DEVELOPMENT AND VALIDATION OF ENZYME LINKED IMMUNOSORBENT ASSAYS FOR DETECTION OF EQUINE ENCEPHALOSIS ANTIBODY AND ANTIGEN

BY

JAN ERNST CRAFFORD

A DISSERTATION SUBMITTED TO THE FACULTY OF VETERINARY SCIENCE OF THE UNIVERSITY OF PRETORIA IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MAGISTER SCIENTIAE (VETERINARY SCIENCE)

Date submitted: November 2001

Acknowledgements

I wish to acknowledge and express my sincere appreciation to the following people:

Chris Hamblin, for all the patience, guidance and time spent in training me in ELISA techniques and for guidance during the project and writing of this dissertation. Thank you for the enthusiasm and for your friendship.

Professor Alan Guthrie, for the foresight and management that made this project possible. Thank you for guidance, motivation and friendship.

Professor Peter Howell for support and encouragement.

Peter Mertens and Nick Burroughs, for teaching me the purification of virus particles and for providing the purified virus for this project.

Magdalena Groenewald, Carina Visagé, Monique ter Linde and Sue Graham for assistance in the laboratory.

I am grateful to the UK/SOUTH AFRICA SCIENCE AND TECHNOLOGY RESEARCH FUND for the grant that made this work possible.

A special word of thanks to **Professor Moritz van Vuuren**, my promoter, for your guidance and friendship throughout this project and to all my colleagues in the Department of Veterinary Tropical Diseases, for providing the positive, stimulating and happy environment in which we work.

D e c l a r a t i o n

I, *Jan Ernst Crafford*, do hereby declare that during the course of this study the purification of equine encephalosis virus particles was done by J N Burroughs and P P C Mertens from the Institute for Animal Health, Pirbright, UK. C Hamblin assisted with ELISAs and D Groenewald and M ter Linde assisted with the virus neutralisation tests on South African horse sera. Except where acknowledgements indicate otherwise and for the advice from my supervisors, this dissertation representsp my own original work. Neither the full dissertation nor any part of it has been, is being, or is to be submitted for another degree at this or any other University.

This dissertation is presented in partial fulfilment of the requirements for the degree of Magister Scientiae (Veterinary Science) in the Department of Veterinary Tropical Diseases, University of Pretoria.

Abstract

DEVELOPMENT AND VALIDATION OF ENZYME LINKED IMMUNOSORBENT ASSAYS FOR DETECTION OF EQUINE ENCEPHALOSIS VIRUS ANTIBODY AND ANTIGEN

BY

JAN ERNST CRAFFORD

PROMOTER: Prof M van Vuuren
CO-PROMOTERS: Prof A J Guthrie, Mr C Hamblin
DEPARTMENT: Department of Veterinary Tropical Diseases
Faculty of Veterinary Science, University of Pretoria
DEGREE: Magister Scientiae (Veterinary Science)

The main purpose of this work was to develop rapid and reliable techniques that will prove valuable in epidemiological studies of equine encephalosis virus (EEV), the detection and identification of the virus for the laboratory confirmation of the clinical diagnosis and for the differential diagnosis between EEV and African horsesickness virus (AHSV). Two enzyme linked immunosorbent assays (ELISA) were developed. A polyclonal antibody-based, group-specific, indirect sandwich ELISA for the detection of EEV antigen was developed. The design of the assay was based on the methods currently used for the detection of AHSV. The cut-off value (absorbance of 0.15) was determined using populations of known negative specimens. No cross-reactions were recorded with viruses from other orbivirus serogroups or from other arboviruses. The assay proved to be sensitive and specific for the rapid detection of EEV and viral antigens in cell culture and mouse brain preparations.

A polyclonal antibody-based, group-specific, competitive ELISA for the detection of antibodies to EEV was developed. No cross-reactions were recorded with the reference sera prepared against other orbivirus serogroups or other arboviruses. The cut-off (29.5% inhibition) value was determined using populations of known positive and negative sera. Analysis of the data showed the assay to be highly repeatable, sensitive and specific.

Table of Contents

List of Figures

List of Tables

List of abbreviations

AGID	agar gel immunodiffusion
AHS	African horsesickness
AHSV	African horsesickness virus
BEFV	bovine ephemeral fever virus
BHK	baby hamster kidney
BHK-21	baby hamster kidney cells clone 21
BT	bluetongue
BTV	bluetongue virus
C-ELISA	competitive enzyme linked immunosorbent assay
CFT	complement fixation test
CPE	cytopathic effect
CV	coefficient of variation
DSp	diagnostic specificity
DVTD	Department of Veterinary Tropical Diseases
EE	equine encephalosis
EEV	equine encephalosis virus
EHDV	epizootic haemorrhagic disease of deer virus
EIA	enzyme immunoassays
ELISA	enzyme linked immunosorbent assay
EU	European Union
FCA	Freund's complete adjuvant
FCS	foetal calf serum
FICA	Freund's incomplete adjuvant
HRPO	horseradish peroxidase
IAH	Institute for Animal Health
ISVP	infectious sub-viral particles
KNP	Kruger National Park

MB	mouse brain
Mr	relative molecular weight
OIE	*Office International des Epizooties* (The World Organization for Animal Health)
OVI	Onderstepoort Veterinary Institute
p.i.	post inoculation
PBS	phosphate buffered saline
PBST	phosphate buffered saline supplemented with 0.05 % (v/v) Tween 20
PCR	polymerase chain reaction
PI	percentage inhibition
ROC	receiver operator characteristic
Se	sensitivity
S-ELISA	sandwich enzyme linked immunosorbent assay
Sp	specificity
TG-ROC	two-graph receiver operator characteristic
UK	United Kingdom
VERO	African green monkey kidney cells
VNT	virus neutralisation test
VP	virus protein

Chapter 1

GENERAL INTRODUCTION

Introduction

International movement of horses for trade, competition and breeding has increased markedly during the last decade. Because most of these animals are now transported by air, the risk of them either incubating or carrying exotic infectious agents has also increased. The primary aim of this project was to develop sensitive and specific assays for the detection of equine encephalosis virus (EEV) antigen and antibody. Provision of such assays is important, not only to study the epidemiology of EEV but also to rapidly differentiate EEV and African horsesickness virus (AHSV), the latter which is the only *Office International des Epizooties* (The World Organization for Animal Health) (OIE) list A disease that exclusively infects equids. Both of these virus groups belong to the family *Reoviridae,* genus *Orbivirus* and both are believed to be transmitted by the same insect vectors. One of the major reasons for including AHSV and EEV in this study is that, in some cases, horses infected with either virus may exhibit clinical signs resembling African horsesickness fever[8]. In the absence of rapid differential diagnostic tests to distinguish between these two viruses, there could be a delay in the laboratory confirmation, which could also delay the implementation of appropriate control

measures, particularly in countries where neither disease has been previously encountered.

African horsesickness (AHS) is considered the most lethal and economically important viral disease affecting horses and as such has been included in the OIE's A list of important trans-boundary diseases. Mortality can exceed 95 % in naïve horses. Live attenuated vaccines prepared against different AHSV's are available and are regularly used to help control the spread of AHSV in endemic and epizootic areas.

Before 1997, the occurrence of AHS in South Africa had precluded the export of horses into the European Union (EU) and other countries that conformed to the EU regulations. However, these restrictions were lifted in 1997 by Commission Decision 97/10/EC, when the principle of regionalization was applied and part of the Western Cape Province in South Africa was identified as an AHS-free zone[4].

Equine encephalosis (EE), on the other hand, is perceived as an emerging disease, having first been identified in 1967[9]. Unlike AHS, the epidemiology and clinical diagnosis of EE are poorly documented. According to the available literature, mortality in endemic regions is usually less than 5 % although morbidity can be in excess of 74%[26].

Limited serological and virological surveys have shown that EEV is prevalent in South Africa, Botswana, Namibia, Zimbabwe and Kenya[5,8,26]. However, since the suspected

midge vectors are widely distributed between latitudes 40° N and 35° S, it is highly likely that EEV has a much wider distribution.

The exact status of EEV in the Western Cape Province is currently unknown although the virus has been isolated from animals within this region. In addition, vector surveillance studies have identified the presence of the suspected *Culicoides* vectors. Since no control strategies have been implemented to help eradicate or control EEV in the Western Cape, it is likely that these viruses are circulating freely. Although the quarantine restrictions, which are applied to AHSV, would equally apply to EEV, the continual presence of EEV in the AHS-free zone does increase the risk of exporting EEV from the Western Cape to countries free of the virus.

The EU requires horses to be tested for antibodies against EEV before export from South Africa, but the OIE nevertheless does not recognise EE as a disease of concern. Horses from AHSV infected areas are allowed into the United States of America provided they complete 60 days postarrival quarantine in the vector protected animal facility in New York. Furthermore, no control strategies are practised and there are no vaccines available. The possibility that this virus can spread to EEV-free countries cannot be excluded and needs further investigation. In addition, the consequences of importing this disease into EEV-free countries remain unknown and warrant further investigation.

The virus neutralisation test (VNT) used for the serotyping of EEVs is time consuming and requires each virus isolate to be tested with antisera against each individual virus serotype. In addition, the VNT is serotype-specific and will not identify any new EEV serotypes. Therefore, there is a need for rapid, reliable, sensitive, group-specific assays for detection of virus and antibody.

A group-specific, indirect sandwich enzyme linked immunosorbent assay (ELISA) has already been developed and the methods published for the detection of AHSV and viral antigen. This assay has been shown to be rapid, sensitive and specific for the identification of AHSV with experimental and field specimens[1,18], and with pools of processed insect vectors[17]. Similar assays have also been developed for bluetongue virus (BTV) and epizootic haemorrhagic disease of deer virus (EHDV)[31], both of which are members of the genus *Orbivirus*. Applying the same methodologies for EEV antigen detection would have distinct advantages particularly for the differential diagnosis between EEV and AHSV.

Competitive ELISAs have also been developed for the detection of antibody against AHS[16], bluetongue (BT)[2] and epizootic haemorrhagic disease of deer (EHD)[30,32] viruses. Each of these assays has been shown to be serogroup-specific and sensitive, providing a reliable assessment of the antibody status of an animal within three hours. Application of similar ELISA methodologies for the detection of EEV antibodies would offer significant advantages in terms of performance, rapidity, and standardisation of the various tests, especially when comparative tests for AHSV and EEV are required.

Objectives

The main objective of this project was to design, develop and validate two different ELISA assays for the rapid detection of EEV antigen and antibody and that are reliable and relatively simple to perform.

The assays were validated using panels of known positive and negative specimens, and thereafter specimens obtained from the field.

The ELISA assays for EEV that are described in this dissertation, were based on standardised methods that have already been accepted internationally by regulatory authorities and Ministries of Agriculture for use in the detection of viral antigen and antibody, for example AHSV, BTV and EHDV. Some of these assays are already listed in the OIE "Manual of Standards for Diagnostic tests and Vaccines"[3] as either designated or recommended tests for international trade.

Application

On completion of the validation, these assays will be used to:

1. Determine the geographic and host species distribution of EEV in Africa and elsewhere throughout the world
2. Study the epidemiology of EEV in vertebrate and invertebrate hosts
3. Rapidly differentiate between field isolates of EE and AHS viruses
4. Rapidly test sera from animals destined for international trade
5. Study EE virus/antibody interactions (multiple serotype infections) in equids.

Chapter 2

REVIEW OF THE LITERATURE

SECTION A: EQUINE ENCEPHALOSIS

Introduction

Equine encephalosis (EE) is an acute arthropod-borne viral infection that causes a mild or subclinical disease in horses. Contrary to the name, the most common clinical signs include inappetence, fever and congestion to mild icterus of the mucous membranes.

The virus was first isolated in March 1967 from a 13-year old Thoroughbred mare (Cascara) on a stud farm near Kimberley in the Northern Cape Province. The mare presented with signs of acute nervous system involvement and frenzy. Congestion and oedema were the only pathological changes observed in the brain. Although no other microscopic changes consistent with viral encephalitis were observed, the isolated agent was named equine encephalosis virus (EEV). The same virus was also isolated from horses that died during the same year in other parts of South Africa. These animals were reported to have shown similar clinical signs and lesions.

Results of a small serological survey carried out at that time suggested that EEV (Cascara) infection had been widespread during the first 3 months of 1967[9]. With the exception of the relatively large epidemics that occurred in the former Transvaal between

1976 and 1978 and in the Cape Province in 1990, only sporadic cases or limited outbreaks of EE were diagnosed in South Africa between 1967 and 1994[8].

Aetiology

EEV is classified in the genus *Orbivirus* of the family *Reoviridae*[12]. The morphology of EEV and the cytopathic effects it produces in cell cultures are usually indistinguishable from those of African horsesickness (AHSV). The virus may be isolated from tissue homogenates, washed lysed blood and homogenated insect suspensions by intracerebral inoculation of day-old mice or by inoculation of baby hamster kidney (BHK) cell cultures. The inoculated mice usually show signs of encephalitis on the first passage and die from the seventh day postinoculation (p.i.). However, on subsequent mouse brain passages the mice become sick and die as early as 48 hours p.i. The BHK cell cultures show a distinct cytopathic effect that results in an increased refractivity and shrinking of cells from the third day p.i. Initially these changes are seen as discrete foci but by the sixth day p.i. they progress to complete destruction of the cell monolayer[9]. EEV may also be propagated in *Aedes albopticus* C6/36 cell monolayers[33] and in African green monkey (VERO) cell monolayers[26].

The virions are 73 nm in diameter[22]. The virus genome comprises 10 linear segments of dsRNA. Each of these segments represents a single gene that codes for a different viral protein. The EEV dsRNA profiles are different from those of epizootic haemorrhagic disease of deer virus (EHDV) and bluetongue virus (BTV) but EEV and

AHSV dsRNA profiles are very similar. It is therefore necessary to distinguish between the two viruses by another method. The assembly of the viral proteins (VP) appears to be typical for orbiviruses with four major capsid proteins (VP2, VP3, VP5, and VP7) and at least three minor protein components (VP1, VP4 and VP6) that vary in size with relative molecular weights (Mr) of 36 000 to 120 000 Daltons.[34]. The virus particle is made up of three concentric layers of proteins (the outer capsid, comprising VP2 and VP5, the core, comprising VP3 and VP7 and the subcore, comprising VP3) that are regarded as two distinct capsid shells (outer capsid and core).

Early hybridisation studies using probes for genomic segments three and five of the Cascara serotype, suggested that EEV is more closely related to BTV and EHDV than to AHSV. However, since most of the probes used were not full-length DNA copies, the results were not necessarily a true reflection of the full genome[34].

To date, seven serotypes of EEV have been reported in the literature. The serotypes were originally named after either the horse or the location within South Africa from which the virus was isolated. The first EEV serotype isolated, Cascara, was obtained from the liver, spleen, brain and blood of a mare of that name, which was euthanased after showing severe nervous signs[9]. In 1971, the second serotype was isolated from the blood of a horse named Gamil that exhibited a febrile reaction, inappetence and slight depression. The horse subsequently recovered uneventfully[10].

The third EEV serotype, Kaalplaas, was named after a farm just north of the Onderstepoort Veterinary Institute. The virus was isolated in 1974 from the blood of a mare that developed a febrile reaction that lasted four days, severe conjunctivitis and swelling of the eyelids[10].

The fourth serotype, Bryanston, was named after a suburb situated north of Johannesburg. This virus was isolated in 1976 from the spleen, liver and lung of a horse that died acutely with signs of cardiac failure. The Bryanston strain of EEV was also isolated from the internal organs, but not the placenta, of three aborted foetuses at about six months gestation. Two of these foetuses were from the Colesberg district in the Northern Cape Province and the third was from Kimberley[10].

