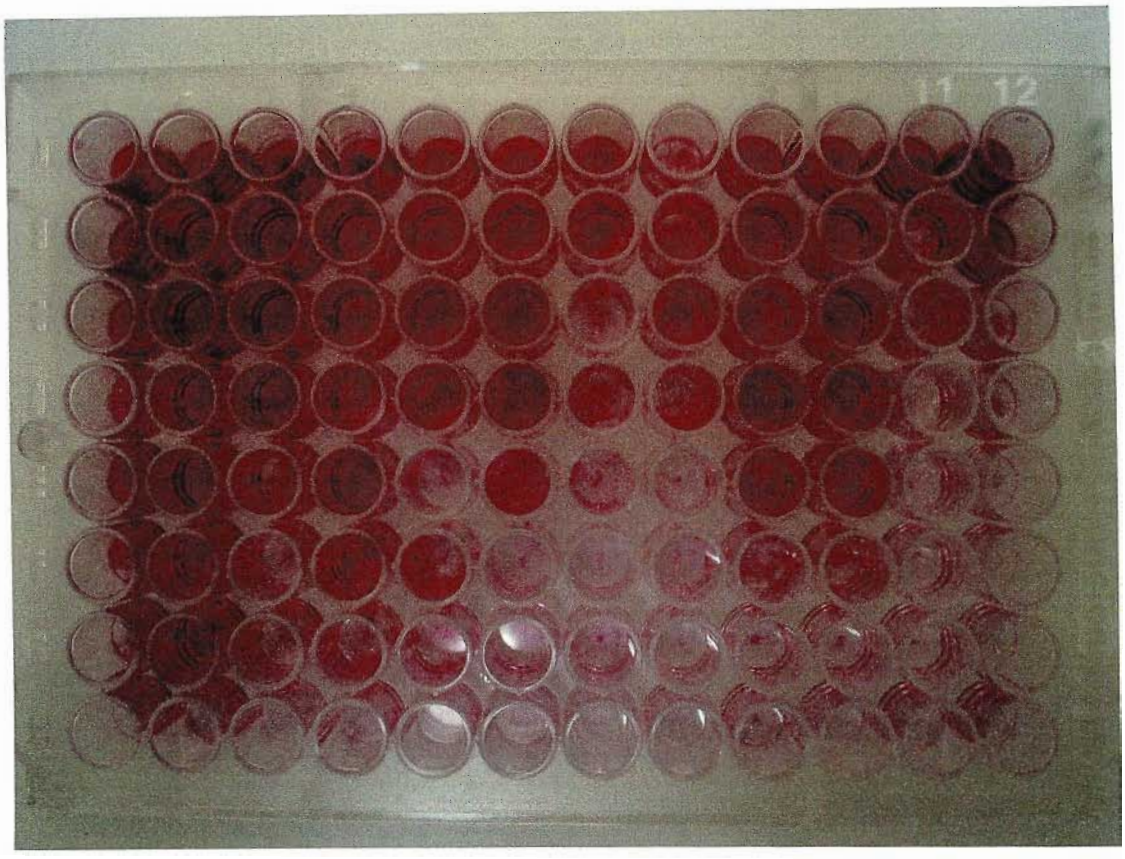

**Figure A5.4** Example of virus neutralisation plate (own photograph)

| Sample Number | 12 | | 12.1 | | 12.2 | | 12.3 | | 12.4 | | 12.5 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Dilution | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | |
| 1:2 | - | - | 2+ | + | - | - | - | - | - | - | - | - | A |
| 1:4 | - | - | 4+ | 4+ | - | - | - | - | - | - | + | + | B |
| 1:8 | - | - | 4+ | 4+ | - | - | - | - | - | 3+ | + | + | C |
| 1:16 | - | - | 4+ | 4+ | - | - | + | + | + | 2+ | + | 2+ | D |
| 1:32 | 3+ | - | 4+ | 4+ | - | - | 2+ | - | 4+ | + | 3+ | 4+ | E |
| 1:64 | + | 4+ | 4+ | 4+ | + | . | + | + | 4+ | 3+ | 3+ | 3+ | F |
| 1:128 | - | 4+ | 4+ | 4+ | - | - | 4+ | + | 4+ | 4+ | 4+ | 4+ | G |
| 1:256 | - | - | 4+ | 4+ | . | . | 4+ | 4+ | 2+ | + | 4+ | 3+ | H |
| Result | 1:16 | | <1:2 | | 1:32 | | 1:16 | | 1:4 | | 1:8 | | |

**Figure A5.5**: Example of results for a plate.

## A5.3. Calculations

$TCID_{50}$ = tissue culture infective dose. Not all virus particles are infective and titration methods only recognise infective particles. If a culture is inoculated with at least one infectious unit (IU), it will be killed. There is, of course, a random distribution of infectious particles in aliquots of a suspension. When this has been diluted to the point where, on average, one half of the aliquots of 1 ml contain at least one infectious unit, then the suspension is said to contain one $TCID_{50}$, (Reed & Muench, 1938).

The $TCID_{50}$ titre is determined by using virus titration. Four replications of titrations from -1 to -7 are used. The plates are read after 7 days. An example of the final calculation is 3 - ½ + 9/4 = 4.75 where:

3 is the highest dilution with 4 wells determined to be $4^+$ wells

1 is the dilution difference and

9 is the number of wells with CPE (five plus the row of four used for determining the highest dilution)

Working dilution or $100TCID_{50}$ = The logarithm of the $TCID_{50}$ titre plus two equals the logarithm dilution of the virus suspension containing $100TCID_{50}$.
For example, if $TCID_{50} = 10^{-4.75}$ per 0.1 ml of suspension, then the log dilution of the virus suspension containing $100TCID_{50}$ in a volume of 0.1 ml = $10^{-2.75}$.
The working dilution is $10^{-2.75}$.

Cell preparation: Each plate needs 11 ml (100 µl / well) of the cells, EMEM and FCS mix.

Example: One roller bottle supplies enough cells for 60 plates, one roux bottle supplies enough cells for 10 plates and one 75 cm vero bottle enough cells for 3 plates.

60 plates * 11 ml = 660 ml

Use 700 ml as the final volume to allow for some spillage and other losses. The final calculation of quantities if 8% FCS is needed is therefore as follows: 56 ml FCS + 644 ml MEM.

Virus needed: 16 ml of virus solution is needed per serotype. The calculation to determine the amount of virus and amount of EMEM in the final solution is as follows:

Working dilution = $10^{-2.6}$

A $10^{-2}$ solution is made first by adding 40 µl of virus to 3960 µl EMEM (4 ml of $10^{-2}$ solution results).

To dilute this to a $10^{-2.6}$ solution, 1 part of the $10^{-2}$ solution is added to 3 parts EMEM (from Table A5.1). The final mix is 4 ml of the $10^{-2}$ solution plus 12 ml of EMEM to make 16 ml of $10^{-2.6}$ solution.

**Table A5.1**: Ratios for the preparation of virus dilutions

| Dilution required | Ratio (solution:EMEM) | Parts (solution + EMEM) |
|---|---|---|
| 0.1 | 1:1.2 | 1 + 0.2 |
| 0.2 | 1:1.6 | 1 + 0.6 |
| 0.3 | 1:2.0 | 1 + 1 |
| 0.4 | 1:2.5 | 1 + 1.5 |
| 0.5 | 1:3.2 | 1 + 2.2 |
| 0.6 | 1:4 | 1 + 3 |
| 0.7 | 1:5 | 1 + 4 |
| 0.8 | 1:6.3 | 1 + 5.3 |
| 0.9 | 1:8 | 1 + 7 |

# APPENDIX 6

## VIRUS NEUTRALISATION RESULTS FOR NINE AHS SEROTYPES

The following tables show the virus neutralisation results for each of the nine African horse sickness serotypes, with the first born (older) foals on the right hand side of each table. Each column represents one foal, with the sequential samples running down the column. The blue-coloured blocks indicate positive antibody titres and the yellow blocks represent negative antibody titres (an antibody titre of less than 1:32). The white squares show where no samples were taken. The rows labelled Vaccination 1 and Vaccination 2 illustrate when the foals were vaccinated with the vaccine combination containing the relevant serotype. The second vaccine combination would have been three weeks later for Serotypes 1, 3 and 4, and three weeks earlier for Serotypes 2, 6, 7 and 8. Table A6.1 gives the birth dates of the foals.

The mares were all vaccinated on the 13 July 2000 and 3 August 2000 with AHS vaccine combinations 1 and 2 respectively. The foals were all vaccinated on 1 February 2001 and 26 February 2001 with AHS vaccine combinations 1 and 2 respectively for the first time. The foals were vaccinated again on 6 August 2001 and 28 August with AHS vaccine combinations 1 and 2 respectively for the second time.

**Table A6.1** Foal birth dates

| Foal Number | Date Born |
|---|---|
| 1 | 28-Nov-00 |
| 2 | 24-Nov-00 |
| 3 | 19-Nov-00 |
| 4 | 10-Nov-00 |
| 5 | 07-Nov-00 |
| 6 | 02-Nov-00 |
| 7 | 28-Oct-00 |
| 8 | 25-Oct-00 |
| 9 | 19-Oct-00 |
| 10 | 18-Oct-00 |
| 11 | 16-Oct-00 |
| 12 | 16-Oct-00 |
| 13 | 02-Oct-00 |
| 14 | 26-Sep-00 |
| 15 | 24-Sep-00 |
| 16 | 23-Sep-00 |
| 17 | 20-Sep-00 |
| 18 | 15-Sep-00 |
| 19 | 11-Sep-00 |
| 20 | 25-Aug-00 |
| 21 | 24-Aug-00 |
| 22 | 22-Aug-00 |
| 23 | 17-Aug-00 |
| 24 | 15-Aug-00 |

## **Table A6.2** Virus neutralisation results for AHS Serotype 1

| ZST result | + | + | + | Weak + | Weak + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | - | - | + | + | Weak + |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| GC result | + | + | + | Weak + | + | + | + | + | + | + | Weak + | + | + | + | + | + | + | + | Weak + | + | + | | | | | |
| **Foal number** | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** | **13** | **14** | **15** | **16** | **17** | **18** | **19** | **20** | **21** | **22** | **23** | **24** |
| Days: mare vac to foaling | 138 | 134 | 129 | 120 | 117 | 112 | 107 | 104 | 98 | 97 | 95 | 95 | 81 | 75 | 73 | 72 | 69 | 64 | 60 | 43 | 42 | 40 | 35 | 33 |
| Foal age at vac 1 (days) | 65 | 69 | 74 | 83 | 86 | 91 | 96 | 99 | 105 | 106 | 108 | 108 | 122 | 128 | 130 | 131 | 134 | 139 | 143 | 160 | 161 | 163 | 168 | 170 |
| Mare | 1:16 | 1:256 | 1:32 | 1:64 | 1:256 | 1:128 | 1:256 | 1:128 | 1:32 | 1:8 | 1:128 | 1:256 | 1:256 | 1:16 | 1:64 | 1:256 | 1:256 | 1:128 | 1:256 | 1:256 | 1:64 | 1:256 | 1:256 | 1:128 |
| Day 0 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 |
| Day 1 | 1:256 | 1:256 | 1:32 | 1:128 | 1:2 | 1:256 | 1:256 | 1:256 | 1:128 | 1:2 | 1:64 | 1:256 | 1:256 | 1:32 | 1:64 | | 1:256 | | | | | | | |
| vac 1 - 3.5 months | | | | | | | | | | | | | | | | | | | 1:128 | 1:32 | | | 1:8 | |
| vac 1 - 3 months | | | | | | | | | | | | | 1:256 | 1:8 | 1:32 | 1:64 | 1:8 | 1:64 | | | | | | 1:2 |
| vac 1 - 2.5 months | | | | | | | | | 1:16 | 1:2 | 1:32 | 1:256 | | | 1:8 | | 1:8 | 1:16 | 1:64 | | 1:2 | | 1:2 | |
| vac 1 - 2 months | | | | | 1:16 | 1:256 | | 1:32 | | | | | 1:128 | | | 1:8 | | | | 1:16 | | 1:4 | | |
| vac 1 - 1.5 months | | 1:256 | 1:128 | 1:128 | 1:2 | | 1:64 | 1:8 | 1:4 | 1:8 | 1:4 | 1:256 | | 1:4 | 1:8 | 1:4 | 1:8 | 1:4 | 1:32 | | 1:2 | 1:4 | 1:2 | 1:2 |
| vac 1 - 1 month | 1:64 | 1:256 | | | | | 1:32 | | | | | | | 1:2 | | | | | | 1:2 | | | | |
| vac 1 - 0.5 months | 1:32 | | 1:16 | 1:32 | | 1:64 | 1:32 | 1:8 | 1:8 | 1:8 | 1:2 | 1:256 | 1:64 | 1:2 | 1:4 | 1:8 | 1:256 | 1:4 | 1:4 | 1:2 | 1:2 | 1:4 | 1:2 | 1:2 |
| Vaccination 1 | **1:32** | **1:256** | **1:4** | **1:16** | **1:256** | **1:8** | **1:16** | **1:8** | **1:4** | **1:2** | **1:2** | **1:256** | **1:16** | **1:2** | **1:8** | **1:8** | **1:4** | **1:4** | **1:8** | **1:2** | **1:2** | **1:2** | **1:2** | **1:2** |
| vac 1 + 0.5 months | | 1:128 | 1:4 | | 1:2 | | | 1:16 | | | | | | | 1:8 | 1:2 | 1:2 | | | | 1:2 | 1:2 | | |
| vac 1 + 1 month | 1:4 | | | 1:16 | | 1:32 | 1:16 | | 1:2 | 1:4 | 1:8 | 1:256 | 1:32 | 1:256 | | | | 1:32 | 1:16 | 1:16 | | | 1:2 | 1:2 |
| vac 1 + 1.5 months | | | | | | | | | | | | | | | | | | | | | | | | |
| vac 1 + 2 months | 1:2 | 1:16 | 1:2 | | 1:2 | 1:2 | 1:2 | 1:32 | 1:2 | 1:256 | 1:32 | 1:256 | 1:8 | 1:256 | 1:256 | 1:256 | 1:64 | 1:256 | 1:128 | | 1:256 | 1:64 | 1:256 | 1:2 |
| vac 1 + 2.5 months | | 1:8 | | 1:16 | | | | 1:256 | | | | | | | 1:256 | | | | | 1:256 | | | | |
| vac 1 + 3 months | 1:2 | | 1:2 | 1:8 | 1:2 | 1:4 | 1:2 | | 1:256 | 1:256 | 1:64 | 1:256 | 1:8 | 56 | | 1:128 | 1:256 | 1:256 | 1:256 | 1:256 | 1:256 | 1:256 | 1:64 | 1:2 |
| vac 1 + 3.5 months | | | | | | | | | | | | | | | | | | | | | | | | |
| vac 1 + 4 months | | 1:16 | 1:2 | | 1:2 | | | 1:64 | 1:8 | | | | | | 1:256 | 1:256 | 1:256 | | | | 1:256 | 1:256 | | |
| vac 1 + 4.5 months | 1:2 | | | 1:2 | | 1:2 | 1:4 | | | 1:256 | 1:256 | 1:256 | 1:8 | 1:256 | | | | 1:256 | 1:256 | 1:256 | | | 1:256 | 1:2 |
| vac 1 + 5 months | | 1:8 | 1:2 | | 1:8 | | | 1:128 | 1:16 | | | | | | 1:256 | 1:64 | 1:256 | | | | 1:256 | 1:256 | | |
| vac 1 + 5.5 months | 1:2 | | | 1:2 | | 1:4 | 1:2 | | | 1:256 | 1:256 | 1:256 | 1:2 | 1:256 | | | | 1:256 | 1:256 | 1:256 | | | 1:64 | 1:2 |
| vac 1 + 6 months | | 1:2 | 1:2 | | 1:2 | 1:2 | | 1:256 | 1:32 | 1:256 | 1:256 | 1:256 | 1:2 | | 1:256 | 1:32 | 1:256 | 1:256 | | | 1:256 | 1:256 | 1:32 | 1:2 |
| vac 1 + 6.5 months | 1:2 | | | 1:2 | | | 1:2 | | | | | | | 1:128 | | | | | 1:256 | 1:256 | | | | |
| | | | | | | | | | | | | | | | | | | | | | | | | |
| Vaccination 2 | **1:2** | **1:2** | **1:2** | **1:2** | **1:2** | **1:2** | **1:2** | **1:256** | **1:32** | **1:256** | **1:256** | **1:256** | **1:2** | **1:128** | **1:256** | **1:32** | **1:256** | **1:256** | **1:256** | **1:256** | **1:256** | **1:256** | **1:32** | **1:2** |
| vac 2 + 0.5 month | 1:2 | 1:4 | | 1:4 | 1:4 | | 1:8 | 1:64 | | | | | | 1:256 | 1:256 | 1:256 | 1:256 | | 1:256 | 1:128 | 1:256 | 1:256 | | |
| vac 2 + 1 month | | | 1:2 | | | 1:16 | | | 1:256 | 1:256 | 1:256 | 1:256 | 1:256 | | | | | 1:256 | | | | | 1:64 | 1:2 |
| vac 2 + 1.5 months | 1:2 | 1:2 | 1:2 | 1:256 | 1:8 | | 1:4 | 1:64 | | | | | | 1:256 | 1:256 | 1:128 | 1:256 | | 1:256 | 1:128 | 1:128 | 1:256 | | |
| vac 2 + 2 months | | | | | | 1:8 | | | 1:64 | 1:256 | 1:128 | 1:256 | 1:256 | | | | | 1:256 | | | | | 1:128 | 1:2 |
| vac 2 + 2.5 months | 1:256 | 1:2 | 1:2 | 1:256 | 1:2 | 1:4 | 1:4 | 1:128 | 1:16 | 1:256 | 1:32 | 1:256 | 1:256 | 1:256 | 1:256 | 1:256 | 1:256 | 1:256 | 1:256 | 1:128 | 1:128 | 1:256 | 1:128 | |
| vac 2 + 3 months | | | | | | | | | | | | | | | | | | | | | | | | 1:2 |
| vac 2 + 3.5 months | 1:256 | 1:2 | 1:2 | | 1:2 | | 1:4 | | | | | | | 1:128 | 1:256 | | 1:256 | | 1:256 | | 1:64 | 1:256 | | |
| vac 2 + 4 months | | | | 1:256 | | 1:2 | | 1:128 | 1:32 | 1:256 | 1:32 | 1:256 | 1:64 | | | 1:128 | | 1:256 | | 1:32 | | | 1:32 | 1:2 |