The fifth serotype, Kyalami, was isolated in 1974 but only identified positively as a new serotype of EEV in 1976. The original isolate was made from a horse from Onderstepoort that exhibited acute liver atrophy. The virus was named in 1976 when isolations of the same serotype were made from a number of horses in the Kyalami area that were febrile and showed mild icterus[26].

In 1991, five isolations of the sixth serotype, named Potchefstroom, were made from blood taken from febrile horses at a military base near Potchefstroom. Infected horses showed signs of depression inappetence and jaundice[11].

A seventh serotype, E21/20 was recovered from blood collected in 2000 from a 6-year old gelding at St Lucia, KwaZulu Natal. This horse had a high fever (41.6 °C) but no other clinical signs were reported[19].

All the available isolates of the Langeberg virus, originally reported as a strain of EEV[8], were shown to be AHSV type 5 by Howell *et al* (2001)[19].

In 1978, Erasmus *et al* reported cross-reactions between serotypes Cascara, Gamil, Kaalplaas and Bryanston in a complement fixation test (CFT) adapted from McIntosh (1956)[23]. These serotypes did not react with other orbiviruses such as BTV, AHSV, corriparta virus, palyam viruses (d'aguilar and abadina), warrego virus (CH 9935), Mitchell river virus (MRM 10434) or EHDV[9,10]. Later, Gerdes and Pieterse (1993) also used this CFT to aid in the identification of the Potchefstroom serotype[11].

In a recent study, Howell *et al* (2001)[19] confirmed the identity of the seven distinct serotypes by serum-virus cross-neutralisation tests incorporating all seven antigens and their homologous antisera. They also proposed that the serotypes be assigned a numerical identity with the original isolates as prototypes. New isolates should be identified with sequential numbers.

The proposed numerical identities of all the named serotypes are represented in Table 2.1 and will be used throughout this dissertation when referring to the various serotypes.

Table 2.1 Serotype numbers that were assigned to each of the prototype EEV isolates in alphabetical order.

Prototype virus name	Serotype no.
Bryanston	1
Cascara	2
Gamil	3
Kaalplaas	4
Kyalami	5
Potchefstroom	6
E21/20	7

Epidemiology

Several serological surveys have been carried out in recent years. Although often limited in the numbers tested, the results do suggest that EEV is endemic in equids in most parts of South Africa, in Botswana, Namibia, Zimbabwe and in Kenya [5,8,26]. Horses of all age groups and of both sexes appear to be susceptible to infection.

Virus neutralising antibodies against EEV type 1 and EEV type 5 have been found in the sera of zebra *(Equus burchelli)* from Namibia[8]. Group-specific, indirect, enzyme-linked immunosorbent assay (ELISA) antibodies against EEV have also been reported in sera from zebra in the Kruger National Park (KNP)[6]. In a later survey, in which sera from twenty-four species of southern African wildlife were tested, group-specific antibodies against EEV were found in zebra (28/117) and elephant (4/49)[5].

Williams *et al* (1993)[35] used an ELISA to test a small number of South African horse, donkey and zebra sera. The donkey sera were collected in the former Eastern Transvaal and the zebra sera in the KNP. Antibodies against both AHSV and EEV were detected in horse (1/12) and zebra (2/9) sera. Most of the donkey sera (10/13) were also positive for antibodies to both of the viruses.

In another survey, 74% of horse sera (449/604) tested in South Africa between 1994 and 1997 were positive for antibodies to EEV[26]. Similarly, 85% (164/193) of the donkey sera received from Zimbabwe in 1995 tested positive for antibodies to EEV[26].

Virus isolates from *Culicoides* biting midges collected during the summer months of 1969 were reported to have cross-reacted with the EE virus group[29] in a CFT adapted from McIntosh (1956)[23]. Isolates of EEV were also reported from *Culicoides imicola* between January 1979 and May 1985[25]. None of these isolates were serotyped. Experimental studies have shown that *C. imicola* can become infected, and will support the replication of EEV type 1 following *in vitro* feeding on virus-infected blood[33]. Infections of EEV, like AHSV and BTV, usually occur in late summer and autumn in years when the climatic conditions favour an abundance of *Culicoides*.

The epidemiology of EEV is poorly understood. However, because AHSV and EEV belong to the same virus genus and are believed to circulate between the same vertebrate and invertebrate hosts, it is possible that much of the epidemiology of AHSV and EEV is similar. Obviously there may be significant differences that will need to be addressed,

for example: a) the duration and level of the viraemia in different equid species – this will influence the transmission of the virus to the vectors; b) the absence of any EEV vaccine and the consequential spread of the uncontrolled virus – there is no interference of vaccine-induced antibodies on the geographic distribution of seropositive animals; c) possible vertebrate and invertebrate host differences – unidentified hosts and vectors.

Pathogenesis

Very little is known about the pathogenesis of EE. Experimental infection of horses usually produces a febrile reaction, with or without depression, but no serious illness. Although all isolations of the original serotypes were made from clinically sick horses or from fatal cases, it appears difficult to fulfil Koch's postulates. In fact, fatal disease has only been reproduced successfully on one occasion[10]. In this case study, a horse was inoculated intravenously with the original EEV type 2 (Cascara) that had been passaged once in suckling mice and twice in BHK cells. The horse developed a febrile reaction that lasted from the second to the seventh day pi. Apart from slight inappetence, no clinical signs were evident. However, during the afternoon of the tenth day the animal became hyperexcitable and reacted violently. After about 30 minutes, it was completely comatose and died four hours later. The necropsy findings closely resembled those observed in field cases of the disease from which EEV type 2 had been isolated. Virus could be isolated from the blood from the first day after inoculation until the day of death, and from most of the organs and lymph nodes[10]. Further studies on the

pathogenesis of EE are therefore required to determine the duration and level of viraemia as well as the tissue tropism of the virus.

Clinical signs

Published and field reports of the clinical signs associated with EEV infections are inconclusive. Coetzer *et al* (1994)[8] have described clinical signs that ranged from a mild febrile disease to abortion and death with few preceding signs. Most affected horses showed either a slightly elevated rectal temperature (39 °C) that persisted for one or two days or a high fever (40 to 41 °C) with increased respiration and pulse rates that lasted for between one and five days.

Other clinical signs that have been observed less frequently included varying degrees of swelling of the eyelids, the supraorbital fossa and even the entire face. Signs of central nervous system involvement such as mild to severe ataxia, reluctance to walk and stiffness, a wild expression in the eyes, and changes in temperament and/or convulsions have also been attributed to EEV infections. Respiratory distress sometimes accompanied by a frothy, clear or slightly blood tinged nasal discharge, petechiae in the conjunctiva and signs of acute heart failure have also been reported. Many of these observations were cited by Erasmus as personal communication[8].

In a retrospective study of 59 EEV isolations made since 1983, it was suggested by Paweska *et al* (1998)[26] that the disease could be divided into four "groups" according to the clinical signs. These were abortion, acute death, AHS-like syndrome and febrile

cases. These authors also suggested that EEV should not be implicated as a significant cause of nervous system disease in the horse.

A recent serological survey of 518 horse sera from well-managed stud farms in South Africa showed that 56.9% of the sera were positive for neutralizing antibodies to EEV (Howell *et al* 2001)[19]. These authors concluded that most EEV infections were either mild or subclinical and that if clinical signs were evident they would closely resemble what has been described by Theiler (1921)[28] as AHS fever.

Pathology

The lesions described in the first ever reported case of EE (the mare Cascara, 1967, Kimberley) included venous congestion, particularly of the liver, kidneys and sub-cutaneous tissues. The liver also showed signs of fatty degeneration. Sharply demarcated areas of catarrhal enteritis were observed in the distal half of the small intestine. The brain was congested and oedematous with an excessive amount of cerebrospinal fluid[9].

Other lesions that have been associated with fatal cases of EE include, varying degrees of lung oedema and hydropericardium, slight hepatomegaly and splenomegaly, hyperaemia of the glandular part of the stomach and petechiae in serosal surfaces (particularly of the small intestine)[8].

The pathological changes reported in cases with liver involvement include a diffuse cloudy swelling and hydropic degeneration of hepatocytes, which is evident microscopically and mild to moderate infiltration predominantly of lymphocytes in the portal triads. Reported cases with nervous involvement have exhibited congestion, oedema and perivascular lymphocytic infiltration in the periventricular areas of the brain. Extensive fibrosis of the myocardium has also been described in horses that died of acute heart failure[8]. No descriptions of foetal lesions are available.

Because of the high prevalence of infection and the apparent subclinical nature of this disease, it is possible that some virus isolations made from sick animals were, and could be purely coincidental.

Diagnosis

Virus detection

The OIE Manual of Standards for Diagnostic Tests and Vaccines identifies several tests for the detection of AHSV and viral antigen[3]. These include the isolation of virus in cell culture and by intracerebral inoculation of day-old mice, ELISA and the polymerase chain reaction (PCR)[27]. No procedures or assay methods are described in the manual for the isolation and identification of EEV.

Currently, only laboratories from the Onderstepoort Veterinary Institute (OVI), South Africa, the Department of Veterinary Tropical Diseases (DVTD) in the Faculty of Veterinary Science, University of Pretoria, South Africa and the Institute for Animal

Health (IAH), Pirbright, United Kingdom (UK) routinely attempt the isolation of EEV. These laboratories are using conventional methods that are used for AHSV isolations, namely cell cultures and intracerebral inoculation of day-old mice. Currently EEV identification is only being carried out in southern Africa. Isolated viruses are subsequently identified using a group-specific CFT. Thereafter, the virus serotype is determined using the serum-virus neutralisation test (VNT)[9]. The VNT method used for serotyping EEV is time consuming and requires each virus isolate to be tested with antisera produced in sheep against each of the individual virus serotypes. This test will however, not identify new or emerging EEV serotypes.

Group-specific genomic probes for the detection of EEV have been described[34] but there are no reports of this technology having been implemented in diagnostic laboratories.

The indirect sandwich ELISA (S-ELISA) described for AHSV[18] has been successfully applied to other orbiviruses including BTV and EHDV[31]. However, this methodology has not yet been applied to the detection of EEV and viral antigen.

Antibody detection

The CFT and ELISA are both recommended by the OIE as approved tests for the detection of serogroup specific antibodies to AHSV[27]. Agar gel immunodiffusion (AGID), CFT, ELISA and VNT are routinely used for the detection and identification of antibodies to AHSV at the World Reference Centre for AHS, OVI[35], in Regional

Reference laboratories around the world and at the DVTD. Similar tests are routinely used for the detection and identification of antibodies against EEV.

Although the agar gel diffusion test has been used successfully for many years for the detection of AHS, BT and EHD virus antibodies, the test is now considered not sensitive enough for international trade. In addition, there are some doubts about the specificity of the test.

The CFT has been used for many years but it is well known that the interpretation of CFT results from different laboratories are subject to variation. The assay primarily detects IgM and is therefore ideal for the detection of a primary antibody response. However, the anti-complementary effects of some animal sera can affect the assay. This is of particular relevance in zebra, mules and donkeys where up to 80% of sera may be anti-complementary.

A group-specific indirect ELISA has been described for the detection of EEV antibody[35]. This assay measures antibodies directed against a concentrated, cell-extracted EEV antigen, and the detecting serum is an anti-horse conjugate. It should be noted that the assay can only be used with equine sera and that there may be a difference in the efficiency of the conjugate to detect donkey, mule and zebra sera.

A competitive ELISA (C-ELISA) for the detection of AHSV antibody has been developed and standardised by Hamblin *et al* [15,16]. This assay has been shown to be

rapid, sensitive and specific and has been implemented internationally in a number of diagnostic and reference laboratories.

Anti-complementary sera do not affect the ELISA and because the standardised assay measures inhibition of guinea pig antisera, the test can be used to detect antibodies in any species except guinea pig and related species.

SECTION B: ENZYME IMMUNOASSAYS

Introduction

Enzyme immunoassays (EIA) directed against viral agents have contributed significantly to the study of infectious diseases, and assisted with the safe movement of animals and animal products for international trade. EIAs have provided highly sensitive and specific assays for the estimation of biological parameters. They can be used to measure antibody responses, to identify causative agents and to study the epidemiology of disease. These assay systems are versatile and rapid with the added advantage that they can be used to test large numbers of specimens.

Principles of EIA

EIAs operate on the principle that one of the test reagents is conjugated to an enzyme. The subsequent addition of the appropriate substrate and chromogen results in a colour change that can be detected both visually and spectrophotometrically.

The fact that carbohydrates and proteins can be passively adsorbed to plastic has led to the development of the enzyme linked immunosorbent assay (ELISA). These assay systems usually utilise plastic 96-well microplates as the solid phase or support for the reactions.

There are many variations in the methodologies and in the names given to the type of ELISA used. Since ELISA methods have already been designed and developed for orbiviruses, including AHS, BT and EHD viruses, it is appropriate that similar

methodologies are adopted for the design and development of ELISAs for the detection of EEV antigen and antibody. This will benefit the user in terms of standardisation and ease of performance and will facilitate more reliable and comparative testing. Therefore, only the indirect sandwich- and the competitive ELISA will be discussed here.

Indirect sandwich ELISA (S-ELISA)

This assay can be used for the detection of specific antigen. A constant amount of specific antibody (capture antibody) is passively adsorbed to the solid phase of an ELISA plate. After incubation, unbound antibody is removed by washing. Suspect specimens are diluted and added to the plate in a blocking buffer designed to prevent non-specific binding of proteins to any available sites remaining on the plate. Plates are again incubated and washed to remove unbound antigen.

A constant amount of a second specific antibody, produced in a different vertebrate species and diluted in blocking buffer, is then added. After incubation and washing, an enzyme-labelled species-specific antiserum (conjugate) directed against the antibody from the second species is added. Colour develops after the addition of chromogen and substrate. This reaction is stopped and absorbance is measured spectrophotometrically. A colour change indicates a positive antigen reaction while no colour change indicates a negative antigen reaction (Figure 2.1 a and b).

Competitive ELISA (C-ELISA)

This assay can be used for the detection of group specific antibody in serum samples. Antigen is passively adsorbed onto the solid phase. The plates are incubated and

unbound antigen is then washed away. The test sera and a constant amount of specific antibody (control antibody) are added simultaneously to the antigen-coated plates. The presence and concentration of specific competing antibody in the test serum will influence the binding of the control antibody to the antigen.

After incubation and washing, an enzyme-conjugated antiserum directed against the control antibody is added. Unreacted conjugate is removed after incubation by washing. Colour develops after the addition of chromogen and substrate. This reaction is stopped and the absorbance is measured spectrophotometrically.

The control antibody must be from a different species to the test specimens otherwise the conjugate will react with both. If the test serum contains antibody it will compete with the control antibody. This will result in a decrease in colour when compared to the control wells in the absence of competitor. If the test serum does not contain specific antibody then there will be no competition when compared with the control wells in the absence of competitor (Figure 2.2 a and b).

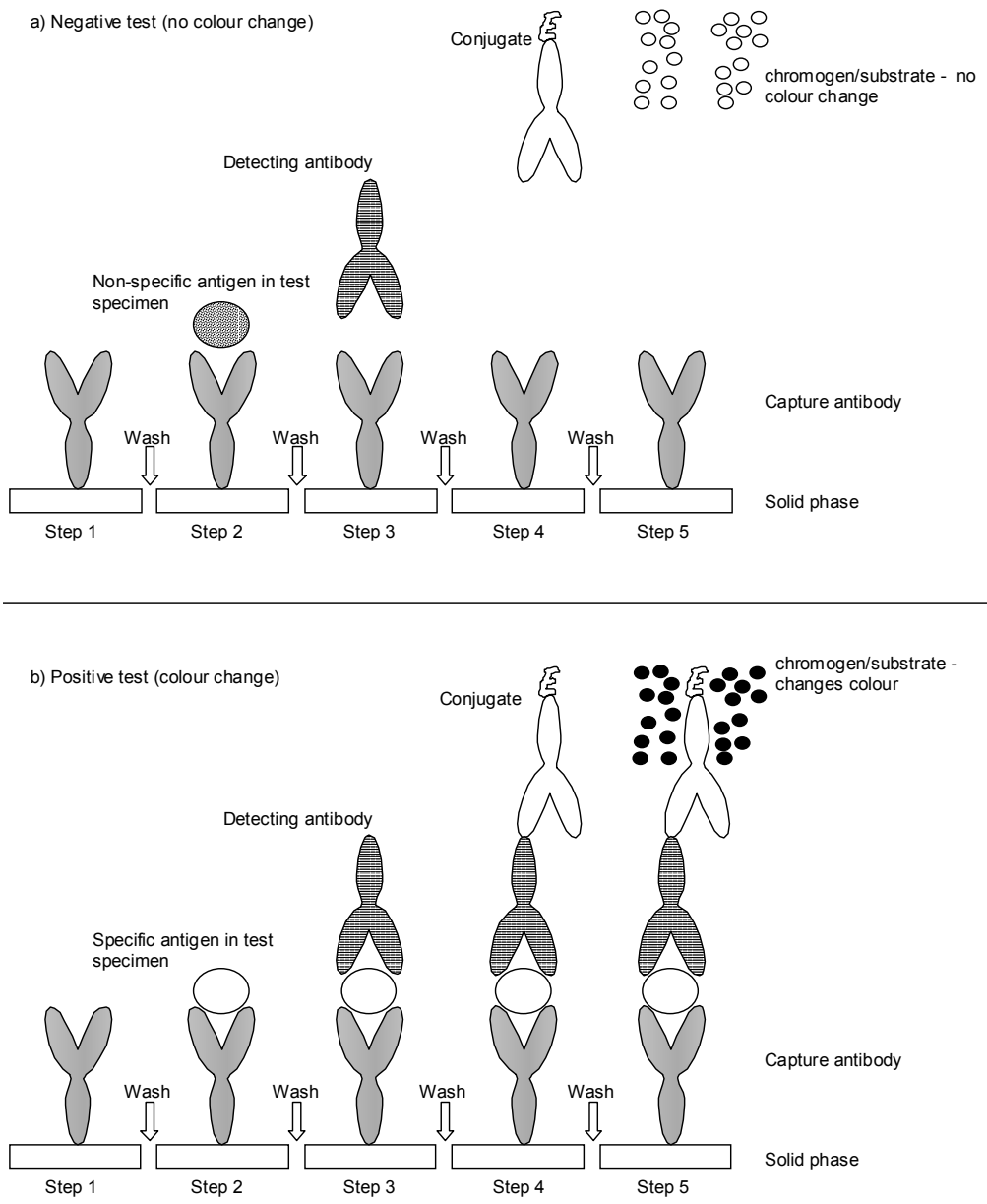

Figure 2.1 Diagrammatic representation of the indirect S-ELISA indicating the various steps and reagents. a) Negative test where there is no specific antigen in the test specimen to bind to the capture antibody, showing no colour development. b) Positive test with specific antigen in test specimen bound to the capture antibody and the subsequent development of colour.

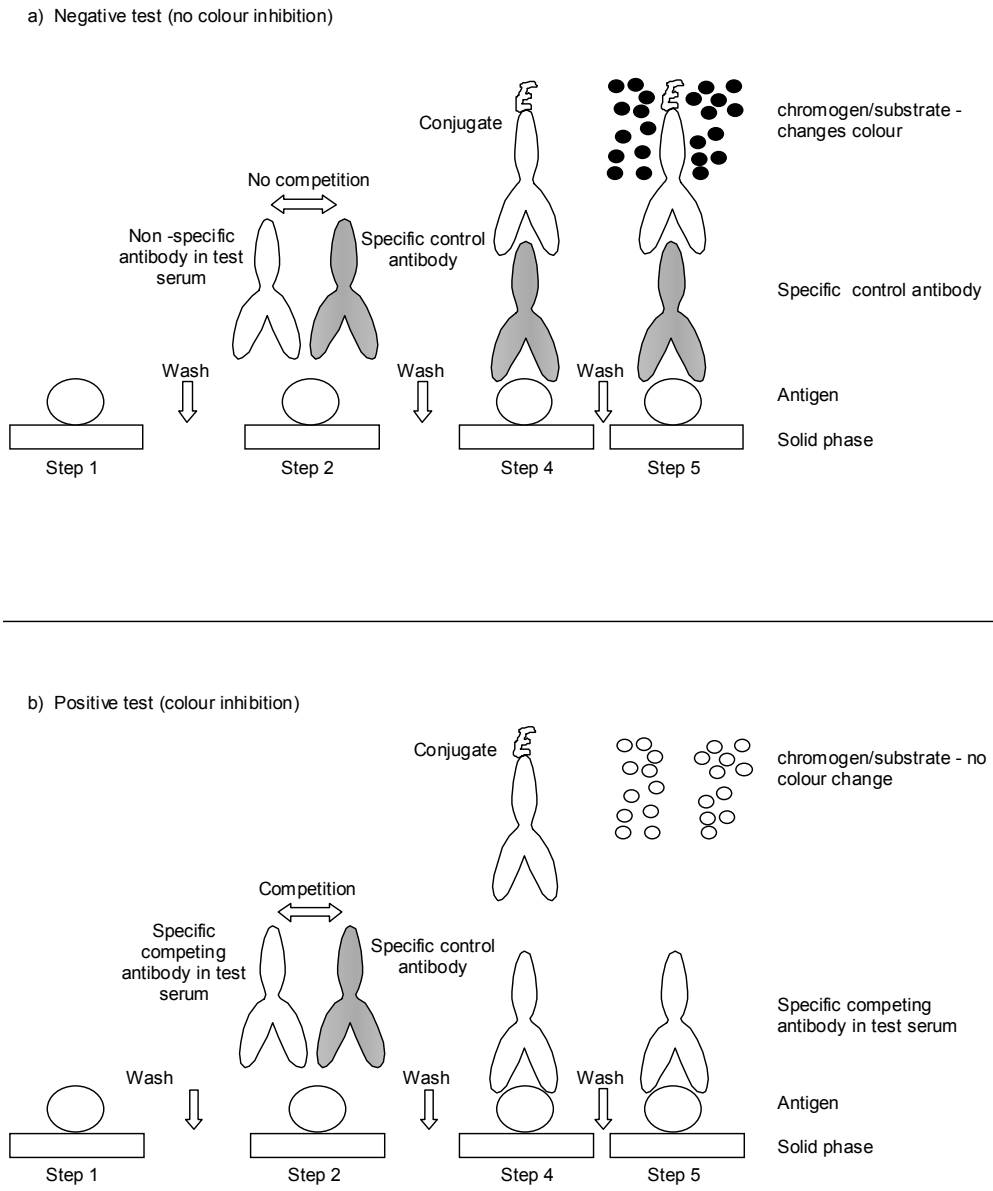

Figure 2.2 Diagrammatic representation of the indirect antibody C-ELISA indicating the various steps and reagents. a) Negative test with no competition between antibody from the test serum and antibody from specific guinea pig antiserum, showing the subsequent development of colour. b) Positive test with competition between specific antibody from the test serum and antibody from specific guinea pig antiserum, showing no colour development.

Validation of assays

Validation is the evaluation of a process to determine its fitness for a particular use. A validated assay yields test results that identify the presence of a particular analyte (e.g. an antibody or antigen) and allows predictions to be made about the status of the test subject[3].

Development and validation of an assay is an incremental process consisting of two principal parts. The first part is to establish parameters and characteristics of the assay through the following methods: a) determination of the feasibility of the method, b) development of the assay through choice, optimisation and standardisation of reagents and protocols and c) determination of the performance characteristics of the assay.

The second part is necessary to assure constant validity of test results and requires the following two processes: a) continuous monitoring of assay performance to assure that the status of the validated assay is merited and b) maintenance and enhancement of validation criteria during routine use of the assay[20].

Various procedures on the validation of serological assays[20] and in particular relating to expression of ELISA data, primary reference standards, quality assurance and diagnostic validation have all been published[36].

Chapter 3

A GROUP-SPECIFIC, INDIRECT SANDWICH ELISA FOR THE DETECTION OF EQUINE ENCEPHALOSIS VIRUS ANTIGEN

Abstract

A polyclonal antibody-based, group-specific, indirect, sandwich ELISA (S-ELISA) for the detection of equine encephalosis virus (EEV) antigen was developed. Purified EEV particles were titrated in the S-ELISA and the limit of detection was approximately 9.0ng of antigen per millilitre (0.45 ng/well). Positive S-ELISA reactions were recorded with seven serologically distinct EEV serotypes. No cross-reactions were recorded with other arboviruses including: African horsesickness serotypes 1 to 9, bluetongue serotypes 1 to 24, epizootic haemorrhagic disease of deer serotypes 1 to 8 and isolate 318, and selected isolates of palyam, eubenangee, corriparta, warrego, akabane and bovine ephemeral fever viruses. The assay proved to be sensitive and specific for the rapid detection of EEV in cell cultures and in homogenated baby mouse brain. The data generated in this study suggests that the ELISA will be valuable for epidemiological studies of EEV and will assist in making a reliable differential diagnosis for AHSV.

Introduction

Group-specific, indirect S-ELISAs have already been developed for the detection and identification of African horsesickness virus (AHSV)[18], bluetongue virus (BTV) and

epizootic haemorrhagic disease of deer virus (EHDV)[31]. These assays are highly sensitive and provide a rapid and reliable confirmation of specific virus antigen within four hours. The same approach was followed in developing an ELISA for the detection of equine encephalosis virus (EEV) antigen. This chapter describes the development and validation of a polyclonal antibody-based, serogroup-specific, indirect, sandwich ELISA (S-ELISA) for the detection of EEV antigen.

Materials and methods

Virus propagation in cell culture for detection in the S-ELISA

Prototype strains of EEV representing six serotypes (type 1 to 6) were selected for this investigation. The Langeberg virus considered to be a strain of EEV[8] was not included, as preliminary unpublished data had indicated a discrepancy in the identification of the original isolate. Stock cultures of the first six identified serotypes of EEV were obtained from the Department of Veterinary Tropical Diseases (DVTD), University of Pretoria, South Africa. The seventh serotype of EEV was only received towards the end of the study and was therefore only included in determining the diagnostic specificity (DSp) of the EEV S-ELISA. The identities of these isolates were confirmed using western blot and serum-virus cross neutralisation techniques as described by Howell *et al* (2001)[19]. Several other arboviruses (Table 3.1) were obtained from the Institute for Animal Health (IAH), Pirbright, United Kingdom (UK).

Table 3.1 Arboviruses that were used to evaluate the diagnostic specificity of the EEV S-ELISA.

Virus	Serotype/strain
Equine encephalosis	1 to 7
African horsesickness	1 to 9
Bluetongue	1 to 24
Epizootic haemorrhagic disease of deer	1 to 8, Ibaraki, isolate 318
Palyam	SU3843, SU48, GP18, D'Aguilar
Eubenangee	Pata, AB1327, Tilligery, Eubenangee
Akabane	Akabane
Warrego	Mitchell River MRM10434, CH9935
Bovine ephemeral fever	Bovine ephemeral fever

All viruses were propagated in baby hamster kidney cells clone 21 (BHK-21). Monolayer cell cultures of BHK-21 were prepared in plastic tissue culture flasks (Falcon, UK Ltd). The cells were grown in BHK-21 medium (GLASGOW MEM, Invitrogen, Gibco BRL, Life Technologies, Paisley, Scotland) supplemented with 5% foetal calf serum (FCS) (Invitrogen) and incubated at 37 °C in a 5% CO_2 atmosphere.

A 175 cm^2 flask supporting a confluent monolayer culture of BHK-21 cells was washed with 25 mℓ of calcium and magnesium free phosphate buffered saline (PBS minus). The flask was flooded with 20 mℓ of 0.25% Trypsin-EDTA solution (Sigma) and left horizontally on the bench at ambient temperature (20 to 26 °C) for 30 minutes. Trypsinised cells were re-suspended by shaking the flask briskly. The cell suspension was decanted into a conical-bottomed, plastic universal bottle containing 5.0 mℓ of FCS and centrifuged at 225 × g for 10 minutes. The cell pellet was re-suspended in BHK-21

medium containing 5% FCS to give a final cell concentration of approximately 2×10^5 cells/mℓ. New 175 cm^2 flasks were seeded with 50 mℓ of the re-suspended cells. Flasks were incubated for three days at 37 °C.

When confluent monolayers had developed, the growth media was discarded and replaced with 2.0 mℓ of one of the respective viruses diluted 1/10 in phosphate buffered saline (PBS). Virus was adsorbed at ambient temperature (20 to 26 °C) for 20 minutes. Twenty-five millilitre of BHK-21 medium supplemented with 1% FCS was subsequently added to each infected flask and incubated at 37 °C. Flasks were examined microscopically and infectious supernatant fluids collected when the cells showed 90 to 100% cytopathic effect (CPE). Each virus was aliquoted and dispensed into cryotubes and stored at minus 70 °C until required. The presence of viral antigen was confirmed for each of the orbivirus preparations using group-specific ELISAs for AHSV,[18] BTV and EHDV[31] before testing them in the EEV S-ELISA.

Virus propagation in embryonating chicken eggs for detection in the S-ELISA

Six groups of three, ten-day old embryonating chicken eggs were each inoculated intravenously with 0.1 mℓ of EEV, types 1 to 6 respectively, diluted 1/10 in BHK-21 medium. The eggs were examined regularly for seven days using a candling lamp. Embryos that died within 24 hours were discarded. Chick embryos that died between days two and seven were collected and stored at 4 °C. Embryo hearts were removed and homogenised separately using a mortar and pestle with sterile sand and 1.0 mℓ of BHK-

21 medium. Each virus type was passaged for a second time in embryonating chicken eggs. Embryo hearts from the first eggs were also passaged in BHK-21 cells.

Virus propagation in mouse brain for detection in the S-ELISA

Six groups of five, one to two day-old mice were each inoculated intracerebrally with 0.03 mℓ of EEV types 1 to 6 respectively, diluted 1/10 in BHK-21 medium. The mice were examined daily for seven days. Mice that died within 24 hours were discarded. The heads of sick and dead mice were collected between days two and seven and stored at 4ºC until processed. Brain tissue from each mouse was removed and resuspended separately in 1.0 mℓ amounts of BHK-21 medium. Mouse brain suspensions were then homogenised by sonication for 30 seconds at an amplitude of 18 micron using a probe sonicator.

Negative test specimens used to establish the baseline for a negative population in the S-ELISA

Brain specimens from 100 uninfected day-old mice were collected and prepared as previously described. One hundred flasks with BHK-21 cell cultures that served as negative controls were collected and stored at minus 20 °C. Forty-two spleen specimens collected from horses in Spain during an outbreak of African horsesickness (1987 to 1990) together with 100 spleen specimens from horses resident in the UK collected at an abattoir (1999) were used. Horse spleens were ground in a mortar as previously described for chicken embryo hearts (10% w/v in PBS).