**Table A6.3** Virus neutralisation results for AHS Serotype 2

| ZST result | + | + | + | Weak + | Weak + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | - | - | + | + | Weak + |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| GC result | + | + | + | Weak + | + | + | + | + | + | + | Weak + | + | + | + | + | + | + | Weak + | + | + | | | | |
| Foal number | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 |
| Days: mare vac to foaling | 138 | 134 | 129 | 120 | 117 | 112 | 107 | 104 | 98 | 97 | 95 | 95 | 81 | 75 | 73 | 72 | 69 | 64 | 60 | 43 | 42 | 40 | 35 | 33 |
| Foal age at vac 1 (days) | 65 | 69 | 74 | 83 | 86 | 91 | 96 | 99 | 105 | 106 | 108 | 108 | 122 | 128 | 130 | 131 | 134 | 139 | 143 | 160 | 161 | 163 | 168 | 170 |
| Mare | 1:2 | 1:256 | 1:16 | 1:128 | 1:16 | 1:32 | 1:8 | 1:16 | 1:16 | 1:2 | 1:8 | 1:128 | 1:64 | 1:2 | 1:256 | 1:32 | 1:32 | 1:256 | 1:256 | 1:2 | 1:8 | 1:16 | 1:16 | 1:8 |
| Day 0 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 |
| Day 1 | 1:4 | 1:256 | 1:8 | 1:128 | 1:2 | 1:32 | 1:16 | 1:64 | 1:16 | 1:16 | 1:8 | 1:128 | 1:256 | 1:32 | 1:64 | | 1:16 | | | | | | | |
| vac 1 - 5 months | | | | | | | | | | | | | | | | | | | | | | | | 1:2 |
| vac 1 - 4.5 months | | | | | | | | | | | | | | | | | | | 1:32 | 1:4 | 1:2 | 1:2 | 1:2 | |
| vac 1 - 4 months | | | | | | | | | | | | | 1:64 | 1:4 | | | | 1:2 | | | | | | 1:2 |
| vac 1 - 3.5 months | | | | | | | | | 1:16 | 1:16 | 1:2 | 1:32 | | | 1:8 | 1:4 | 1:2 | 1:2 | 1:4 | | | | 1:2 | |
| vac 1 - 3 months | | | | | | 1:64 | | | | | | | 1:16 | | 1:8 | | 1:2 | | | 1:2 | 1:2 | | | |
| vac 1 - 2.5 months | | | | 1:32 | 1:2 | | 1:2 | 1:16 | 1:2 | 1:2 | 1:2 | 1:8 | | 1:2 | | 1:2 | | 1:2 | 1:2 | | | 1:2 | 1:2 | 1:2 |
| vac 1 - 2 months | 1:2 | 1:128 | 1:4 | | 1:2 | | 1:2 | 1:8 | | | | | | 1:2 | 1:4 | 1:2 | 1:2 | | | 1:2 | 1:2 | 1:2 | | |
| vac 1 - 1.5 months | 1:2 | 1:256 | | 1:4 | | 1:32 | 1:2 | | 1:2 | 1:2 | 1:2 | 1:4 | 1:4 | 1:2 | | | | 1:2 | 1:2 | 1:2 | | | 1:2 | 1:2 |
| vac 1 - 1 month | 1:2 | | 1:2 | 1:2 | | 1:4 | 1:2 | 1:8 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:64 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 |
| vac 1 - 0.5 months | | 1:128 | 1:2 | | 1:16 | | | 1:4 | | | | | | | 1:2 | 1:2 | 1:2 | | | | 1:2 | 1:2 | | |
| **Vaccination 1** | **1:2** | **1:16** | **1:4** | **1:4** | **1:2** | **1:8** | **1:2** | **1:4** | **1:2** | **1:2** | **1:2** | **1:2** | **1:2** | **1:4** | **1:4** | **1:2** | **1:2** | **1:2** | **1:2** | **1:2** | **1:2** | **1:2** | **1:2** | **1:2** |
| vac 1 + 0.5 months | | | | | | | | | | | | | | | | | | | | | | | | |
| vac 1 + 1 month | 1:2 | | | | | 1:2 | 1:2 | | 1:2 | 1:2 | 1:2 | 1:64 | 1:2 | 1:16 | | | | 1:4 | 1:8 | | | | 1:4 | 1:2 |
| vac 1 + 1.5 months | | 1:16 | 1:2 | 1:2 | 1:2 | | | 1:4 | | | | | | | 1:16 | 1:2 | 1:2 | | | 1:4 | 1:8 | 1:2 | | |
| vac 1 + 2 months | 1:2 | 1:2 | | 1:2 | | 1:2 | 1:2 | 1:128 | 1:4 | 1:2 | 1:4 | 1:32 | 1:2 | 1:16 | 1:8 | | | 1:256 | 1:256 | 1:4 | | | 1:2 | 1:2 |
| vac 1 + 2.5 months | | | 1:2 | | 1:2 | | | | | | | | | | | 1:2 | 1:16 | | | | 1:32 | 1:8 | | |
| vac 1 + 3 months | | | | | | | | | 1:2 | | | | | | | | | | | | | | | |
| vac 1 + 3.5 months | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:16 | | 1:256 | 1:16 | 1:256 | 1:2 | 1:8 | 1:256 | 1:16 | 1:8 | 1:64 | 1:64 | 1:32 | 1:16 | 1:2 | 1:8 | 1:2 |
| vac 1 + 4 months | | | | | | | | | 1:2 | | | | | | | | | | | | | | | |
| vac 1 + 4.5 months | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:64 | | 1:256 | 1:64 | 1:256 | 1:2 | 1:4 | 1:256 | 1:2 | 1:256 | 1:16 | 1:16 | 1:32 | 1:8 | 1:128 | 1:2 | 1:2 |
| vac 1 + 5 months | | | | | | 1:2 | | | 1:2 | 1:128 | 1:128 | 1:256 | 1:2 | | | | | 1:16 | | | | | 1:2 | 1:2 |
| vac 1 + 5.5 months | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | | 1:2 | 1:32 | | | | | | 1:4 | 1:64 | 1:2 | 1:256 | | 1:8 | 1:32 | 1:4 | 1:64 | | |
| vac 1 + 6 months | 1:2 | 1:2 | | 1:2 | 1:2 | 1:2 | 1:2 | 1:32 | 1:4 | 1:64 | 1:16 | 1:256 | 1:64 | 1:4 | 1:64 | 1:2 | 1:32 | 1:32 | 1:8 | 1:2 | 1:4 | 1:64 | 1:2 | 1:2 |
| vac 1 + 6.5 months | | | 1:2 | | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | | | | | |
| **Vaccination 2** | **1:2** | **1:2** | **1:2** | **1:2** | **1:2** | **1:2** | **1:2** | **1:32** | **1:4** | **1:64** | **1:16** | **1:256** | **1:64** | **1:4** | **1:64** | **1:2** | **1:32** | **1:32** | **1:8** | **1:2** | **1:4** | **1:64** | **1:2** | **1:2** |
| vac 2 + 0.5 months | | | 1:2 | | | | | | | | | | | | | | | | | | | | | |
| vac 2 + 1 month | 1:2 | 1:2 | | 1:32 | 1:2 | 1:4 | 1:2 | 1:16 | 1:16 | 1:64 | 1:8 | 1:32 | 1:128 | 1:32 | 1:32 | 1:8 | 1:64 | 1:32 | 1:8 | 1:4 | 1:2 | 1:32 | 1:2 | 1:2 |
| vac 2 + 1.5 months | | | 1:2 | | | 1:2 | | | 1:2 | 1:16 | 1:4 | 1:32 | 1:128 | | | | | 1:8 | | | | | 1:4 | |
| vac 2 + 2 months | 1:8 | 1:2 | | 1:64 | 1:2 | | 1:2 | 1:32 | | | | | | 1:8 | 1:64 | 1:2 | 1:64 | | 1:8 | 1:2 | 1:2 | 1:32 | | 1:2 |
| vac 2 + 2.5 months | | | 1:2 | | | | | | | | | | | | | | | | | | | | | |
| vac 2 + 3 months | 1:16 | 1:2 | | | 1:2 | 1:2 | 1:2 | | 1:8 | 1:16 | 1:2 | 1:8 | 1:32 | 1:8 | 1:32 | | 1:128 | 1:8 | 1:32 | | 1:2 | 1:16 | 1:2 | 1:2 |
| vac 2 + 3.5 months | | | | 1:32 | | | | 1:32 | | | | | | | | 1:8 | | | | 1:4 | | | | |

## Table A6.4 Virus neutralisation results for AHS Serotype 3

| ZST result | + | + | + | Weak + | Weak + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | - | - | + | + | Weak + |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| GC result | + | + | + | Weak + | + | + | + | + | + | + | Weak + | + | + | + | + | + | + | + | Weak + | + | + | | | | |
| **Foal number** | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** | **13** | **14** | **15** | **16** | **17** | **18** | **19** | **20** | **21** | **22** | **23** | **24** |
| Days: mare vac to foaling | 138 | 134 | 129 | 120 | 117 | 112 | 107 | 104 | 98 | 97 | 95 | 95 | 81 | 75 | 73 | 72 | 69 | 64 | 60 | 43 | 42 | 40 | 35 | 33 |
| Foal age at vac 1 (days) | 65 | 69 | 74 | 83 | 86 | 91 | 96 | 99 | 105 | 106 | 108 | 108 | 122 | 128 | 130 | 131 | 134 | 139 | 143 | 160 | 161 | 163 | 168 | 170 |
| Mare | 1:16 | 1:32 | 1:128 | 1:64 | 1:64 | 1:128 | 1:32 | 1:32 | 1:64 | 1:4 | 1:16 | 1:16 | 1:128 | 1:2 | 1:128 | 1:16 | 1:128 | 1:16 | 1:32 | 1:32 | 1:256 | 1:256 | 1:32 | 1:16 |
| Day 0 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 |
| Day 1 | 1:16 | 1:32 | 1:16 | 1:8 | 1:2 | 1:128 | 1:32 | 1:128 | 1:64 | 1:32 | 1:16 | 1:64 | 1:128 | 1:4 | 1:256 | | 1:64 | | | | | | | |
| vac 1 - 3.5 months | | | | | | | | | | | | | | | | | | | 1:8 | 1:2 | | | 1:2 | |
| vac 1 - 3 months | | | | | | | | | | | | | 1:128 | 1:4 | 1:64 | 1:4 | 1:4 | 1:2 | | | | | | 1:2 |
| vac 1 - 2.5 months | | | | | | | | | 1:32 | 1:16 | 1:2 | 1:8 | | | 1:8 | | 1:2 | 1:2 | 1:4 | | 1:2 | | 1:2 | |
| vac 1 - 2 months | | | | | 1:2 | 1:256 | | 1:8 | | | | | 1:16 | | | 1:2 | | | | 1:2 | | 1:2 | | |
| vac 1 - 1.5 months | | 1:16 | 1:256 | 1:8 | 1:2 | | 1:2 | 1:16 | 1:8 | 1:4 | 1:2 | 1:2 | | 1:2 | 1:4 | 1:2 | 1:2 | 1:2 | 1:2 | | 1:2 | 1:2 | 1:2 | 1:2 |
| vac 1 - 1 month | 1:16 | 1:16 | | | | | 1:2 | | | | | | | 1:2 | | | | | | 1:2 | | | | |
| vac 1 - 0.5 months | 1:2 | | 1:32 | 1:2 | | 1:32 | 1:2 | 1:4 | 1:2 | 1:2 | 1:2 | 1:2 | 1:4 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 |
| **Vaccination 1** | **1:2** | **1:8** | **1:4** | **1:2** | **1:256** | **1:8** | **1:2** | **1:4** | **1:2** | **1:2** | **1:2** | **1:2** | **1:4** | **1:2** | **1:2** | **1:2** | **1:2** | **1:2** | **1:2** | **1:2** | **1:2** | **1:2** | **1:2** | **1:2** |
| vac 1 + 0.5 months | | 1:2 | 1:4 | | 1:2 | | | 1:4 | | | | | | | 1:2 | 1:2 | 1:2 | | | | 1:2 | 1:2 | | |
| vac 1 + 1 month | 1:2 | | | 1:2 | | 1:8 | 1:2 | | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | | | | 1:2 | 1:2 | 1:2 | | | 1:2 | 1:2 |
| vac 1 + 1.5 months | | | | | | | | | | | | | | | | | | | | | | | | |
| vac 1 + 2 months | 1:2 | 1:2 | 1:2 | | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | | 1:2 | 1:2 | 1:2 | 1:2 |
| vac 1 + 2.5 months | | 1:2 | | 1:2 | | | | 1:2 | | | | | | | 1:2 | | | | | 1:2 | | | | |
| vac 1 + 3 months | 1:2 | | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 |
| vac 1 + 3.5 months | | | | | | | | | | | | | | | | | | | | | | | | |
| vac 1 + 4 months | | 1:2 | 1:2 | | 1:2 | | | 1:2 | 1:2 | | | | | | 1:2 | 1:2 | 1:2 | | | | 1:2 | 1:2 | | |
| vac 1 + 4.5 months | 1:2 | | | 1:2 | | 1:2 | 1:2 | | | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | | | | 1:2 | 1:2 | 1:2 | | | 1:2 | 1:2 |
| vac 1 + 5 months | | 1:2 | 1:2 | | 1:2 | | | 1:2 | 1:2 | | | | | | 1:2 | 1:2 | 1:2 | | | | 1:2 | 1:2 | | |
| vac 1 + 5.5 months | 1:2 | | | 1:2 | | 1:2 | 1:2 | | | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | | | | 1:2 | 1:2 | 1:2 | | | 1:2 | 1:2 |
| vac 1 + 6 months | | 1:2 | 1:2 | | 1:2 | 1:2 | | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | | 1:2 | 1:2 | 1:2 | 1:2 | | | 1:2 | 1:2 | 1:2 | 1:2 |
| vac 1 + 6.5 months | 1:2 | | | 1:2 | | | 1:2 | | | | | | | 1:2 | | | | | 1:2 | 1:2 | | | | |
| **Vaccination 2** | **1:2** | **1:2** | **1:2** | **1:2** | **1:2** | **1:2** | **1:2** | **1:2** | **1:2** | **1:2** | **1:2** | **1:2** | **1:2** | **1:2** | **1:2** | **1:2** | **1:2** | **1:2** | **1:2** | **1:2** | **1:2** | **1:2** | **1:2** | **1:2** |
| vac 2 + 0.5 month | 1:2 | 1:2 | | 1:2 | 1:2 | | 1:2 | 1:2 | | | | | | 1:4 | 1:256 | 1:2 | 1:2 | | 1:2 | 1:2 | 1:2 | 1:16 | | |
| vac 2 + 1 month | | | 1:2 | | | 1:2 | | | 1:2 | 1:2 | 1:2 | 1:32 | 1:4 | | | | | 1:2 | | | | | 1:2 | 1:2 |
| vac 2 + 1.5 months | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | | 1:2 | 1:2 | | | | | | 1:4 | 1:128 | 1:4 | 1:2 | | 1:256 | 1:2 | 1:2 | 1:4 | | |
| vac 2 + 2 months | | | | | | 1:256 | | | 1:2 | 1:2 | 1:2 | 1:256 | 1:256 | | | | | 1:2 | | | | | 1:2 | 1:4 |
| vac 2 + 2.5 months | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:64 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:256 | 1:128 | 1:4 | 1:128 | 1:2 | 1:2 | 1:2 | 1:128 | 1:2 | 1:2 | 1:2 | 1:2 | |
| vac 2 + 3 months | | | | | | | | | | | | | | | | | | | | | | | | 1:2 |
| vac 2 + 3.5 months | 1:2 | 1:2 | 1:2 | | 1:2 | | 1:2 | | | | | | | 1:2 | 1:64 | | 1:2 | | 1:128 | | 1:2 | 1:2 | | |
| vac 2 + 4 months | | | | 1:2 | | 1:32 | | 1:2 | 1:2 | 1:2 | 1:2 | 1:64 | 1:16 | | | 1:2 | | 1:4 | | 1:2 | | | 1:2 | 1:2 |