Purification of EEV serotype 1 for production of capture antibody

Fifty 850cm² roller bottle, monolayer cell cultures of BHK-21 cells were infected with 2.0 mℓ of diluted EEV type 1 as described earlier. Each roller bottle was overlayed with 100 ml of maintenance medium. Infected cells were harvested between 48 and 72 hours, when showing 100% CPE, and then centrifuged at 800 × g for 30 minutes. Virus was recovered from both the infected cells and the infectious supernatant fluids exactly as described for BTV by Mertens *et al* (1987)[24] and later AHSV by Burroughs *et al* (1994)[7].

A cytoplasmic extract was prepared from the cell pellet by extracting the nuclear material through homogenisation and centrifugation. The cellular pellet was resuspended in TNET buffer (50 mM Tris/HCL, pH 8.0, 0.2 M NaCl, 5 mM EDTA, 0.5% Triton X-100) and homogenised in a glass homogeniser (10 strokes). The nuclei were separated from the cell homogenate by centrifugation at 800 × g for 10 minutes. The pellet was resuspended in the same buffer and centrifuged for a second time. Both the supernatants, representing the cytoplasmic extract, were collected and kept on ice.

Virus was recovered from the tissue culture supernatant through ammonium sulphate precipitation. The supernatant was mixed with an equal volume of saturated ammonium sulphate solution in 0.04 M sodium phosphate buffer, pH 7.6 and centrifuged at 3 000 × g for 1 hour at 4 °C. The pellet was resuspended in a small volume of TNET buffer.

The recovered virus and cytoplasmic extract was pooled and concentrated by one cycle of centrifugation (85 000 × g for 3 hours at 4 °C in 38ml polyacrilamide tubes) on a

discontinuous sucrose gradient (6 mℓ.66% w/w sucrose, 10 mℓ of 40% w/v sucrose in 0.2 M Tris/HCL, pH 8.0, 24 mℓ of sample). For further purification, the method described for BTV by Mertens *et al* (1987)[24] was adapted.

The discs from the interface of the sucrose solutions (resuspended in 0.2 M Tris, pH 8.0) were made 1% with 3-(dimethyldodecylammonio)propanesulphonate (Fluka) and 40 µg/mℓ chymotrypsin (TLCK treated, Sigma) and held at 37 °C for 2.25 hours. Dithiothreitol was then added to a final concentration of 20 mM and again incubated at 37 °C for 2.25 hours.

The product was then centrifuged at 850 × g for 15 minutes, giving a large pellet and a clear supernatant. The supernatant was centrifuged on discontinuous CsCl gradients (2.5 mℓ 44% CsCl, R.I. 1.3792, 2.5 mℓ 34% CsCl, R.I. 1.3657 and 1mℓ 40% w/v sucrose) in Beckman SW 40 tubes at 140 000 × g for 1.5 hours at 4 °C. Three bands were obtained: 1) white aggregated material at the top of the CsCl; 2) a blue band of infectious sub-viral particles (ISVP) half way down the gradient; 3) a less defined grey band of lower density with a profile between ISVP and empty core particles. The blue and grey bands were recovered and dialysed separately, twice against 2.0 ℓ changes of 0.1 M Tris/HCl, pH 8.0. The material from bands two and three were used for inoculation of rabbits and guinea pigs.

Production of antisera (capture antibody and viral detection antibody)

Vaccine emulsions were prepared by mixing equal volumes of purified EEV antigen with Freund's complete adjuvant (FCA) and Freund's incomplete adjuvant (FICA). Each of two rabbits was inoculated subcutaneously, into four sites, with 0.25 mℓ/site of emulsified ISVP antigen at a concentration of 30 µg/mℓ in FCA. The vaccination was repeated with two more rabbits using core antigen. After 28 days, the rabbits received a second subcutaneous inoculation of the vaccine emulsion in FICA. Rabbits were exsanguinated 10 days after the second inoculation and the serum collected.

Five guinea pigs were each inoculated once, subcutaneously, into four sites, with 0.25 mℓ/site of emulsified core antigen at a concentration of 15 µg/mℓ in FCA. Another five guinea pigs were vaccinated using ISVP antigen instead. Guinea pigs were exsanguinated after 28 days and the serum collected.

Evaluation of guinea pig antisera for use as "detecting antibody" in the S-ELISA

The reactivity of each guinea pig antiserum was evaluated by titration against a constant amount of viral antigen. Briefly, polystyrene 96-well "MaxiSorb", flat-bottomed, ELISA plates (Nunc, UK) were coated with 50 µℓ/well of rabbit anti-ISVP antiserum diluted 1/6 000 in 0.05 M carbonate-bicarbonate buffer, pH 9.6 (Sigma) (coating buffer). The plates were incubated overnight at 4 °C. Unbound reagent was removed after incubation by emptying and flooding the wells three times with PBS and then blotting on adsorbent paper (washing).

Fifty microlitres of EEV type 1 stock virus diluted 1/5 in PBS supplemented with 0.05% (v/v) Tween 20 (BDH), (PBST) was added to all the wells on the pre-coated plates. The plates were incubated at 37 ºC for one hour on an orbital shaker (Luckhams, Rotatest) and washed as before.

Each guinea pig antiserum was titrated (1/400 to 1/51 200) with PBST supplemented with 5% (w/v) skimmed milk powder (Cadbury's Marvel) (blocking buffer), down eight rows of the plates. The plates were incubated and washed as before and 50 µℓ/well of rabbit anti-guinea pig immunoglobulin conjugated to horseradish peroxidase (HRPO-conjugate) (Dako, Denmark) diluted 1/1 000 in blocking buffer (based on previous experience with the AHSV S-ELISA) was added to all the wells. After incubation and washing, 50 µℓ/well of ortho-phenylenediamine (chromogen) (Sigma) at a concentration of 0.4 mg/mℓ, and containing 0.05% H_2O_2 (30% v/v) (substrate), was added to each well. Colour was developed at ambient temperature and was stopped after 10 minutes by the addition of an equal volume of 1.0 M H_2SO_4 (stopping solution). The plates were read spectrophotometrically at 492 nm.

Checkerboard titration of rabbit- and guinea pig antisera for use in the S-ELISA.

Each of the rabbit antisera was evaluated in a checkerboard titration using a constant amount of viral antigen and guinea pig antiserum. A two-fold dilution series (1/400 to 1/819 200) of each rabbit antiserum was prepared in coating buffer, in eight replicate wells (50 µℓ/well) across the 12 columns of an ELISA plate. The plates were incubated at 4 °C overnight. After washing, 50 µℓ/well of an excess of EEV type 1 stock virus

(1/5) in PBST was added to all the wells. The plates were incubated at 37 ºC for one hour on an orbital shaker and then washed as before. A two-fold dilution series (1/400 to 1/51 200) of guinea antiserum was prepared in blocking buffer in 12 replicate wells (50 µℓ/well) down the eight rows of each plate.

After incubation and washing, 50 µℓ/well of HRPO-conjugate diluted 1/1 000 in blocking buffer was added. After a further incubation and washing step, 50 µℓ/well of chromogen/substrate were added as before. The reaction was stopped after 10 minutes by the addition of an equal volume of 1.0 M H_2SO_4. The plates were read spectrophotometrically at 492 nm.

Rabbit and guinea pig antisera that recorded similar reactions in the ELISA for each of the inoculated antigens were pooled separately. Each batch of pooled antisera was diluted 1 in 10 with PBS, dispensed into 1.0 mℓ aliquots, freeze-dried and then stored at minus 20 °C. Before use, each vial of freeze-dried reagent was reconstituted in 1.0 mℓ of sterile distilled water.

Titration of conjugate for use in the S-ELISA

Fifty microlitres per well of optimally diluted hyperimmune rabbit antisera (in coating buffer) was passively adsorbed onto the solid phase of an ELISA plate and incubated overnight at 4 ºC. The plate was washed and blotted dry. A quadruplicate two-fold dilution series (50 µℓ/well) of an EEV positive control antigen and a negative control antigen was prepared in PBST across columns one to 11 (1/1 to 1/1 024) of the plate.

The positive control antigen dilutions were made in rows A, C, E and G and the negative control antigen dilutions were made in rows B, D, F and H. Column 12, the blank control wells, received 50 µℓ/well of PBST alone. The plate was incubated at 37ºC for one hour on an orbital shaker.

After washing, 50 µℓ of optimally diluted immune guinea-pig antisera was added to each well. The plate was incubated at 37 ºC for one hour on an orbital shaker. After washing 50 µℓ per well of HRPO-conjugate diluted in blocking buffer (1/1 000 to rows A and B; 1/2 000 to rows C and D; 1/4 000 to rows E and F; and 1/8 000 to rows G and H) was added. The plate was incubated at 37 ºC for one hour on an orbital shaker and then washed. Fifty microlitres of chromogen/substrate was added to each well. The reaction was stopped after 10 minutes by the addition of an equal volume of 1.0 M H_2SO_4. The plate was read spectrophotometrically at 492 nm. The mean absorbance value obtained for the blank control wells was subtracted from the corresponding test wells for each HRPO-conjugate dilution.

Sandwich ELISA (S-ELISA)

MaxiSorb, flat-bottomed, ELISA plates were used as the solid phase for all assays. Volumes of 50 µℓ/well were used throughout the test for all reagents. After the addition of reagents, plates were covered and incubated at 37 °C for 1 hour on an orbital shaker. Unbound reagent was removed after each incubation step by emptying and flooding the wells three times with PBS and then blotting on adsorbent paper.

Hyperimmune rabbit antiserum, optimally diluted in coating buffer, was passively adsorbed on ELISA plates overnight at 4 °C.

The plates were washed and a two-fold dilution series of each test sample from undiluted to 1/128 was prepared in PBST across eight wells of single columns, numbers 1 to 10. A similar two-fold dilution series of the positive (undiluted to 1/128) and negative (undiluted to 1/32) antigen controls were prepared across columns 11 and 12, respectively. Wells A12 and B12 were designated blank wells and contained blocking buffer alone (Figure 3.1).

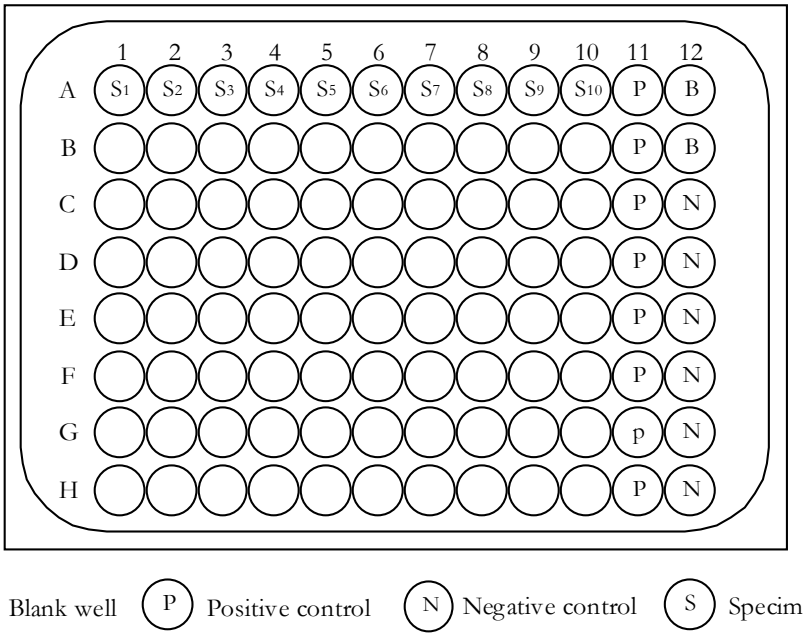

Figure 3.1 Diagrammatic representation of a 96-well microtitre plate indicating the organisation of the wells in the S-ELISA.

Plates were incubated and washed, and specific guinea pig antiserum optimally diluted in blocking buffer was added to each well. After incubation and washing, rabbit anti-guinea pig immunoglobulin HRPO-conjugate, optimally diluted in blocking buffer, was added to all the wells. After a further incubation and washing, chromogen/substrate was added to each well. The reaction was stopped after 10 minutes by addition of 1.0 M H_2SO_4. The plates were read spectrophotometrically at a wavelength of 492 nm using an ELISA reader.

Selection of plates

Various ELISA plates were evaluated for day-to-day variation. A 1/5 dilution from stock virus of each of the individual EEV serotypes was tested on four consecutive days with the S-ELISA, using three different types of plates: 1) PVC Microtitre® "U" Bottom Plates (Dynex Technologies - catalogue number 2101), 2) Dynex plates from the same catalogue number but from a previous mould, 3) Polystyrene MaxiSorb Nunc-immuno flat bottom plates (Nalge Nunc International - batch number 049001).

Analytical sensitivity and specificity of the S-ELISA

The infectivity of the purified EEV type 1 ISVP was determined by titration, using eight replicate wells per dilution, on established monolayers of BHK-21 cells. The plates were examined daily for five days for the development of CPE. Virus titres were calculated according to the method of Kärber (1931)[21]. The analytical sensitivity of the S-ELISA was determined by an endpoint titration of the purified ISVP. The analytical specificity

of the assay was analysed by testing the EEV prototype strains and comparing their absorbance values to those obtained with 55 other arboviruses (Table 3.1).

Repeatability

The plate-to-plate variation within a single S-ELISA test was determined by titration of EEV type 1 stock virus in 10 separate MaxiSorb Nunc ELISA plates.

Results

Evaluation of guinea pig antisera

The serum of each vaccinated guinea pig was evaluated separately. There was an expected degree of variation in the reactivity of the serum from individual guinea pigs within the vaccinated groups.

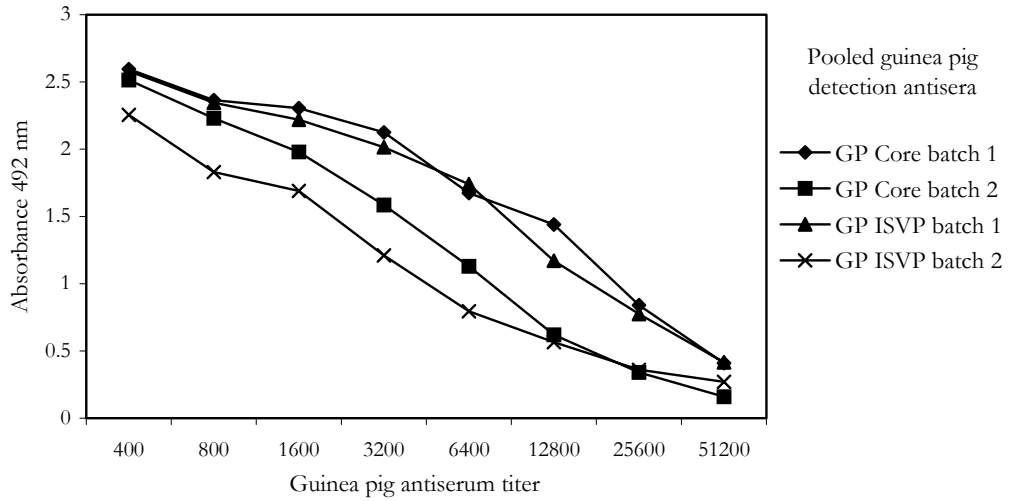

Figure 3.2 Titration of pooled guinea pig antisera against a standard rabbit serum. Plates were coated with rabbit antiserum at a dilution of 1/6 000. A constant amount of virus was added to all the wells. The various pools of guinea pig antiserum were then titrated from a 1/400 to 1/51 200 dilution. Each point on the graph represents the mean absorbance value of two wells.

The individual guinea pig sera were pooled into batches according to the strongest reactors. This resulted in two batches of serum for each of the ISVP and core antigens respectively. Batch 1 contained the stronger S-ELISA reacting sera while batch 2 contained the less S-ELISA reactive sera. Both the batches of ISVP and core guinea pig antisera had similar reactivity (Figure 3.2).