## Table A6.5 Virus neutralisation results for AHS Serotype 4

| ZST result | + | + | + | Weak + | Weak + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | - | - | + | + | Weak + |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| GC result | + | + | + | Weak + | + | + | + | + | + | + | Weak + | + | + | + | + | + | + | Weak + | + | + | | | | |
| **Foal number** | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** | **13** | **14** | **15** | **16** | **17** | **18** | **19** | **20** | **21** | **22** | **23** | **24** |
| Days: mare vac to foaling | 138 | 134 | 129 | 120 | 117 | 112 | 107 | 104 | 98 | 97 | 95 | 95 | 81 | 75 | 73 | 72 | 69 | 64 | 60 | 43 | 42 | 40 | 35 | 33 |
| Foal age at vac 1 (days) | 65 | 69 | 74 | 83 | 86 | 91 | 96 | 99 | 105 | 106 | 108 | 108 | 122 | 128 | 130 | 131 | 134 | 139 | 143 | 160 | 161 | 163 | 168 | 170 |
| Mare | 1:32 | 1:256 | 1:32 | 1:256 | 1:256 | 1:32 | 1:32 | 1:32 | 1:8 | 1:128 | 1:32 | 1:64 | 1:256 | 1:16 | 1:32 | 1:64 | 1:64 | 1:32 | 1:256 | 1:128 | 1:128 | 1:256 | 1:128 | 1:16 |
| Day 0 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 |
| Day 1 | 1:32 | 1:128 | 1:16 | 1:64 | 1:2 | 1:64 | 1:32 | 1:32 | 1:32 | 1:256 | 1:16 | 1:128 | 1:128 | 1:32 | 1:64 | | 1:64 | | | | | | | |
| vac 1 - 3.5 months | | | | | | | | | | | | | | | | | | | 1:256 | 1:16 | | | 1:4 | |
| vac 1 - 3 months | | | | | | | | | | | | | 1:32 | 1:2 | 1:4 | 1:8 | 1:8 | 1:8 | | | | | | 1:2 |
| vac 1 - 2.5 months | | | | | | | | | 1:8 | 1:128 | 1:8 | 1:32 | | | 1:8 | | 1:4 | 1:8 | 1:64 | | 1:4 | | 1:2 | |
| vac 1 - 2 months | | | | | 1:2 | 1:32 | | 1:2 | | | | | 1:8 | | | 1:2 | | | | 1:16 | | 1:8 | | |
| vac 1 - 1.5 months | | 1:64 | 1:32 | 1:32 | 1:2 | | 1:32 | 1:2 | 1:4 | 1:32 | 1:4 | 1:4 | | 1:2 | 1:2 | 1:2 | 1:4 | 1:2 | 1:32 | | 1:2 | 1:8 | 1:2 | 1:2 |
| vac 1 - 1 month | 1:32 | 1:64 | | | | | 1:4 | | | | | | | 1:2 | | | | | | 1:4 | | | | |
| vac 1 - 0.5 months | 1:16 | | 1:16 | 1:16 | | 1:8 | 1:4 | 1:4 | 1:2 | 1:8 | 1:2 | 1:16 | 1:4 | 1:2 | 1:2 | 1:2 | 1:8 | 1:2 | 1:8 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 |
| **Vaccination 1** | **1:4** | **1:64** | **1:2** | **1:8** | **1:128** | **1:4** | **1:2** | **1:4** | **1:2** | **1:32** | **1:2** | **1:4** | **1:4** | **1:2** | **1:2** | **1:2** | **1:4** | **1:2** | **1:4** | **1:2** | **1:2** | **1:2** | **1:2** | **1:2** |
| vac 1 + 0.5 months | | 1:4 | 1:2 | | 1:2 | | | 1:2 | | | | | | | 1:2 | 1:16 | 1:2 | | | | 1:2 | 1:2 | | |
| vac 1 + 1 month | 1:2 | | | 1:4 | | 1:2 | 1:2 | | 1:2 | 1:16 | 1:2 | 1:2 | 1:2 | 1:2 | | | | 1:2 | 1:2 | 1:2 | | | 1:2 | 1:2 |
| vac 1 + 1.5 months | | | | | | | | | | | | | | | | | | | | | | | | |
| vac 1 + 2 months | 1:2 | 1:4 | 1:2 | | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:8 | 1:4 | 1:4 | 1:2 | 1:2 | 1:2 | | 1:2 | 1:2 | 1:2 | 1:2 |
| vac 1 + 2.5 months | | 1:2 | | 1:2 | | | | 1:2 | | | | | | | 1:2 | | | | | 1:2 | | | | |
| vac 1 + 3 months | 1:2 | | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:256 | | 1:4 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 |
| vac 1 + 3.5 months | | | | | | | | | | | | | | | | | | | | | | | | |
| vac 1 + 4 months | | 1:2 | 1:2 | | 1:2 | | | 1:2 | 1:2 | | | | | | 1:8 | 1:8 | 1:2 | | | | 1:2 | 1:2 | | |
| vac 1 + 4.5 months | 1:2 | | | 1:2 | | 1:2 | 1:2 | | | 1:2 | 1:2 | 1:2 | 1:2 | 1:32 | | | | 1:2 | 1:2 | 1:2 | | | 1:2 | 1:2 |
| vac 1 + 5 months | | 1:2 | 1:2 | | 1:2 | | | 1:2 | 1:2 | | | | | | 1:2 | 1:16 | 1:2 | | | | 1:2 | 1:2 | | |
| vac 1 + 5.5 months | 1:2 | | | 1:2 | | 1:2 | 1:2 | | | 1:2 | 1:2 | 1:2 | 1:2 | 1:16 | | | | 1:2 | 1:2 | 1:2 | | | 1:2 | 1:2 |
| vac 1 + 6 months | | 1:2 | 1:2 | | 1:2 | 1:2 | | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | | 1:2 | 1:4 | 1:2 | 1:2 | | | 1:2 | 1:2 | 1:2 | 1:2 |
| vac 1 + 6.5 months | 1:2 | | | 1:2 | | | 1:2 | | | | | | | 1:32 | | | | | 1:2 | 1:2 | | | | |
| | | | | | | | | | | | | | | | | | | | | | | | | |
| **Vaccination 2** | **1:2** | **1:2** | **1:2** | **1:2** | **1:2** | **1:2** | **1:2** | **1:2** | **1:2** | **1:2** | **1:2** | **1:2** | **1:2** | **1:32** | **1:2** | **1:4** | **1:2** | **1:2** | **1:2** | **1:2** | **1:2** | **1:2** | **1:2** | **1:2** |
| vac 2 + 0.5 month | 1:2 | 1:2 | | 1:16 | 1:2 | | 1:32 | 1:2 | | | | | | 1:64 | 1:8 | 1:16 | 1:2 | | 1:2 | 1:16 | 1:32 | 1:8 | | |
| vac 2 + 1 month | | | 1:2 | | | 1:2 | | | 1:2 | 1:2 | 1:2 | 1:8 | 1:32 | | | | | 1:8 | | | | | 1:16 | 1:8 |
| vac 2 + 1.5 months | 1:2 | 1:2 | 1:2 | 1:32 | 1:2 | | 1:8 | 1:2 | | | | | | 1:32 | 1:4 | 1:16 | 1:8 | | 1:8 | 1:4 | 1:4 | 1:2 | | |
| vac 2 + 2 months | | | | | | 1:16 | | | 1:2 | 1:2 | 1:2 | 1:8 | 1:16 | | | | | 1:4 | | | | | 1:2 | 1:2 |
| vac 2 + 2.5 months | 1:2 | 1:2 | 1:2 | 1:64 | 1:2 | 1:4 | 1:4 | 1:2 | 1:2 | 1:2 | 1:2 | 1:8 | 1:8 | 1:32 | 1:2 | 1:4 | 1:16 | 1:4 | 1:2 | 1:4 | 1:4 | 1:4 | 1:8 | |
| vac 2 + 3 months | | | | | | | | | | | | | | | | | | | | | | | | 1:2 |
| vac 2 + 3.5 months | 1:2 | 1:2 | 1:2 | | 1:2 | | 1:4 | | | | | | | 1:8 | 1:2 | | 1:8 | | 1:4 | | 1:4 | 1:4 | | |
| vac 2 + 4 months | | | | 1:32 | | 1:2 | | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | | | 1:8 | | 1:4 | | 1:4 | | | 1:2 | 1:2 |