Checkerboard titration of rabbit- and guinea pig antisera

The two rabbit sera were pooled and used in a checkerboard titration against the guinea pig core 1 antisera to determine the working dilution for the rabbit and guinea pig antisera (Figure 3.3). A dilution of 1/6 000 rabbit anti-ISVP serum was used to coat the plates giving slight antibody excess. The guinea pig antiserum was used at a 1/2 000 dilution to obtain a relatively strong absorbance value.

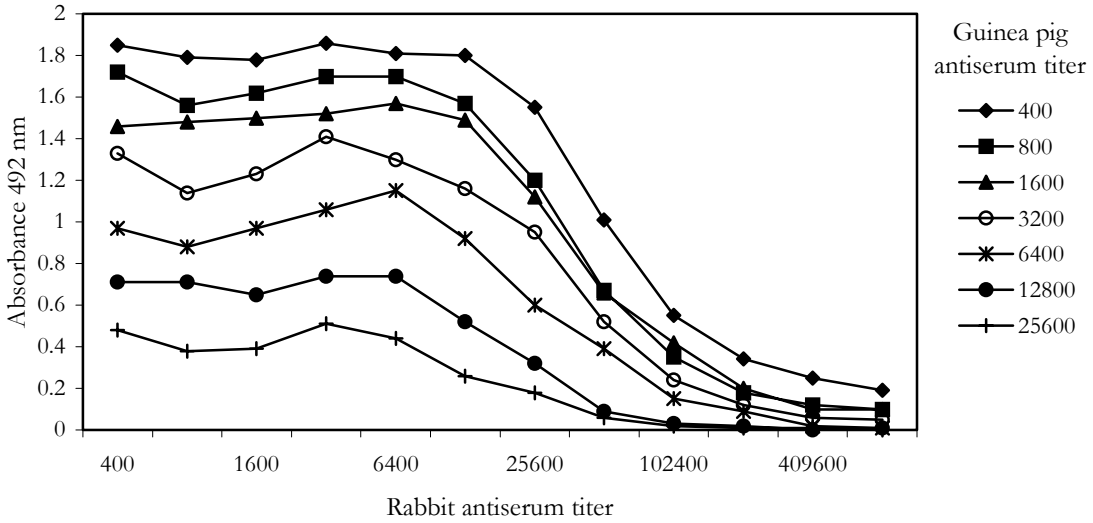

Figure 3.3 Checkerboard titration of pooled rabbit ISVP antiserum against guinea pig core antisera. Rabbit antiserum was diluted across the plate from 1/400 to 1/819 200. After incubation and washing a constant amount of virus was added to all wells. After further incubation and washing, guinea pig antiserum was diluted down from 1/400 to 1/51 200.

Titration of conjugate

The 1/1 000 and 1/2 000 conjugate dilutions both recorded an endpoint of 1/64 for the positive control antigen with similar absorbance values. The conjugate was used at a 1/1000 dilution giving an optical density between 1.0 and 2.0 in the first wells of the positive control antigen, while maintaining a clear differentiation, with minimum background, between the positive and negative control sera. This procedure was repeated for each new batch of conjugate.

Selection of plates

The mean optical densities for each of the EEV serotypes, after testing them in different ELISA plates for day-to-day variation are compared in Table 3.2. The Nunc MaxiSorb plates produced the most consistent results and were selected for all subsequent assays.

Table 3.2 Summary of the mean absorbance values obtained from testing replicates of EEV serotypes at a 1/2 dilution over four days using different ELISA plates in the EEV S-ELISA.

	Mean absorbance (± 1 standard deviation) obtained after four consecutive runs		
Specimen	Nunc	Dynex*	Dynex
EEV type 1	1.66 ± 0.056	1.33 ± 0.308	1.37 ± 0.477
EEV type 2	1.37 ± 0.404	1.12 ± 0.317	1.16 ± 0.215
EEV type 3	1.91 ± 0.112	1.92 ± 0.277	1.79 ± 0.579
EEV type 4	1.73 ± 0.214	1.47 ± 0.126	1.24 ± 0.436
EEV type 5	1.59 ± 0.250	1.50 ± 0.268	1.19 ± 0.609
EEV type 6	1.77 ± 0.119	1.46 ± 0.139	1.33 ± 0.376
Average	1.67 ± 0.192	1.46 ± 0.239	1.35 ± 0.449

* Dynex plates with the same catalogue number but from a different mould.

Analytical sensitivity and specificity

The purified ISVP preparation tested contained approximately 600 µg/mℓ virus particles and had a titre of $10^{9.5}$ $TCID_{50}$/mℓ. The endpoint for the virus titration was at a dilution of 9.2E+00, which is in the order of 9.0 ng/mℓ (0.45 ng/well) (Figure 3.4). This weight of antigen was equivalent to $10^{4.683}$ $TCID_{50}$/mℓ.

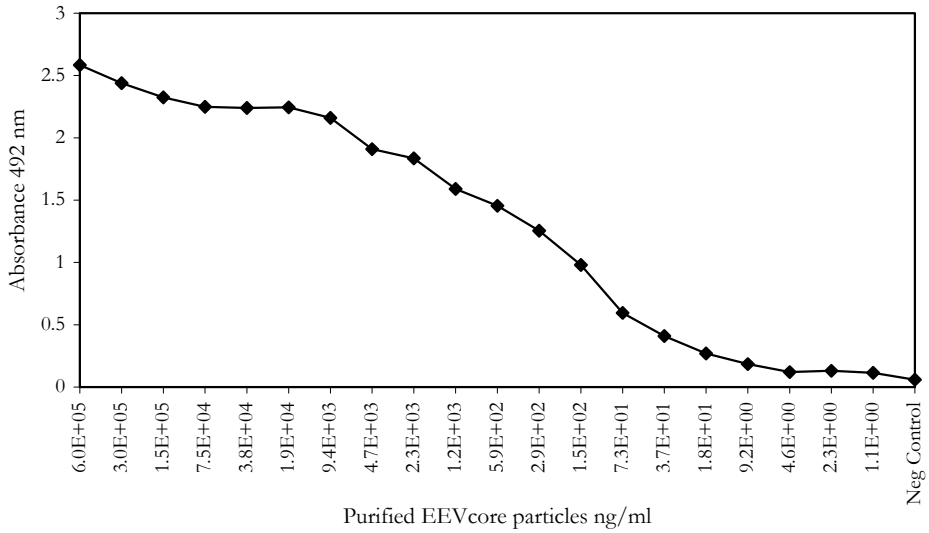

Figure 3.4 Results of the titration of a preparation containing approximately 600 µg/mℓ of virus particles with the aid of the EEV S-ELISA. The limit of detection of the virus particles was in the order of 9 ng/mℓ. Each point on the graph represents the mean absorbance of two wells.

All the orbivirus serotypes, other than EEV, were checked for antigen using their respective S-ELISAs (Figure 3.5). The EEV S-ELISA detected viral antigens in all seven EEV serotypes propagated in BHK cells. No cross-reactions were detected with any of the other 55 viruses tested (Figure 3.6).

.

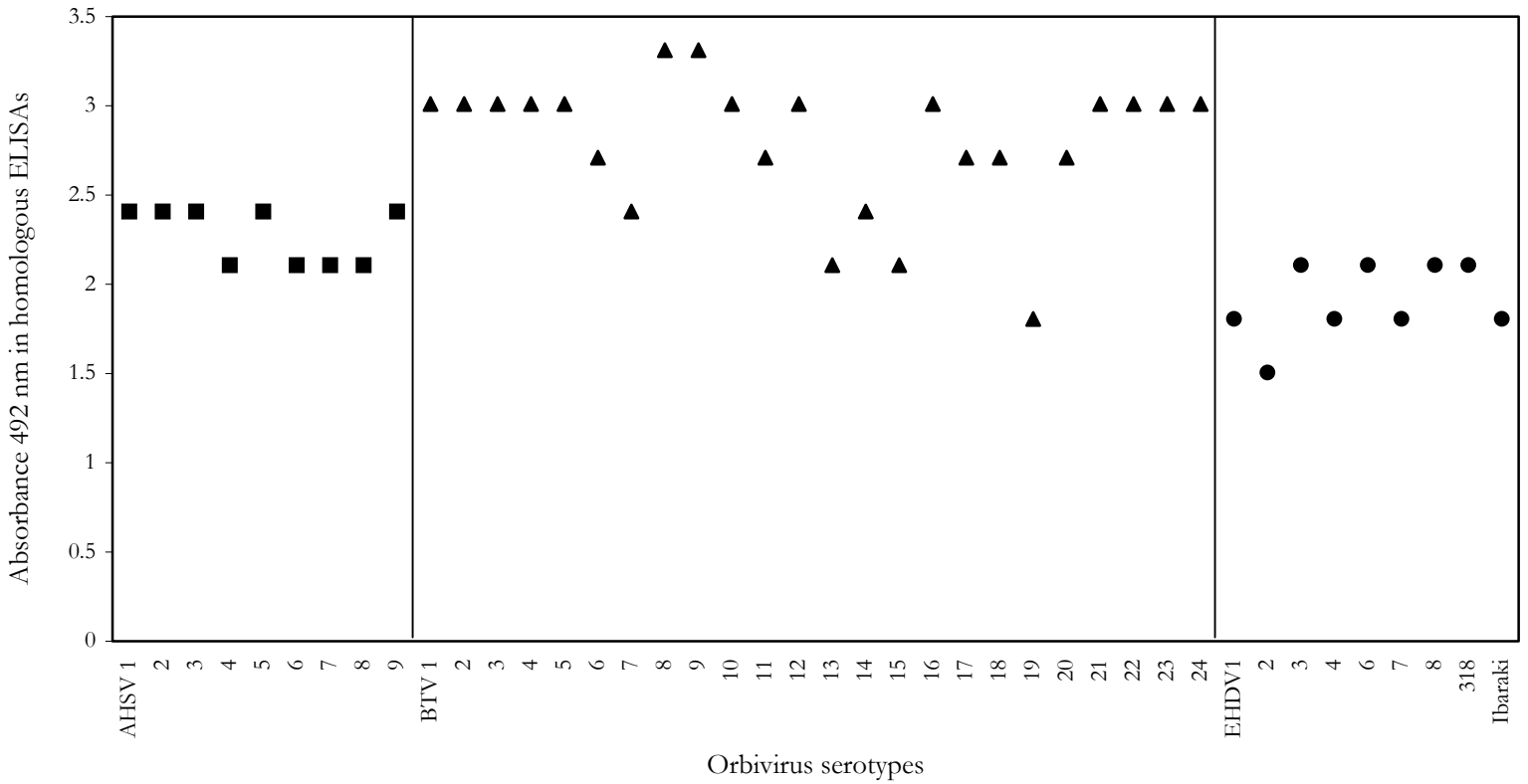

Figure 3.5 Results obtained after testing stock virus specimens of EHDV, BTV and AHSV in their respective S-ELISAs. The results of each ELISA are represented separately.

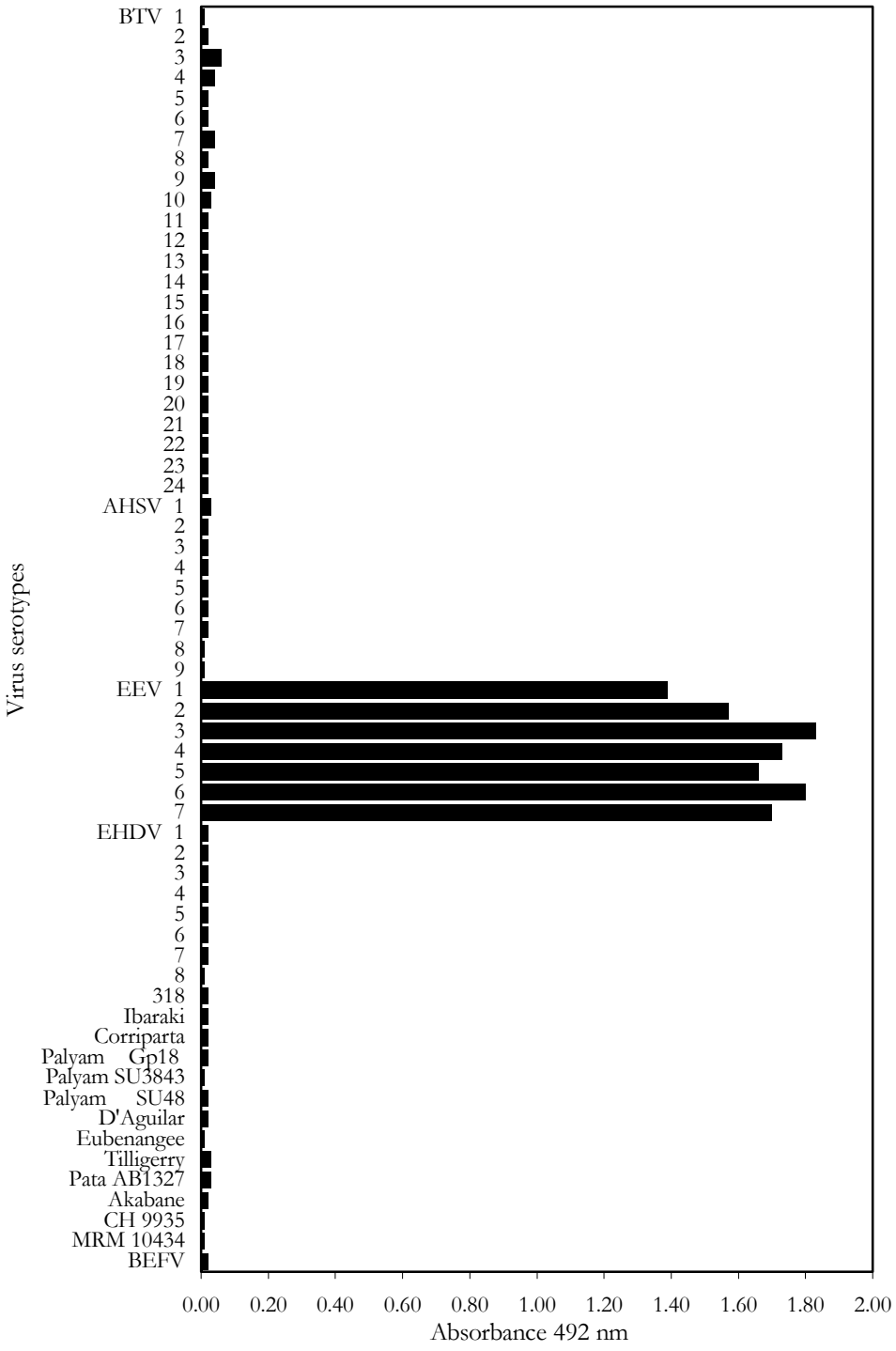

Figure 3.6 EEV S-ELISA results after testing 1/2 dilutions from various stock virus preparations. The ELISA detected seven EEV serotypes. No cross-reactions were observed with any of the other viruses that were tested.

The six EEV serotypes that were passaged in suckling mouse brains were titrated in a two-fold dilution series by S-ELISA to determine the level of antigen detection. All specimens tested gave strong colour reactions up to a dilution of 1/8 and thereafter titrated to at least 1/128 (Figure 3.7).

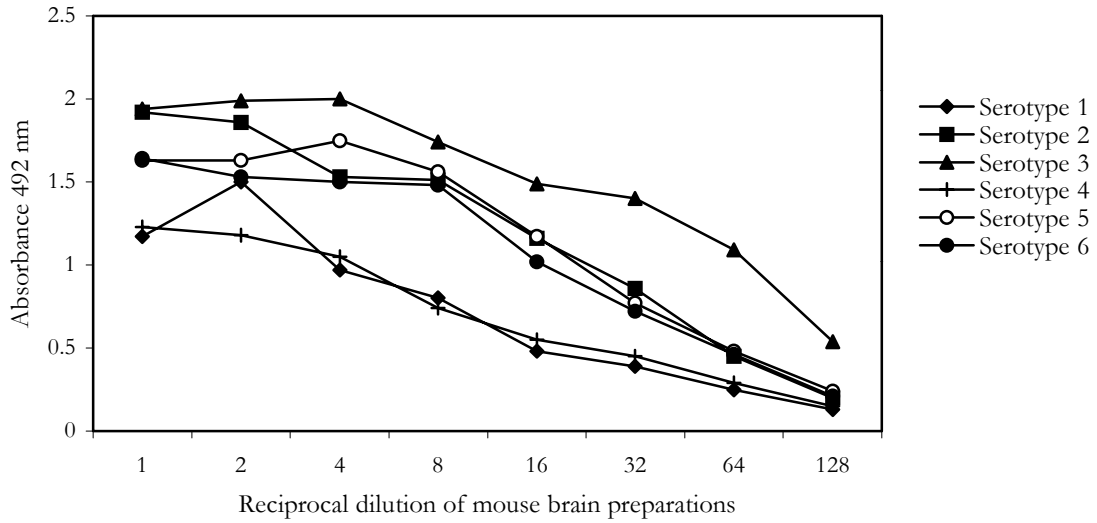

Figure 3.7 EEV S-ELISA results after titrating individual EEV serotypes from mouse brain preparations. All specimens that were tested contained viral antigen at a dilution of 1/128 or higher.

In most cases, all the EEV inoculated chicken embryos (3/3) died between days three and six. The only exception was for EEV type 2 where 2/3 embryos died. All embryos died between days three and six. Although the embryos showed typical lesions (oedema, haemorrhage) and/or death, which are often characteristic of an orbivirus infection, the homogenised embryo hearts were recorded negative by S-ELISA. On a second passage in embryonating hens eggs, fewer embryos died: EEV type 1 (4/5); EEV type 2 (3/5); EEV type 3 (0/5); EEV type 4 (1/5); EEV type 5 (1/5) and EEV type 6 (0/5).

A weak positive signal was recorded by S-ELISA with the homogenate from the second egg passage of EEV type 1. Homogenated embryo hearts for all other second egg passages were negative. However, when homogenised embryo hearts from the first egg passage were inoculated onto BHK-21 cells, a CPE was observed. The supernatant fluids from the BHK cell cultures were positive by S-ELISA. Strong positive signals were also obtained by S-ELISA for homogenates of suckling mouse brain the first and second passages of the six EEV types (Table 3.3)

Table 3.3 Results from growing EEV serotypes in chicken embryos, mouse brains (MB) and BHK-21 cells. Each one of these preparations was also tested with the EEV S-ELISA.

Passage history	Egg_1		Egg_2		Egg_1/ BHK_1	BHK_1	MB_1	MB_2
Test results	†/3	ELISA	†/5	ELISA	ELISA			
Serotype 1	3	-	4	+	++	+++	+++	+++
Serotype 2	2	-	3	-	++	+++	+++	+++
Serotype 3	3	-	0	ND	++	+++	+++	+++
Serotype 4	3	-	1	-	++	+++	+++	+++
Serotype 5	3	-	1	-	++	+++	+++	+++
Serotype 6	3	-	0	ND	++	+++	+++	+++

† Number of chicken embryos that died after intravenous inoculation. ND Not done

Negative/positive threshold

The frequency distributions of the absorbance values obtained from testing undiluted 100 negative cell culture specimens and 100 homogenates of suckling mouse brain are shown in Figure 3.8.

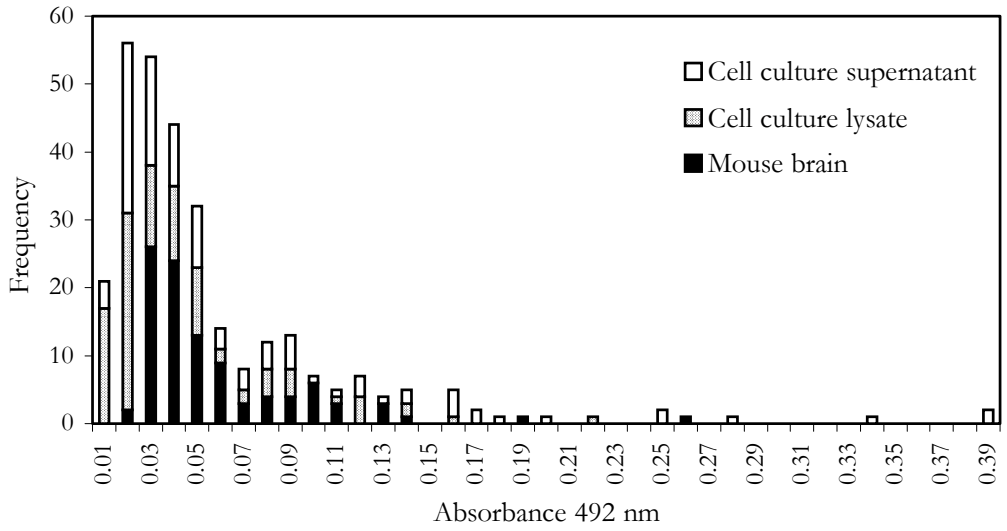

Figure 3.8 A frequency distribution plot of the absorbance values for EEV negative mouse brain and cell culture preparations. The cut-off was selected at an absorbance of 0.15, which represented the 97th percentile.

The cut-off was selected at an absorbance of 0.15, which represented the 97th percentile and a DSp of 97%. None of these samples titrated over three consecutive wells yielded values above the threshold of 0.15 (data not shown).

A frequency distribution plot of S-ELISA absorbance values obtained after testing EEV negative horse spleen homogenates from Spain and the UK is shown in Figure 3.9. A large proportion of these preparations reacted non-specifically in the S-ELISA (DSp = 20%)

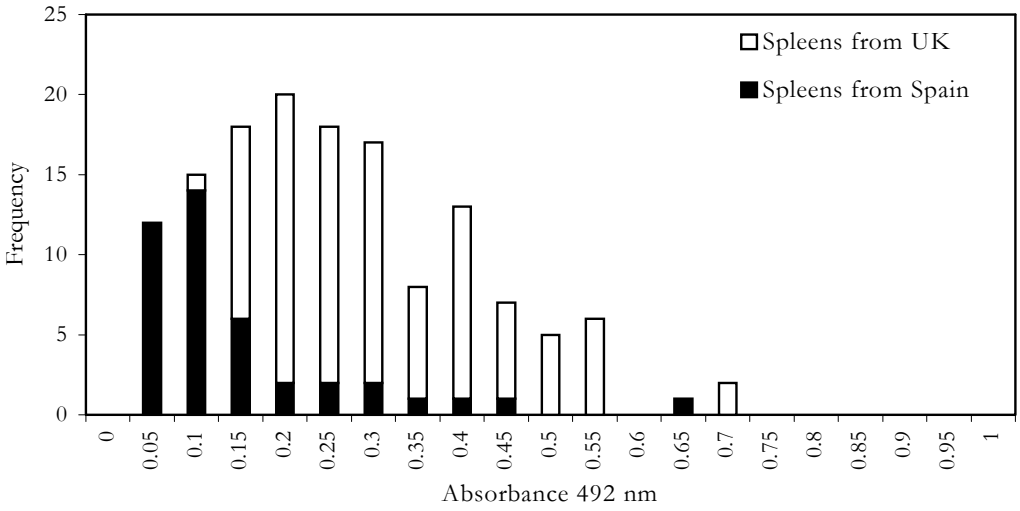

Figure 3.9 A frequency distribution plot of EEV S-ELISA absorbance values after testing EEV negative spleen suspensions from horses from Spain (n = 42) and horses from the UK (n = 100).

Repeatability

Ten replicates (on separate Nunc MaxiSorb plates) of an EEV type 1 stock virus preparation produced a mean absorbance value (± 1 standard deviation) of 1.63 ± 0.08 within a single run of the S-ELISA. The coefficient of variation (CV) for these replicates was 4.93% (Figure 3.10).

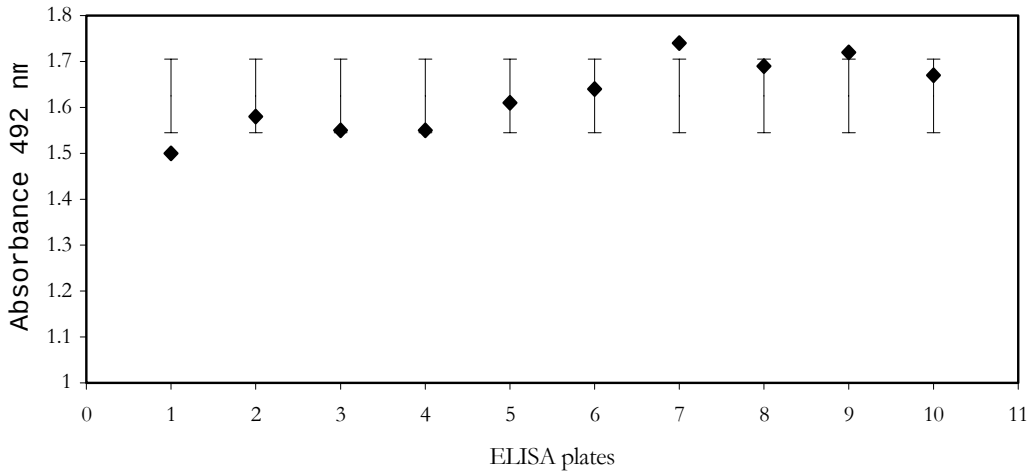

Figure 3.10 Comparison of the absorbance values from a single EEV type 1 specimen repeated in 10 plates during a single run of the S-ELISA. The error bars represent one standard deviation from the mean.

Discussion

This study reports the development and validation of a polyclonal antibody-based, group-specific, indirect sandwich ELISA (S-ELISA) that will help in the rapid laboratory confirmation of EEV infection and the differential diagnosis of EEV and AHSV.

EEV serotype 1 was chosen as the candidate for purification and antiserum production, because it is one of the most widely distributed and frequently isolated viruses within this serogroup. Furthermore, this strain produces high titres of infectious virus, which is advantageous for the production of antigen. The amount of purified EEV obtained following purification was lower than expected when compared to similar methods used for AHSV[18] and BTV[24]. The yield might be improved with further refinement of the method.

The S-ELISA was designed using a hyperimmune rabbit anti-ISVP serum as the capture antibody coated directly onto the plates. The specific antibody produced in a hyper-immunised rabbit should be at a high concentration due to the exponential amplification of antibody during the secondary immune response. These antibodies would also have matured in their affinity towards the more immuno-dominant epitopes on the virus particles that are presented to the B-lymphocytes in the secondary lymphoid follicles. These antibodies would therefore have the capacity to capture virus particles with the highest possible avidity and specificity. Since this S-ELISA is group-specific, it is presumed that the majority of antibodies in this assay are directed against epitopes on group-specific proteins e.g. VP 7. The second antibody used to detect any captured virus was produced in guinea pigs that were immunised once with core antigen. The specific antibodies that are produced during a late primary immune response are relatively immature in their specificity. These antibodies would therefore be directed against the widest possible range of epitopes on the group-specific proteins, that is the VP7/VP3. This would increase the group specificity of the detecting antiserum.

The mass of antigen detected by the EEV S-ELISA corresponds with that previously reported for the AHSV S-ELISA[18]. This S-ELISA detected virus of all six EEV serogroups from infected cell cultures and suckling mouse brains and has been shown to be specific, repeatable and sensitive. The results from all 42 orbiviruses tested with this S-ELISA confirm previous reports that there are no serological cross-reactions between EEV and other viruses in this genus. Also, no cross reactions were recorded against the

other arboviruses tested. The EEV isolates that were used in this study were all well adapted to BHK-21 cells and mouse brain and therefore yielded sufficient concentrations of progeny to be detected in the S-ELISA. When these isolates were passaged in chicken embryos, typical signs of orbivirus infection could be observed macroscopically but the viral concentration in the embryo hearts was beyond the range of detection for the S-ELISA.

Based on a 1/5 dilution, an absorbance value of 0.15 was determined as the baseline for negative samples (DSp=97%). The data obtained from titrating specimens from un-infected cell cultures and brain homogenates from uninfected suckling mice showed that negative samples did not titrate over three or more consecutive wells above an absorbance value of 0.15. Hence, a specimen is only recorded positive if it titrates at an absorbance value above 0.15 over three or more consecutive dilutions.

Homogenated spleen specimens are not suitable for use in the S-ELISA (DSp = 20%). Fortunately, this is not considered a problem because in practice spleen tissue need only be tested to provide a rapid confirmation of AHSV. If negative, virus isolation attempts will be made. Isolated viruses are then assayed in parallel for EEV and AHSV. In addition, a large proportion of equids in endemic areas have circulating antibody to one or more of the EEV serotypes[26]. These antibodies would form immune complexes with virus present in organ suspensions and could potentially interfere with the binding of virus in the S-ELISA. This is also true in countries where multiple AHSV serotypes are circulating or in countries where multiple AHSV serotype vaccines are used.

A further application of the S-ELISA could also be used to detect EEV in infected tissue cultures on microtitre plates particularly when cell sheets are difficult to read due to poor growth or contamination. This can be achieved by transferral of the culture supernatants into the S-ELISA micro-well plate

Chapter 4

A COMPETITIVE ELISA FOR THE DETECTION OF GROUP-SPECIFIC ANTIBODY TO EQUINE ENCEPHALOSIS VIRUS

Abstract

A polyclonal antibody-based, group-specific, competitive ELISA (C-ELISA) for the detection of antibodies to equine encephalosis virus (EEV) was developed. This assay measures the competition between a specific guinea pig antiserum and a test serum for a pre-titrated EEV antigen. The C-ELISA detected antibodies to seven EEV serotypes. No cross-reactions were observed with any of the other reference antisera to arboviruses that were tested. Negative sera from horses in the United Kingdom were used to assist in establishing a baseline for a negative population. Virus neutralisation tests were used to select a negative and a positive population from South African horses. A cut-off value (29.5%) was calculated by two-graph receiver operator characteristic (TG-ROC) analysis and yielded equal test parameters (Sensitivity ≅ Specificity ≅ 100%). The TG-ROC plot can be used to select a cut-off with the required Se and Sp depending on the application of the test. The data generated in this study was highly repeatable and suggests that the assay will be useful for epidemiological studies of EEV by providing an effective and efficient group-specific method for detecting antibodies to EEV.

Introduction

Traditionally, the complement fixation test (CFT) and agar gel immunodiffusion (AGID) test were used for the detection of group-specific antibody to EEV.

Interpretation of the CFT can be subjective and anti-complementary effects, particularly with zebra and donkey sera seriously affect the reliability and efficiency of the test. The AGID test uses a concentrated antigen prepared from infected cell cultures. This antigen can react with antibodies against cell proteins in the test sample to give a non-specific line of precipitation. There are also concerns about the specificity of this test with related serogroups of the genus orbivirus and, because of the weight of antibody required, the sensitivity.

A group-specific indirect enzyme linked immunosorbent assay (ELISA) for the detection of EEV antibody has been described by Williams *et al*[35]. It should be noted that this assay measures antibody directly in horse serum and therefore can only be used with equine sera. There may also be a difference in the efficiency of the anti-horse conjugate to detect donkey, mule and zebra sera.