**Table A6.6** Virus neutralisation results for AHS Serotype 5 (note: Serotype 5 not included in AHS vaccination)

| ZST result | + | + | + | Weak + | Weak + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | - | - | + | + | Weak + |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| GC result | + | + | + | Weak + | + | + | + | + | + | + | Weak + | + | + | + | + | + | + | + | Weak + | + | + | | | | |
| **Foal number** | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** | **13** | **14** | **15** | **16** | **17** | **18** | **19** | **20** | **21** | **22** | **23** | **24** |
| Days: mare vac to foaling | 138 | 134 | 129 | 120 | 117 | 112 | 107 | 104 | 98 | 97 | 95 | 95 | 81 | 75 | 73 | 72 | 69 | 64 | 60 | 43 | 42 | 40 | 35 | 33 |
| Foal age at vac 1 (days) | 65 | 69 | 74 | 83 | 86 | 91 | 96 | 99 | 105 | 106 | 108 | 108 | 122 | 128 | 130 | 131 | 134 | 139 | 143 | 160 | 161 | 163 | 168 | 170 |
| Mare | 1:32 | 1:256 | 1:64 | 1:256 | 1:256 | 1:128 | 1:128 | 1:256 | 1:256 | 1:64 | 1:256 | 1:256 | 1:128 | 1:16 | 1:256 | 1:128 | 1:256 | 1:256 | 1:256 | 1:256 | 1:256 | 1:256 | 1:128 | 1:256 |
| Day 0 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 |
| Day 1 | 1:64 | 1:256 | 1:64 | 1:256 | 1:256 | 1:256 | 1:128 | 1:256 | 1:256 | 1:256 | 1:256 | 1:256 | 1:64 | 1:32 | | | 1:256 | | | | | | | |
| 1 month | 1:32 | 1:256 | 1:256 | 1:64 | 1:128 | 1:256 | | | 1:128 | 1:256 | 1:256 | 1:256 | 1:16 | 1:2 | | | | | 1:256 | | | | | |
| 1.5 months | 1:16 | 1:64 | | | 1:32 | | 1:128 | 1:128 | | | | | | | 1:128 | 1:64 | 1:64 | 1:128 | | 1:32 | 1:2 | 1:16 | | 1:8 |
| 2 months | 1:4 | | 1:32 | 1:64 | | | 1:32 | 1:128 | 1:64 | 1:8 | 1:64 | 1:128 | 1:2 | | 1:128 | | 1:32 | 1:64 | 1:128 | | | | 1:2 | |
| 2.5 months | | 1:64 | 1:2 | 1:16 | | 1:64 | 1:16 | | | | | | | 1:2 | | 1:8 | | | | | | | | 1:2 |
| 3 months | 1:4 | 1:16 | 1:2 | | 1:256 | 1:16 | 1:8 | 1:16 | 1:4 | 1:4 | 1:16 | 1:32 | | 1:2 | 1:32 | 1:8 | 1:8 | 1:32 | 1:64 | 1:16 | 1:2 | | 1:2 | |
| 3.5 months | | | | 1:16 | 1:2 | | | 1:8 | 1:16 | 1:8 | 1:2 | 1:32 | 1:2 | 1:2 | | | | | | | | 1:2 | | |
| 4 months | 1:2 | | | | | 1:16 | 1:4 | 1:4 | | | | | 1:16 | 1:2 | 1:16 | 1:2 | 1:64 | 1:8 | 1:16 | 1:4 | 1:2 | 1:2 | 1:2 | 1:4 |
| 4.5 months | | 1:16 | 1:2 | | | | | | 1:4 | 1:2 | 1:2 | 1:8 | | | 1:16 | 1:2 | 1:8 | 1:4 | 1:8 | 1:4 | | | | |
| 5 months | 1:2 | 1:2 | | 1:2 | 1:2 | 1:2 | 1:2 | | | | | | 1:16 | 1:2 | 1:8 | 1:2 | 1:2 | | | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 |
| 5.5 months | | | 1:2 | 1:8 | | | | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | | | | | | 1:4 | 1:4 | | 1:2 | 1:2 | 1:2 | 1:2 |
| 6 months | | | | | 1:2 | 1:2 | 1:2 | 1:4 | | | | | 1:2 | 1:8 | | | | | | 1:2 | 1:2 | 1:2 | | |
| 6.5 months | 1:2 | 1:2 | 1:2 | | | | | | 1:2 | 1:2 | 1:2 | 1:4 | | | 1:8 | 1:2 | 1:2 | 1:2 | 1:2 | | | | 1:2 | 1:2 |
| 7 months | | | | 1:2 | 1:128 | | | | | | | | 1:2 | 1:256 | 1:2 | | | | | | | | | |
| 7.5 months | 1:2 | 1:2 | 1:2 | | | 1:2 | 1:2 | 1:2 | 1:2 | | | | | | | 1:2 | 1:2 | 1:2 | 1:8 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 |
| 8 months | | | | 1:2 | 1:64 | | | | | 1:2 | 1:2 | 1:2 | | | | | | | | 1:2 | | | | |
| 8.5 months | 1:2 | 1:2 | 1:2 | | | 1:2 | 1:2 | 1:4 | 1:2 | | | | 1:2 | 1:32 | 1:4 | 1:2 | 1:2 | | | | 1:2 | 1:2 | 1:2 | 1:2 |
| 9 months | 1:2 | 1:2 | | 1:2 | 1:32 | 1:2 | | | | 1:2 | 1:2 | 1:2 | | | | | | 1:2 | 1:2 | | | | | |
| 9.5 months | | | 1:2 | 1:4 | 1:64 | | 1:2 | 1:4 | 1:2 | 1:2 | 1:2 | 1:4 | 1:2 | 1:16 | 1:2 | 1:4 | 1:16 | | | 1:4 | 1:2 | 1:2 | | |
| 10 months | 1:2 | 1:2 | 1:2 | | | 1:8 | 1:2 | 1:4 | | | | | 1:2 | | | | | 1:2 | 1:2 | | | | 1:2 | 1:2 |
| 10.5 months | | | | 1:4 | 1:64 | | | | 1:32 | 1:2 | 1:16 | 1:16 | | 1:32 | 1:2 | 1:4 | 1:4 | 1:2 | | 1:8 | 1:2 | 1:2 | | |
| 11 months | 1:32 | 1:2 | 1:2 | | | 1:4 | 1:4 | 1:16 | | | | | 1:256 | 1:64 | 1:4 | 1:128 | 1:2 | | 1:2 | | | | 1:2 | 1:2 |
| 11.5 months | | | | 1:16 | 1:32 | 1:4 | | | 1:2 | 1:2 | 1:4 | 1:32 | | | | | | 1:4 | 1:2 | 1:32 | 1:2 | 1:2 | 1:2 | 1:2 |
| 12 months | 1:32 | 1:4 | 1:2 | | | | 1:2 | 1:8 | 1:2 | 1:2 | 1:2 | 1:4 | 1:256 | 1:32 | 1:16 | 1:64 | 1:32 | | | 1:16 | 1:16 | 1:2 | | |
| 12.5 months | | | | | 1:32 | | | | | | | | 1:128 | | | | | 1:2 | 1:16 | | | | 1:4 | 1:8 |
| 13 months | | | | 1:4 | | 1:2 | 1:2 | | | | | | | 1:32 | 1:2 | 1:32 | 1:128 | 1:2 | | 1:256 | 1:32 | 1:2 | | |
| 13.5 months | | | | | | | | 1:8 | 1:2 | 1:2 | 1:2 | 1:2 | | | | | | | 1:4 | | | | 1:2 | 1:32 |
| 14 months | | | | | | | | | | | | | 1:128 | 1:8 | 1:8 | | 1:32 | | | 1:128 | 1:64 | 1:2 | 1:2 | |
| 14.5 months | | | | | | | | | | | | | | | | 1:16 | | 1:2 | 1:16 | | | | | 1:16 |
| 15 months | | | | | | | | | | | | | | | | | | | | | 1:16 | 1:2 | | |
| 15.5 months | | | | | | | | | | | | | | | | | | | | 1:64 | | | 1:2 | 1:8 |

## Table A6.7 Virus neutralisation results for AHS Serotype 6

| ZST result | + | + | + | Weak + | Weak + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | - | - | + | + | Weak+ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| GC result | + | + | + | Weak + | + | + | + | + | + | + | Weak + | + | + | + | + | + | + | Weak + | + | + | | | | |
| **Foal number** | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** | **13** | **14** | **15** | **16** | **17** | **18** | **19** | **20** | **21** | **22** | **23** | **24** |
| Days: mare vac to foaling | 138 | 134 | 129 | 120 | 117 | 112 | 107 | 104 | 98 | 97 | 95 | 95 | 81 | 75 | 73 | 72 | 69 | 64 | 60 | 43 | 42 | 40 | 35 | 33 |
| Foal age at vac 1 (days) | 65 | 69 | 74 | 83 | 86 | 91 | 96 | 99 | 105 | 106 | 108 | 108 | 122 | 128 | 130 | 131 | 134 | 139 | 143 | 160 | 161 | 163 | 168 | 170 |
| Mare | 1:64 | 1:256 | 1:64 | 1:128 | 1:128 | 1:256 | 1:256 | 1:256 | 1:256 | 1:16 | 1:256 | 1:256 | 1:256 | 1:64 | 1:256 | 1:128 | 1:256 | 1:128 | 1:128 | 1:256 | 1:256 | 1:256 | 1:256 | 1:128 |
| Day 0 | 1:2 | 1:4 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 |
| Day 1 | 1:32 | 1:256 | 1:128 | 1:256 | 1:2 | 1:256 | 1:256 | 1:128 | 1:256 | 1:256 | 1:128 | 1:256 | 1:256 | 1:256 | | | 1:256 | | | | | | | |
| vac 1 - 5 months | | | | | | | | | | | | | | | | | | | | | | | | 1:128 |
| vac 1 - 4.5 months | | | | | | | | | | | | | | | | | | | 1:256 | 1:32 | 1:8 | 1:32 | 1:8 | |
| vac 1 - 4 months | | | | | | | | | | | | | 1:128 | 1:128 | | | | 1:128 | | | | | | 1:32 |
| vac 1 - 3.5 months | | | | | | | | | 1:256 | 1:16 | 1:4 | 1:32 | | | 1:256 | 1:64 | 1:32 | 1:16 | 1:16 | | | | 1:2 | |
| vac 1 - 3 months | | | | | | 1:256 | | | | | | | 1:32 | | 1:256 | | 1:32 | | | 1:8 | 1:2 | | | |
| vac 1 - 2.5 months | | | | 1:32 | 1:2 | | 1:128 | 1:256 | 1:256 | 1:8 | 1:16 | 1:4 | | 1:16 | | 1:256 | | 1:16 | 1:4 | | | 1:2 | 1:2 | 1:2 |
| vac 1 - 2 months | 1:32 | 1:32 | 1:256 | | 1:2 | | 1:32 | 1:64 | | | | | | 1:4 | 1:64 | 1:32 | 1:16 | | | 1:2 | 1:2 | 1:4 | | |
| vac 1 - 1.5 months | 1:16 | 1:64 | | 1:16 | | 1:128 | 1:16 | | 1:64 | 1:8 | 1:2 | 1:2 | 1:8 | 1:2 | | | | 1:8 | 1:2 | 1:2 | | | 1:2 | 1:2 |
| vac 1 - 1 month | 1:16 | | 1:32 | 1:32 | | 1:256 | 1:32 | 1:16 | 1:16 | 1:4 | 1:2 | 1:4 | 1:4 | 1:2 | 1:32 | 1:8 | 1:8 | 1:4 | 1:8 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 |
| vac 1 - 0.5 months | | 1:64 | 1:8 | | 1:2 | | | 1:8 | | | | | | | 1:32 | 1:4 | 1:16 | | | | 1:2 | 1:2 | | |
| **Vaccination 1** | **1:64** | **1:256** | **1:8** | **1:2** | **1:2** | **1:16** | **1:8** | **1:8** | **1:16** | **1:2** | **1:4** | **1:2** | **1:8** | **1:2** | **1:16** | **1:16** | **1:4** | **1:2** | **1:4** | **1:2** | **1:2** | **1:2** | **1:2** | **1:2** |
| vac 1 + 0.5 months | | | | | | | | | | | | | | | | | | | | | | | | |
| vac 1 + 1 month | 1:2 | | | | | 1:8 | 1:4 | | 1:8 | 1:2 | 1:4 | 1:2 | 1:2 | 1:2 | | | | 1:2 | 1:2 | | | | 1:2 | 1:2 |
| vac 1 + 1.5 months | | 1:16 | 1:2 | 1:2 | 1:2 | | | 1:8 | | | | | | | 1:32 | 1:2 | 1:4 | | | 1:2 | 1:4 | 1:2 | | |
| vac 1 + 2 months | 1:2 | 1:8 | | 1:4 | | 1:2 | 1:2 | 1:8 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:4 | | | 1:2 | 1:8 | 1:2 | | | 1:2 | 1:2 |
| vac 1 + 2.5 months | | | 1:16 | | 1:2 | | | | | | | | | | | 1:16 | 1:8 | | | | 1:4 | 1:2 | | |
| vac 1 + 3 months | | | | | | | | | 1:2 | | | | | | | | | | | | | | | |
| vac 1 + 3.5 months | 1:2 | 1:4 | 1:2 | 1:2 | 1:2 | 1:4 | 1:2 | 1:2 | | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:32 | 1:32 | 1:4 | 1:2 | 1:8 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 |
| vac 1 + 4 months | | | | | | | | | 1:4 | | | | | | | | | | | | | | | |
| vac 1 + 4.5 months | 1:2 | 1:8 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:4 | | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:16 | 1:4 | 1:16 | 1:4 | 1:2 | 1:4 | 1:2 | 1:2 | 1:2 | 1:2 |
| vac 1 + 5 months | | | | | | 1:2 | | | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | | | | | 1:4 | | | | | 1:4 | 1:2 |
| vac 1 + 5.5 months | 1:2 | 1:32 | 1:4 | 1:2 | 1:2 | | 1:2 | 1:8 | | | | | | 1:2 | 1:64 | 1:4 | 1:4 | | 1:4 | 1:16 | 1:2 | 1:2 | | |
| vac 1 + 6 months | 1:2 | 1:2 | | 1:2 | 1:2 | 1:8 | 1:2 | 1:8 | 1:64 | 1:2 | 1:2 | 1:2 | 1:128 | 1:2 | 1:64 | 1:16 | 1:2 | 1:4 | 1:2 | 1:4 | 1:16 | 1:2 | 1:8 | 1:8 |
| vac 1 + 6.5 months | | | 1:8 | | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | | | | | |
| **Vaccination 2** | **1:2** | **1:2** | **1:8** | **1:2** | **1:2** | **1:8** | **1:2** | **1:8** | **1:64** | **1:2** | **1:2** | **1:2** | **1:128** | **1:2** | **1:64** | **1:16** | **1:2** | **1:4** | **1:2** | **1:4** | **1:16** | **1:2** | **1:8** | **1:8** |
| vac 2 + 0.5 months | | | 1:4 | | | | | | | | | | | | | | | | | | | | | |
| vac 2 + 1 month | 1:4 | 1:8 | | 1:8 | 1:4 | 1:8 | 1:2 | 1:64 | 1:32 | 1:2 | 1:4 | 1:4 | 1:256 | 1:4 | 1:8 | 1:256 | 1:32 | 1:8 | 1:64 | 1:2 | 1:32 | 1:4 | 1:2 | 1:8 |
| vac 2 + 1.5 months | | | 1:2 | | | 1:8 | | | 1:8 | 1:2 | 1:4 | 1:2 | 1:64 | | | | | 1:2 | | | | | 1:2 | |
| vac 2 + 2 months | 1:4 | 1:16 | | 1:8 | 1:2 | | 1:2 | 1:32 | | | | | | 1:2 | 1:16 | 1:128 | 1:32 | | 1:32 | 1:4 | 1:16 | 1:2 | | 1:4 |
| vac 2 + 2.5 months | | | 1:4 | | | | | | | | | | | | | | | | | | | | | |
| vac 2 + 3 months | 1:4 | 1:8 | | | 1:2 | 1:2 | 1:2 | | 1:4 | 1:2 | 1:4 | 1:4 | 1:64 | 1:2 | 1:16 | | 1:128 | 1:2 | 1:8 | | 1:16 | 1:2 | 1:2 | 1:8 |
| vac 2 + 3.5 months | | | | 1:16 | | | | 1:8 | | | | | | | | 1:128 | | | | 1:4 | | | | |