A competitive ELISA for African horsesickness (AHSV) antibody detection has been developed and standardised by Hamblin *et al*[15,16]. This assay has been shown to be rapid, sensitive and specific and has been implemented internationally in a number of diagnostic and reference laboratories. This chapter describes the development and validation of a similar assay that can be used for the detection of EEV antibodies in the

sera of any species except guinea pigs and closely related species, and may therefore become a valuable aid in the study of the seroepidemiology of EEV.

Materials and methods

Preparation of antigen for use in the C-ELISA

Equine encephalosis virus type 1 was produced in fifty 175 cm^2 monolayer flasks containing monolayers of BHK-21 cells as described in Chapter 3. Antigen for coating the ELISA plates was extracted from the cell harvest using a method described by Hamblin *et al*[16]. Briefly, the cells were harvested at 48 hours, when showing 100% cytopathic effect (CPE). The cell harvest and supernatant fluids were collected and centrifuged at 1 000 × g and the supernatant discarded. The cell pellets were re-suspended in approximately 60 mℓ of 1 % phosphate buffered saline (PBS)-Sarkosyl (30% w/v aqueous solution of sodium laurolyl sarcosinate) (BDH) and sonicated (Soniprep 150, MSE) for 1 minute at maximum amplitude (18 microns). The sonicated preparation was then centrifuged in 50ml centrifuge tubes on an MSE high-speed 18 centrifuge at 10 000 rpm for 10 minutes (using a 8 × 50 rotor head) and the supernatant collected. The remaining pellet was re-suspended in PBS-Sarkosyl (10 mℓ per 50 mℓ centrifuge tube) and clarified by a further three to four cycles of sonication and centrifugation.

The collected supernatant fluid was centrifuged in 38ml polyacrilamide tubes on a 5 mℓ sucrose cushion (40 % w/v sucrose, BDH) at 100 000 × g for 2 hours using a Beckman

Ultra Centrifuge with SW 28 head. The pellet was re-suspended in approximately 10 mℓ PBS and sonicated as before. The preparation was left to stand for 1 minute and the cloudy upper layer collected. A further 10 mℓ PBS was added to the pellet debris and sonicated as above, the cycle was repeated until the entire pellet was re-suspended. The collected supernatant was made up to 100 mℓ in PBS and stored at minus 70 °C.

Production of antisera for use in the C-ELISA

Antisera against purified EEV type 1 ISVP was prepared in guinea pigs according to the method previously described in Chapter 3.

Checkerboard titration of antigen and guinea pig antisera for use in the C-ELISA

PVC Microtitre® "U" Bottom Plates (Dynex Technologies – catalogue no 2101) were used as the solid phase for all assays. Volumes of 50 µℓ/well for all reagents were used throughout the tests. Unless otherwise stated all reagents were diluted using a blocking buffer consisting of PBS supplemented with 0.05% (v/v) Tween 20 (BDH), 5.0% (w/v) skimmed milk powder (Cadbury's Marvel) and 1% (w/v) bovine serum albumin (Sigma). After the addition of the reagents, plates were covered and incubated at 37 °C for 1 hour on an orbital shaker (Luckhams, Rotatest). Unbound reagent was removed after each incubation step by flooding and emptying the wells three times with PBS and then blotting on absorbent paper.

A two-fold dilution series (1/25 to 1/25 600) of EEV antigen in 0.05 M carbonate-bicarbonate buffer, pH 9.6 (Sigma) (coating buffer) was prepared in eight replicate wells

across the 12 columns of an ELISA plate. The plates were incubated overnight at 4 °C and then washed. A two-fold dilution series (1/25 to 1/3 200) of guinea pig antiserum in blocking buffer was prepared in 12 replicate wells down the eight rows of the plate.

After incubation and washing, rabbit anti-guinea pig immunoglobulin conjugated to horseradish peroxidase (HRPO-conjugate) (Dako, Denmark) diluted 1/1000 in blocking buffer was added to all the wells. The plates were incubated, washed, and ortho-phenylenediamine (Sigma) (chromogen), at a concentration of 0.4 mg/mℓ, and containing 0.05% H_2O_2 (30% v/v) (substrate) was added to each well. The reaction was stopped after 10 minutes by the addition of 1.0 M H_2SO_4. The plate was read spectrophotometrically at 492 nm.

Competitive ELISA (C-ELISA)

EEV antigen, optimally diluted in coating buffer was passively adsorbed onto ELISA plates overnight at 4 °C. Plates were washed and test sera were added to duplicate wells. For routine screening of antibodies to EEV, sera were used at a single dilution of 1/5, whereas for quantitative measurement of antibody, sera were titrated in a two-fold dilution series (1/5 to 1/640) across eight wells. A similar two-fold dilution series (1/10 to 1/1 280) of a positive control serum was prepared across column 11. A negative control serum at a dilution of 1/5 was added to wells C12 and D12. Wells E12 to H12 received 50 µℓ blocking buffer instead of test serum and were used as controls (100 % value). Wells A12 and B12 were used as blank wells and only contained blocking buffer (Figure 4.1).

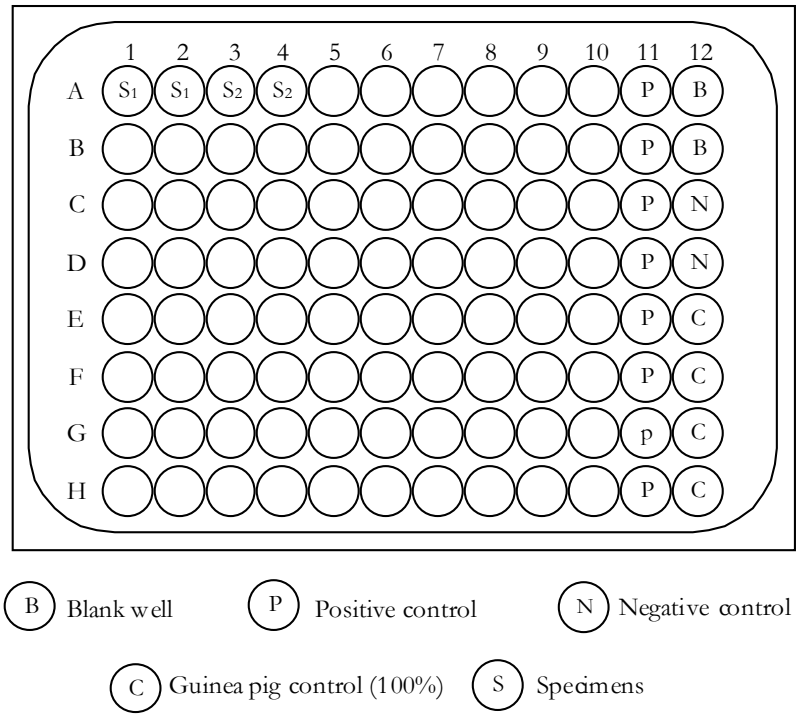

Figure 4.1 Diagrammatic representation of a 96-well microtitre plate indicating the organisation of the wells in the C-ELISA.

Guinea-pig antiserum against EEV type 1 ISVP optimally diluted in blocking buffer was then added to each well of the plate excluding wells A12 and B12 (total volume in each well = 100µℓ). After incubation and washing, HRPO-conjugate optimally diluted (1/1000) in blocking buffer was added to all the wells. Plates were incubated and washed and chromogen/substrate was added to each well. The reaction was stopped after 10 minutes by addition of 1.0 M H_2SO_4 to all the wells. The plates were read spectrophotometrically at a wavelength of 492 nm using an ELISA reader.

The mean percentage inhibition (PI) values for each sample were calculated using the following formula:

$$PI = 100 - \left(\frac{\text{Mean } A_{492} \text{ of test sample}}{\text{Median } A_{492} \text{ of guinea pig antiserum control}} \right) \times 100$$

Test specimens

Reference antisera (produced in sheep) against the seven EEV serotypes were obtained from the Department of Veterinary Tropical Diseases (DVTD), University of Pretoria, South Africa. Reference antisera were obtained from the Institute for Animal Health (IAH), Pirbright, United Kingdom (UK) against the following arboviruses: AHSV (serotypes 1-9), bluetongue virus (BTV) (serotypes 1-24), epizootic haemorrhagic disease of deer virus (EHDV) (serotypes 1-8 and 318), corriparta, ibaraki, tilligery, eubenangee, pata, palyam (B105), akabane and bovine ephemeral fever virus (BEFV). Equine sera (n=13) that were positive in the serum-virus neutralisation test (VNT) to only one of the individual EEV serotypes were selected from the serum banks of the DVTD. Serum specimens (n = 297) from horses that were presumed never to have been in contact with EEV were collected in the UK. Serum specimens (n = 517) were collected at the 1999 National Yearling Sale in South Africa, an EEV endemic country, and provided by the Equine Research Centre, University of Pretoria.

Analytical specificity

The analytical specificity of the assay was determined after testing reference EEV antisera and comparing PI values to those obtained for reference arbovirus antisera. Selected serum specimens from horses that were naturally infected and that tested

positive to only one of the EEV serotypes by VNT, were titrated in the EEV antibody specific C-ELISA.

Diagnostic sensitivity (Se) and specificity (Sp)

Horse sera (n = 297) from the UK, where EEV has never been recorded, were tested to determine the mean and the range of PI values for a negative population. Horse sera (n = 517) from South Africa were tested to determine the distribution and range of PI values recorded for a population from an endemic region.

Repeatability

A strong positive, weak positive, negative control and a blocking buffer control were tested over 16 days by C-ELISA to determine the day-to-day variation. These samples were also repeated as single replicates in corresponding wells of 13 ELISA plates within the same test to determine the plate-to-plate variation.

Results

Checkerboard titration of antigen and guinea pig antisera

The absorbance values recorded for each antigen dilution were plotted against a range of dilutions of guinea pig ISVP antisera (Figure 4.2).

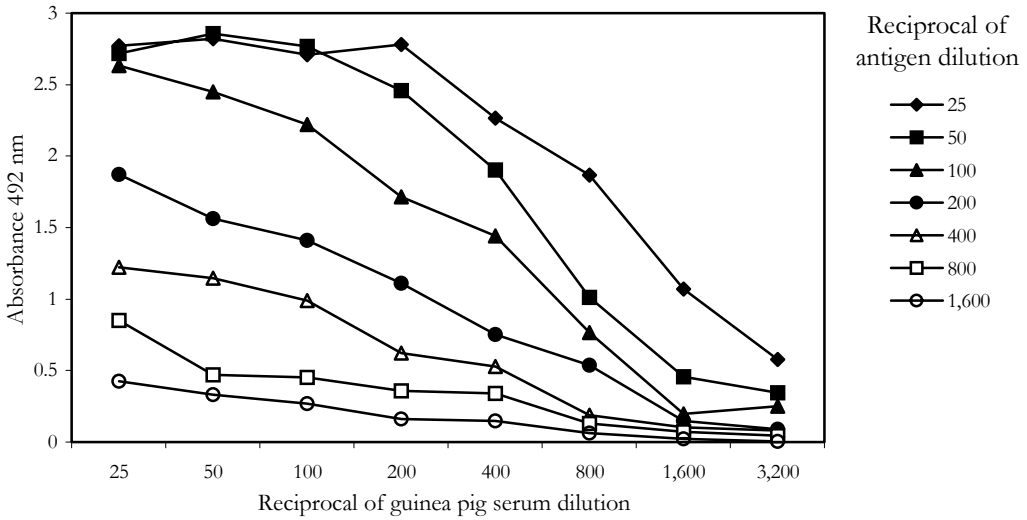

Figure 4.2 Checkerboard titration of immune guinea pig anti-EEV ISVP serum against EEV C-ELISA antigen. An EEV antigen dilution of 1/200 and a guinea pig antiserum dilution of 1/200 were selected.

An antigen dilution (1/200) with maximum absorbance values between 1.5 and 2.0 was selected. A guinea pig antiserum dilution (1/200) that would lead to slight antigen excess was selected accordingly. Working dilutions of antigen and antibody were selected to be economical for both reagents and to give a 100% colour reaction (no inhibition) between absorbance 1.0 and 1.2.

Analytical specificity

The dilution of test serum selected for use in the C-ELISA was 1/5. This dilution allowed direct dilution of the serum into the ELISA plate and corresponds to that which has been shown to be most suitable of separating positive and negative samples in other orbivirus C-ELISAs.

EEV reference antisera prepared in sheep against the seven known serotypes of EEV recorded PI values between 70.0% and 80.5 % and were therefore deemed positive by the C-ELISA. Antisera prepared against 49 other arboviruses recorded inhibition values below 20.0% and were therefore negative (Figure 4.3).

The PI values recorded for the 13 VNT positive horse sera assayed in a two-fold dilution series by C-ELISA are given in (Table 4.1). At a dilution of 1/5 all 13 sera recorded positive PI values between 72 and 93%. The end-point titres recorded for these sera, based on a 50% end-point, were between 1/15 and 1/220.

Table 4.1 Group-specific antibody C-ELISA results obtained from 13 serum specimens collected from South African horses that were naturally infected. All these horses were EEV-positive and each showed a mono-specific antibody response when tested by VNT.

Horse serum identification number.	C-ELISA percentage inhibition values								Serotype detected by virus neutralisation test
	5†	10	20	40	80	160	320	640	
105/94-39	87.9	67.2	40.7	32.6	16.4	21.1	7.2	1.2	EEV type 1
155/94-34	92.9	83.8	69.0	50.3	29.2	25.0	16.4	3.8	EEV type 1
155/94-46	90.4	86.4	75.8	61.0	40.6	35.4	21.9	4.6	EEV type 2
124/95-46	90.8	88.6	74.7	59.5	41.8	22.5	19.9	14.3	EEV type 2
119/94-9	93.3	93.2	91.1	85.4	69.9	54.8	32.0	23.2	EEV type 3
E73/95-15	92.0	88.4	69.6	53.5	32.3	20.7	20.6	1.4	EEV type 4
119/94-7	91.3	87.9	66.8	58.3	37.5	24.0	23.2	4.9	EEV type 4
119/94-6	82.5	76.3	51.1	34.8	26.5	15.3	18.6	6.3	EEV type 5
E73/95-42	88.6	78.6	55.9	38.0	20.8	0.4	10.7	1.1	EEV type 5
19/96-6	90.6	90.2	77.0	62.7	49.3	42.0	27.8	-8.1	EEV type 6
19/96-2	85.7	73.0	49.3	41.4	33.7	27.1	23.0	9.6	EEV type 6
E73/95-26	91.0	93.1	89.5	86.1	73.8	58.1	46.0	25.3	EEV type 7
E73/95-90	71.9	52.5	31.5	26.6	16.5	16.8	8.1	0.2	EEV type 7