## Table A6.8 Virus neutralisation results for AHS Serotype 7

| ZST result | + | + | + | Weak + | Weak + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | - | - | + | + | Weak + |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| GC result | + | + | + | Weak + | + | + | + | + | + | + | Weak + | + | + | + | + | + | + | Weak + | + | + | | | | |
| **Foal number** | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** | **13** | **14** | **15** | **16** | **17** | **18** | **19** | **20** | **21** | **22** | **23** | **24** |
| Days: mare vac to foaling | 138 | 134 | 129 | 120 | 117 | 112 | 107 | 104 | 98 | 97 | 95 | 95 | 81 | 75 | 73 | 72 | 69 | 64 | 60 | 43 | 42 | 40 | 35 | 33 |
| Foal age at vac 1 (days) | 65 | 69 | 74 | 83 | 86 | 91 | 96 | 99 | 105 | 106 | 108 | 108 | 122 | 128 | 130 | 131 | 134 | 139 | 143 | 160 | 161 | 163 | 168 | 170 |
| Mare | 1:32 | 1:128 | 1:128 | 1:256 | 1:128 | 1:32 | 1:256 | 1:256 | 1:128 | 1:32 | 1:128 | 1:256 | 1:256 | 1:2 | 1:128 | 1:32 | 1:128 | 1:128 | 1:128 | 1:32 | 1:256 | 1:256 | 1:64 | 1:64 |
| Day 0 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:4 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 |
| Day 1 | 1:8 | 1:128 | 1:16 | 1:16 | | 1:128 | 1:64 | 1:256 | 1:128 | 1:256 | 1:64 | 1:32 | 1:256 | 1:2 | | | 1:128 | | | | | | | |
| vac 1 - 5 months | | | | | | | | | | | | | | | | | | | | | | | | 1:16 |
| vac 1 - 4.5 months | | | | | | | | | | | | | | | | | | | 1:256 | 1:4 | 1:8 | 1:8 | 1:2 | |
| vac 1 - 4 months | | | | | | | | | | | | | 1:128 | 1:2 | | | | 1:8 | | | | | | 1:2 |
| vac 1 - 3.5 months | | | | | | | | | 1:32 | 1:8 | 1:8 | 1:4 | | | 1:32 | 1:16 | 1:128 | 1:8 | 1:8 | | | | 1:2 | |
| vac 1 - 3 months | | | | | | 1:128 | | | | | | | 1:32 | | 1:16 | | 1:8 | | | 1:2 | 1:4 | | | |
| vac 1 - 2.5 months | | | | 1:32 | 1:2 | | 1:8 | 1:8 | 1:32 | 1:8 | 1:8 | 1:4 | | 1:2 | | 1:2 | | 1:2 | 1:8 | | | 1:2 | 1:2 | 1:2 |
| vac 1 - 2 months | 1:8 | 1:32 | 1:64 | | 1:2 | | 1:2 | 1:16 | | | | | | 1:2 | 1:4 | 1:2 | 1:2 | | | 1:2 | 1:2 | 1:4 | | |
| vac 1 - 1.5 months | 1:2 | 1:8 | | 1:32 | | 1:32 | 1:2 | | 1:16 | 1:2 | 1:8 | 1:4 | 1:8 | 1:2 | | | | 1:2 | 1:2 | 1:2 | | | 1:2 | 1:2 |
| vac 1 - 1 month | 1:4 | | 1:64 | 1:4 | | 1:16 | 1:2 | 1:16 | 1:4 | 1:4 | 1:2 | 1:2 | 1:4 | 1:2 | 1:4 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 |
| vac 1 - 0.5 months | | 1:16 | 1:16 | | 1:256 | | | 1:4 | | | | | | | 1:4 | 1:2 | 1:2 | | | | 1:2 | 1:2 | | |
| **Vaccination 1** | **1:2** | **1:8** | **1:8** | **1:2** | **1:2** | **1:16** | **1:2** | **1:2** | **1:2** | **1:2** | **1:2** | **1:2** | **1:2** | **1:2** | **1:2** | **1:2** | **1:4** | **1:2** | **1:2** | **1:2** | **1:2** | **1:2** | **1:2** | **1:2** |
| vac 1 + 0.5 months | | | | | | | | | | | | | | | | | | | | | | | | |
| vac 1 + 1 month | 1:2 | | | | | 1:2 | 1:2 | | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | | | | 1:2 | 1:2 | | | | 1:2 | 1:2 |
| vac 1 + 1.5 months | | 1:2 | 1:4 | 1:2 | 1:2 | | | 1:2 | | | | | | | 1:2 | 1:2 | 1:2 | | | 1:2 | 1:2 | 1:2 | | |
| vac 1 + 2 months | 1:2 | 1:2 | | 1:2 | | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | | | 1:2 | 1:4 | 1:2 | | | 1:2 | 1:2 |
| vac 1 + 2.5 months | | | 1:2 | | 1:2 | | | | | | | | | | | 1:2 | 1:2 | | | | 1:2 | 1:2 | | |
| vac 1 + 3 months | | | | | | | | | 1:2 | | | | | | | | | | | | | | | |
| vac 1 + 3.5 months | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 |
| vac 1 + 4 months | | | | | | | | | 1:2 | | | | | | | | | | | | | | | |
| vac 1 + 4.5 months | 1:2 | 1:2 | 1:4 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:8 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 |
| vac 1 + 5 months | | | | | | 1:2 | | | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | | | | | 1:2 | | | | | 1:2 | 1:2 |
| vac 1 + 5.5 months | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | | 1:2 | 1:2 | | | | | | 1:2 | 1:2 | 1:2 | 1:2 | | 1:2 | 1:2 | 1:2 | 1:2 | | |
| vac 1 + 6 months | 1:2 | 1:2 | | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:4 | 1:16 | 1:16 | 1:8 | 1:128 | 1:16 | 1:2 | 1:8 | 1:2 | 1:2 | 1:2 | 1:4 | 1:2 | 1:4 |
| vac 1 + 6.5 months | | | 1:2 | | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | | | | | |
| **Vaccination 2** | **1:2** | **1:2** | **1:2** | **1:2** | **1:2** | **1:2** | **1:2** | **1:2** | **1:2** | **1:2** | **1:4** | **1:16** | **1:16** | **1:8** | **1:128** | **1:16** | **1:2** | **1:8** | **1:2** | **1:2** | **1:2** | **1:4** | **1:2** | **1:4** |
| vac 2 + 0.5 months | | | 1:2 | | | | | | | | | | | | | | | | | | | | | |
| vac 2 + 1 month | 1:2 | 1:2 | | 1:2 | 1:2 | 1:128 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:128 | 1:128 | 1:8 | 1:256 | 1:2 | 1:2 | 1:4 | 1:128 | 1:2 | 1:4 | 1:2 | 1:2 | 1:2 |
| vac 2 + 1.5 months | | | 1:2 | | | 1:128 | | | 1:2 | 1:2 | 1:2 | 1:128 | 1:128 | | | | | 1:4 | | | | | 1:2 | |
| vac 2 + 2 months | 1:2 | 1:2 | | 1:2 | 1:2 | | 1:2 | 1:2 | | | | | | 1:8 | 1:64 | 1:2 | 1:2 | | 1:128 | 1:2 | 1:2 | 1:4 | | 1:2 |
| vac 2 + 2.5 months | | | 1:2 | | | | | | | | | | | | | | | | | | | | | |
| vac 2 + 3 months | 1:2 | 1:2 | | | 1:2 | 1:32 | 1:2 | | 1:2 | 1:2 | 1:2 | 1:64 | 1:128 | 1:16 | 1:64 | | 1:2 | 1:4 | 1:32 | | 1:2 | 1:4 | 1:2 | 1:2 |
| vac 2 + 3.5 months | | | | 1:2 | | | | 1:2 | | | | | | | | 1:2 | | | | 1:2 | | | | |

## Table A6.9 Virus neutralisation results for AHS Serotype 8

| ZST result | + | + | + | Weak + | Weak + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | - | - | + | + | Weak + |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| GC result | + | + | + | Weak + | + | + | + | + | + | + | Weak + | + | + | + | + | + | + | Weak + | + | + | | | | |
| **Foal number** | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** | **13** | **14** | **15** | **16** | **17** | **18** | **19** | **20** | **21** | **22** | **23** | **24** |
| Days: mare vac to foaling | 138 | 134 | 129 | 120 | 117 | 112 | 107 | 104 | 98 | 97 | 95 | 95 | 81 | 75 | 73 | 72 | 69 | 64 | 60 | 43 | 42 | 40 | 35 | 33 |
| Foal age at vac 1 (days) | 65 | 69 | 74 | 83 | 86 | 91 | 96 | 99 | 105 | 106 | 108 | 108 | 122 | 128 | 130 | 131 | 134 | 139 | 143 | 160 | 161 | 163 | 168 | 170 |
| Mare | 1:32 | 1:32 | 1:64 | 1:256 | 1:256 | 1:32 | 1:128 | 1:256 | 1:64 | 1:256 | 1:128 | 1:256 | 1:256 | 1:256 | 1:256 | 1:256 | 1:256 | 1:256 | 1:256 | 1:64 | 1:256 | 1:256 | 1:64 | 1:256 |
| Day 0 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 |
| Day 1 | 1:32 | 1:64 | 1:32 | 1:128 | | 1:32 | 1:128 | 1:128 | 1:64 | 1:128 | 1:128 | 1:256 | 1:128 | 1:256 | | | 1:256 | | | | | | | |
| vac 1 - 5 months | | | | | | | | | | | | | | | | | | | | | | | | 1:2 |
| vac 1 - 4.5 months | | | | | | | | | | | | | | | | | | | 1:256 | 1:16 | 1:2 | 1:32 | 1:4 | |
| vac 1 - 4 months | | | | | | | | | | | | | 1:64 | 1:64 | | | | 1:64 | | | | | | 1:4 |
| vac 1 - 3.5 months | | | | | | | | | 1:16 | 1:128 | 1:16 | 1:256 | | | 1:32 | 1:16 | 1:32 | 1:32 | 1:256 | | | | 1:2 | |
| vac 1 - 3 months | | | | | | 1:32 | | | | | | | 1:32 | | 1:32 | | 1:32 | | | 1:4 | 1:2 | | | |
| vac 1 - 2.5 months | | | | 1:64 | 1:4 | | 1:16 | 1:32 | | 1:32 | 1:8 | 1:256 | | 1:64 | | 1:8 | | 1:32 | 1:128 | | | 1:2 | 1:2 | 1:2 |
| vac 1 - 2 months | 1:16 | 1:32 | 1:64 | | 1:4 | | 1:32 | 1:32 | | | | | | 1:32 | 1:32 | 1:4 | 1:16 | | | 1:2 | 1:2 | 1:4 | | |
| vac 1 - 1.5 months | 1:4 | 1:16 | | 1:64 | | 1:8 | 1:32 | | 1:4 | 1:16 | 1:4 | 1:32 | 1:8 | 1:8 | | | | 1:32 | 1:8 | 1:2 | | | 1:2 | 1:2 |
| vac 1 - 1 month | 1:4 | | 1:4 | 1:32 | | 1:2 | 1:8 | 1:16 | 1:2 | 1:8 | 1:2 | 1:16 | 1:8 | 1:8 | 1:32 | 1:2 | 1:16 | 1:2 | 1:8 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 |
| vac 1 - 0.5 months | | 1:8 | 1:4 | | 1:256 | | | 1:8 | | | | | | | 1:16 | 1:2 | 1:8 | | | | 1:2 | 1:2 | | |
| **Vaccination 1** | **1:2** | **1:4** | **1:4** | **1:8** | **1:2** | **1:2** | **1:8** | **1:4** | **1:2** | **1:8** | **1:2** | **1:8** | **1:2** | **1:8** | **1:4** | **1:4** | **1:4** | **1:2** | **1:8** | **1:2** | **1:2** | **1:2** | **1:2** | **1:2** |
| vac 1 + 0.5 months | | | | | | | | | | | | | | | | | | | | | | | | |
| vac 1 + 1 month | 1:2 | | | | | 1:2 | 1:2 | | 1:2 | 1:2 | 1:2 | 1:4 | 1:2 | 1:16 | | | | 1:2 | 1:2 | | | | 1:2 | 1:2 |
| vac 1 + 1.5 months | | 1:2 | 1:2 | 1:8 | 1:2 | | | 1:2 | | | | | | | 1:8 | 1:2 | 1:2 | | | 1:2 | 1:2 | 1:2 | | |
| vac 1 + 2 months | 1:2 | 1:2 | | 1:2 | | 1:2 | 1:2 | 1:2 | 1:2 | 1:4 | 1:2 | 1:2 | 1:2 | 1:32 | 1:2 | | | 1:8 | 1:8 | 1:2 | | | 1:2 | 1:2 |
| vac 1 + 2.5 months | | | 1:2 | | 1:128 | | | | | | | | | | | 1:4 | 1:4 | | | | 1:2 | 1:2 | | |
| vac 1 + 3 months | | | | | | | | | 1:2 | | | | | | | | | | | | | | | |
| vac 1 + 3.5 months | 1:2 | 1:2 | 1:2 | 1:2 | 1:256 | 1:2 | 1:2 | 1:2 | | 1:2 | 1:2 | 1:4 | 1:2 | 1:8 | 1:16 | 1:2 | 1:4 | 1:2 | 1:2 | 1:4 | 1:2 | 1:2 | 1:2 | 1:2 |
| vac 1 + 4 months | | | | | | | | | 1:2 | | | | | | | | | | | | | | | |
| vac 1 + 4.5 months | 1:2 | 1:2 | 1:2 | 1:2 | 1:256 | 1:2 | 1:2 | 1:2 | | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:8 | 1:2 | 1:4 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 |
| vac 1 + 5 months | | | | | | 1:2 | | | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | | | | | 1:2 | | | | | 1:2 | 1:2 |
| vac 1 + 5.5 months | 1:2 | 1:2 | 1:2 | 1:2 | 1:256 | | 1:2 | 1:2 | | | | | | 1:4 | 1:2 | 1:2 | 1:2 | | 1:2 | 1:2 | 1:2 | 1:2 | | |
| vac 1 + 6 months | 1:2 | 1:2 | | 1:2 | 1:256 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:8 | 1:128 | 1:8 | 1:8 | 1:32 | 1:2 | 1:2 | 1:2 | 1:4 | 1:4 | 1:2 | 1:4 | 1:2 |
| vac 1 + 6.5 months | | | 1:2 | | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | | | | | |
| **Vaccination 2** | **1:2** | **1:2** | **1:2** | **1:2** | **1:256** | **1:2** | **1:2** | **1:2** | **1:2** | **1:2** | **1:2** | **1:8** | **1:128** | **1:8** | **1:8** | **1:32** | **1:2** | **1:2** | **1:2** | **1:4** | **1:4** | **1:2** | **1:4** | **1:2** |
| vac 2 + 0.5 months | | | 1:2 | | | | | | | | | | | | | | | | | | | | | |
| vac 2 + 1 month | 1:2 | 1:4 | | 1:2 | 1:256 | 1:2 | 1:4 | 1:8 | 1:2 | 1:4 | 1:8 | 1:4 | 1:128 | 1:256 | 1:8 | 1:16 | 1:2 | 1:4 | 1:32 | 1:16 | 1:16 | 1:2 | 1:4 | 1:2 |
| vac 2 + 1.5 months | | | 1:2 | | | 1:2 | | | 1:2 | 1:2 | 1:2 | 1:4 | 1:64 | | | | | 1:4 | | | | | 1:2 | |
| vac 2 + 2 months | 1:8 | 1:4 | | 1:2 | 1:128 | | 1:2 | 1:32 | | | | | | 1:64 | 1:4 | 1:8 | 1:4 | | 1:16 | 1:16 | 1:8 | 1:2 | | 1:2 |
| vac 2 + 2.5 months | | | 1:2 | | | | | | | | | | | | | | | | | | | | | |
| vac 2 + 3 months | 1:2 | 1:32 | | | 1:64 | 1:2 | 1:2 | | 1:2 | 1:2 | 1:2 | 1:4 | 1:64 | 1:64 | 1:2 | | 1:16 | 1:2 | 1:16 | | 1:8 | 1:2 | 1:2 | 1:2 |
| vac 2 + 3.5 months | | | | 1:2 | | | | 1:32 | | | | | | | | 1:8 | | | | 1:8 | | | | |

**Table A6.10** Virus neutralisation results for AHS Serotype 9 (note: Serotype 9 not included in AHS vaccination)

| ZST result | + | + | + | Weak + | Weak + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | - | - | + | + | Weak + |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| GC result | + | + | + | Weak + | + | + | + | + | + | + | Weak + | + | + | + | + | + | + | Weak + | + | + | | | | | |
| **Foal number** | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** | **13** | **14** | **15** | **16** | **17** | **18** | **19** | **20** | **21** | **22** | **23** | **24** |
| Days: mare vac to foaling | 138 | 134 | 129 | 120 | 117 | 112 | 107 | 104 | 98 | 97 | 95 | 95 | 81 | 75 | 73 | 72 | 69 | 64 | 60 | 43 | 42 | 40 | 35 | 33 |
| Foal age at vac 1 (days) | 65 | 69 | 74 | 83 | 86 | 91 | 96 | 99 | 105 | 106 | 108 | 108 | 122 | 128 | 130 | 131 | 134 | 139 | 143 | 160 | 161 | 163 | 168 | 170 |
| Mare | 1:4 | 1:64 | 1:16 | 1:256 | 1:256 | 1:256 | 1:256 | 1:256 | 1:256 | 1:16 | 1:128 | 1:256 | 1:128 | 1:128 | 1:256 | 1:256 | 1:256 | 1:256 | 1:256 | 1:256 | 1:256 | 1:256 | 1:64 | 1:128 |
| Day 0 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 |
| Day 1 | 1:16 | 1:256 | 1:16 | 1:128 | | 1:128 | 1:256 | 1:256 | 1:128 | 1:256 | 1:128 | 1:256 | 1:256 | 1:256 | | | 1:128 | | | | | | | |
| 1 month | 1:4 | 1:64 | 1:32 | 1:64 | 1:2 | 1:256 | | | 1:64 | 1:64 | 1:16 | 1:128 | 1:128 | 1:8 | | | | | 1:256 | | | | | |
| 1.5 months | 1:4 | 1:32 | | | 1:2 | | 1:16 | 1:256 | | | | | | | 1:256 | 1:32 | 1:64 | 1:128 | | 1:8 | 1:4 | 1:128 | | 1:8 |
| 2 months | 1:2 | | 1:2 | 1:64 | | | 1:32 | 1:256 | | 1:32 | 1:4 | 1:16 | 1:32 | | 1:256 | | 1:32 | 1:64 | 1:256 | | | | 1:4 | |
| 2.5 months | | 1:32 | 1:2 | 1:16 | | 1:128 | 1:16 | | | | | | | 1:2 | | 1:8 | | | | | | | | 1:2 |
| 3 months | 1:2 | 1:4 | 1:4 | | 1:128 | 1:32 | 1:8 | 1:256 | 1:16 | 1:8 | 1:2 | 1:16 | | 1:4 | 1:128 | 1:8 | 1:16 | 1:16 | 1:128 | 1:8 | 1:2 | | 1:2 | |
| 3.5 months | | | | 1:8 | 1:2 | | | 1:32 | 1:16 | 1:4 | 1:2 | 1:8 | 1:8 | 1:2 | | | | | | | | 1:8 | | |
| 4 months | 1:2 | | | | | 1:8 | 1:16 | 1:32 | | | | | 1:8 | 1:2 | 1:128 | 1:4 | 1:8 | 1:8 | 1:128 | 1:8 | 1:2 | 1:4 | 1:2 | 1:2 |
| 4.5 months | | 1:2 | 1:2 | | | | | | 1:2 | 1:2 | 1:2 | 1:4 | | | 1:64 | 1:2 | 1:8 | 1:2 | 1:32 | 1:2 | | | | |
| 5 months | 1:2 | 1:2 | | 1:2 | 1:2 | 1:16 | 1:4 | | | | | | 1:2 | 1:2 | 1:16 | 1:2 | 1:4 | | | 1:2 | 1:2 | 1:4 | 1:2 | 1:2 |
| 5.5 months | | | 1:2 | 1:4 | | | | 1:8 | 1:2 | 1:2 | 1:2 | 1:4 | | | | | | 1:4 | 1:16 | | 1:2 | 1:2 | 1:2 | 1:2 |
| 6 months | | | | | 1:4 | 1:4 | 1:2 | 1:16 | | | | | 1:2 | 1:8 | | | | | | 1:2 | 1:2 | 1:2 | | |
| 6.5 months | 1:2 | 1:2 | 1:2 | | | | | | 1:8 | 1:2 | 1:2 | 1:4 | | | 1:16 | 1:2 | 1:2 | 1:8 | 1:16 | | | | 1:2 | 1:2 |
| 7 months | | | | 1:2 | 1:16 | | | | | | | | 1:2 | 1:32 | 1:8 | | | | | | | | | |
| 7.5 months | 1:2 | 1:2 | 1:2 | | | 1:4 | 1:2 | 1:4 | 1:8 | | | | | | | 1:2 | 1:16 | 1:16 | 1:32 | 1:8 | 1:2 | 1:2 | 1:2 | 1:2 |
| 8 months | | | | 1:2 | 1:16 | | | | | 1:64 | 1:4 | 1:128 | | | | | | | | 1:8 | | | | |
| 8.5 months | 1:2 | 1:2 | 1:2 | | | 1:2 | 1:2 | 1:64 | 1:4 | | | | 1:2 | 1:8 | 1:128 | 1:8 | 1:8 | | | | 1:8 | 1:2 | 1:2 | 1:2 |
| 9 months | 1:2 | 1:2 | | 1:2 | 1:4 | 1:2 | | | | 1:32 | 1:8 | 1:64 | | | | | | 1:16 | 1:16 | | | | | |
| 9.5 months | | | 1:2 | 1:2 | 1:4 | | 1:2 | 1:16 | 1:2 | 1:4 | 1:16 | 1:32 | 1:2 | 1:4 | 1:128 | 1:2 | 1:64 | | | 1:32 | 1:2 | 1:2 | | |
| 10 months | 1:4 | 1:2 | 1:2 | | | 1:2 | 1:2 | 1:16 | | | | | 1:2 | | | | | 1:8 | 1:2 | | | | 1:4 | 1:2 |
| 10.5 months | | | | 1:8 | 1:8 | | | | 1:8 | 1:8 | 1:16 | 1:64 | | 1:2 | 1:128 | 1:2 | 1:64 | 1:16 | | 1:8 | 1:2 | 1:8 | | |
| 11 months | 1:16 | 1:2 | 1:2 | | | 1:4 | 1:2 | 1:16 | | | | | 1:8 | 1:32 | 1:256 | 1:8 | 1:64 | | 1:4 | | | | 1:4 | 1:2 |
| 11.5 months | | | | 1:64 | 1:4 | 1:4 | | | 1:4 | 1:8 | 1:8 | 1:64 | | | | | | 1:128 | 1:2 | 1:32 | 1:2 | 1:16 | 1:2 | 1:2 |
| 12 months | 1:4 | 1:2 | 1:2 | | | | 1:2 | 1:32 | 1:4 | 1:2 | 1:8 | 1:64 | 1:256 | 1:32 | 1:256 | 1:8 | 1:128 | | | 1:16 | 1:4 | 1:8 | | |
| 12.5 months | | | | | 1:8 | | | | | | | | 1:256 | | | | | 1:128 | 1:256 | | | | 1:64 | 1:4 |
| 13 months | | | | 1:64 | | 1:2 | 1:2 | | | | | | | 1:128 | 1:64 | 1:4 | 1:256 | 1:64 | | 1:4 | 1:16 | 1:4 | | |
| 13.5 months | | | | | | | | 1:4 | 1:8 | 1:2 | 1:2 | 1:4 | | | | | | | 1:64 | | | | 1:32 | 1:8 |
| 14 months | | | | | | | | | | | | | 1:64 | 1:32 | 1:128 | | 1:32 | | | 1:4 | 1:16 | 1:8 | 1:32 | |
| 14.5 months | | | | | | | | | | | | | | | | 1:32 | | 1:8 | 1:32 | | | | | 1:4 |
| 15 months | | | | | | | | | | | | | | | | | | | | | 1:2 | 1:4 | | |
| 15.5 months | | | | | | | | | | | | | | | | | | | | 1:2 | | | 1:16 | 1:2 |