† Reciprocal of test serum dilution

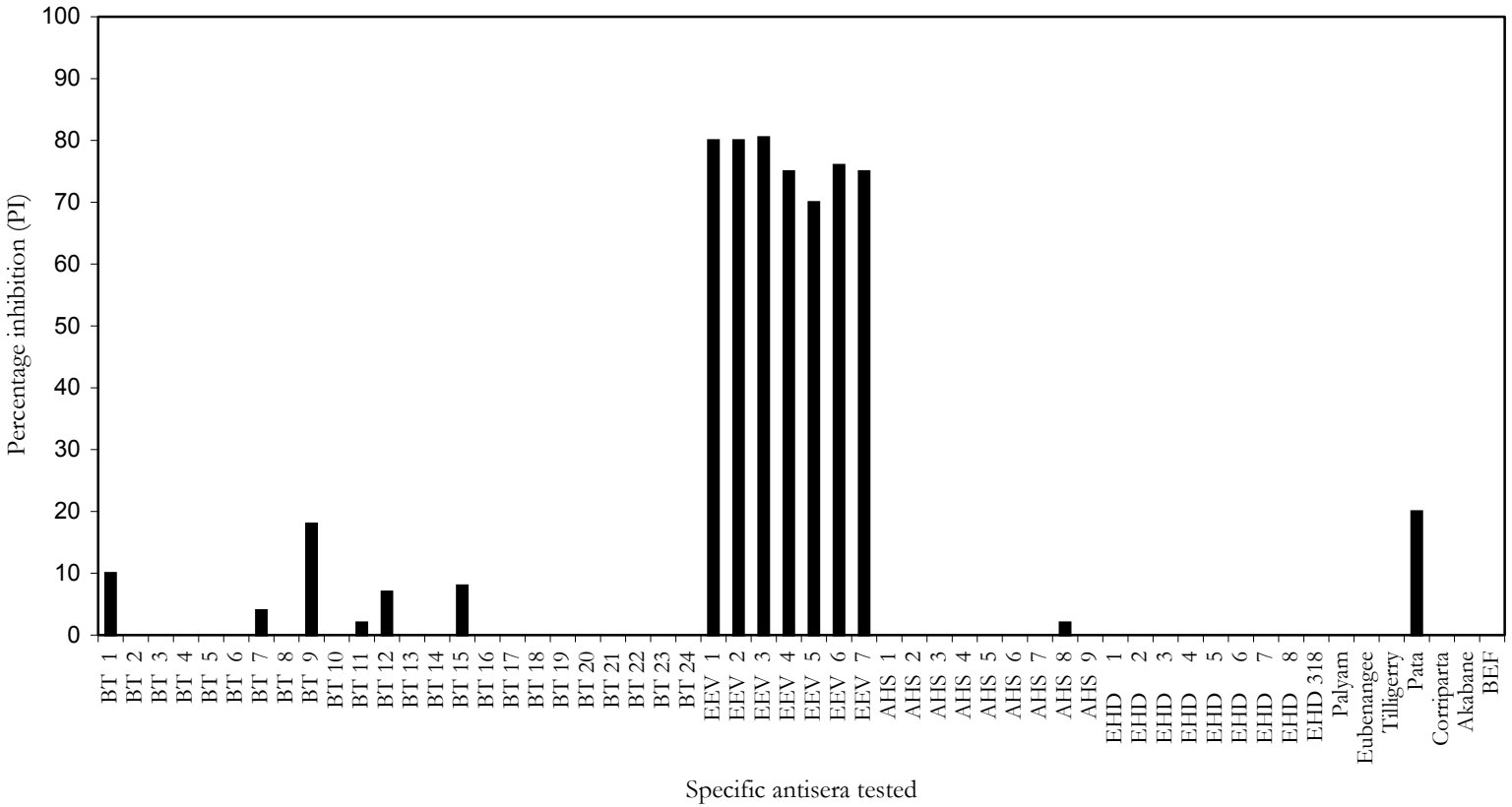

Figure 4.3 Group-specific antibody C-ELISA results obtained from reference antisera at a 1/5 dilution. The ELISA detected antibodies to all seven EEV serotypes. No significant cross-reactions were observed with any of the other antisera that were tested.

Diagnostic sensitivity and specificity

Analysis of the C-ELISA results obtained for negative horse sera collected from a resident population in the UK gave a mean PI value of minus 26.6% with a range between minus 100.0% and 31.8% (Figure 4.4). Two of the specimens were severely contaminated (PI values of 38% and 60%) and were omitted from the calculations.

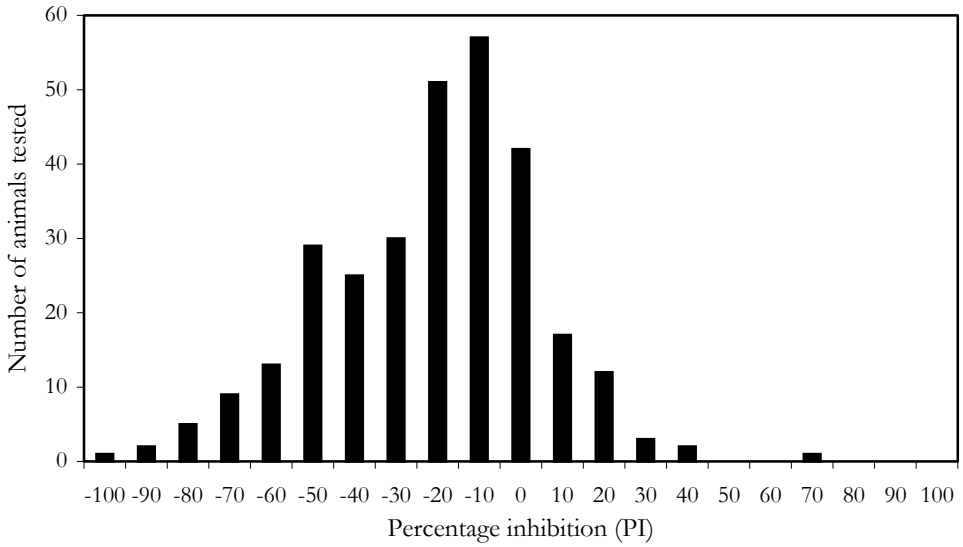

Figure 4.4 A frequency distribution plot showing EEV group-specific antibody C-ELISA results obtained from horse sera (n=297) collected in the UK. The mean PI value was minus 26.6% with a range between minus 100.0% and 31.6%. Two of the specimens were severely contaminated and gave inhibition values of 38% and 60%, respectively.

The results of testing 517 sera collected from horses at the South African yearling sales are presented as a frequency distribution plot in Figure 4.5. The plot shows a clear discrimination between positive and negative sera. All 148 of the sera from South African yearlings that recorded positive in the C-ELISA (PI > 50%) were confirmed

positive to one or more serotypes of EEV by VNT. A representative sample (49) from all the South African yearling sera that gave PI < 20%, and all the sera that gave PI values between 20% and 50%, were also tested by the VNT. The results are presented in Table 4.2.

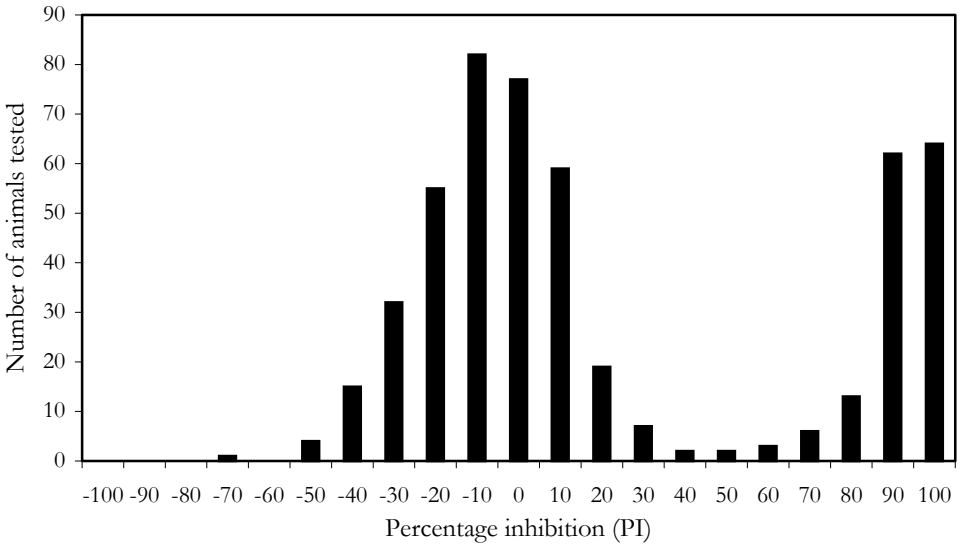

Figure 4.5 Frequency distribution plot showing EEV group-specific antibody C-ELISA results obtained from horse sera (n=517) collected at the 1999 National Yearling Sale in South Africa. The distribution shows a clear discrimination between positive and negative sera.

Table 4.2 Comparison between C-ELISA and VNT results after testing South African yearling sera for the presence of antibodies to EEV.

Range of PI values for sera tested by C-ELISA	Number of specimens that tested either positive or negative for antibodies to EEV by VNT*	
	Positive	Negative
Between minus 70 and 20	1	48
Between 20 and 50	5†	5
Between 50 and 100	148†	0
Total	154	53

* Sera were tested for all EEV serotypes. † All the specimens were positive to EEV type 6.

Two percent (1/49) of the sera that were recorded as clearly negative (PI = 4.6%) by C-ELISA were positive by VNT with a titre > 80. Fifty percent (5/10) of the sera that recorded PI values between 20% and 50% by C-ELISA were positive by VNT to EEV type 6.

Competitive ELISA data from confirmed VNT positive (n = 154) and negative (n = 54) sera collected from South African yearlings was subjected to a two-graph receiver operating characteristic (TG-ROC) analysis[13,14]. The software indicated that data from the negative population showed departure from a normal distribution. Therefore, the non-parametric (distribution-free) method for the estimation of the measures described was used. A cut-off as established by the non-parametric option in the software with sensitivity (Se) ≅ specificity (Sp) ≅ 100% was 29.5% with 95% accuracy (Figure 4.6). Summary statistics for the positive and negative reference population C-ELISA results are presented in Table 4.3.

Table 4.3 Descriptive measures for the results of an EEV group-specific antibody C-ELISA after testing positive and negative reference populations (South African yearlings) that were confirmed by VNT. Data for sera from horses in the UK presumed negative is also shown.

	United Kingdom	South Africa	
Measure	Sera presumed to be negative	Sera negative by VNT*	Sera positive by VNT†
Sample size	297	53	154
Mean	-26.6	-11.6	84.4
Median	-23.8	-10.3	89.1
Standard deviation	25.0	22.6	12.8
Minimum	-100.0	-57.5	4.5
Maximum	31.6	26.3	94.6

* Sera were tested for all the EEV serotypes. † All the specimens were positive to EEV type 6.

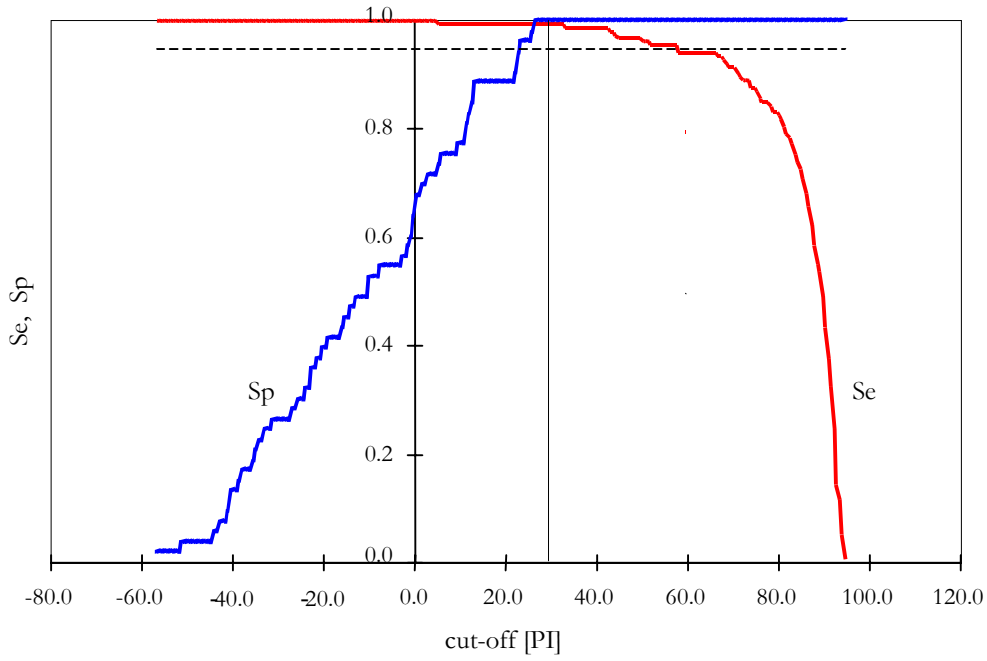

Figure 4.6 TG-ROC analysis of an EEV group-specific antibody C-ELISA. The intersection point of the two graphs indicates the cut-off point (29.5%) at which equivalence (Se ≅ Sp) can be achieved. The dotted line indicates Se and Sp with an accuracy of 95%.

If the cut-off value was calculated by the conventional method of mean plus two-fold standard deviation of the negative population, 33.6% would be obtained for South African yearling sera and 23.4% for the negative population of UK horses.

Repeatability

The analyses of the data obtained after repeated testing of three standard sera by C-ELISA are presented in Table 4.4. Results indicate expected "within test" and "within plate" variations. The coefficients of variation (CVs) for the normalised data obtained from replicates of the strong and weak positive sera were all below 7%, which is below the acceptable value of 10%. CVs for the replicates of the negative sera were not calculated because they are not valid when the mean approaches zero.

Table 4.4 Descriptive measures for the results of an EEV-specific, antibody C-ELISA that was repeated several times for a strong positive, a weak positive and a negative serum specimen respectively.

	C-ELISA results from different runs†			C-ELISA results from a single run*		
Measure	Strong positive	Weak positive	Negative	Strong positive	Weak positive	Negative
Replicates	16	16	16	13	13	13
Mean	96.8	87.0	-2.0	95.0	70.7	-10.4
Median	96.9	87.7	-0.7	95.0	68.6	-9.5
Standard deviation	1.1	4.2	8.6	1.4	4.7	7.0
Minimum	94.0	78.0	-19.9	92.5	65.4	-22.3
Maximum	98.6	92.2	12.7	96.9	80.8	-0.9
Coefficient of variation	1.1	4.8	-	1.5	6.7	-

† Runs were performed on separate days. * Specimens were placed in corresponding wells, on different plates.

Repeatability data for the control wells that were used for the 100% colour reaction is presented in Figure 4.7. With a few exceptions, the replicates remained within ±2 standard deviations of the mean for all runs.

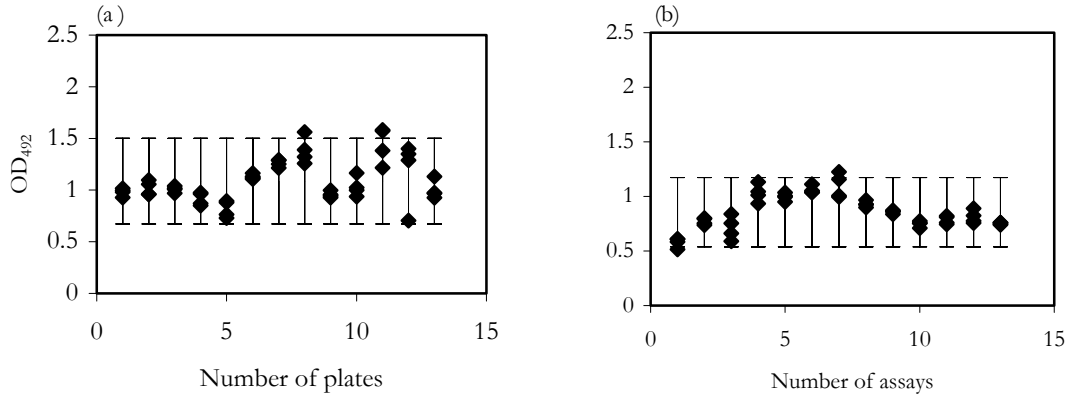

Figure 4.7 Distribution plots of the optical density of the guinea pig control serum that was placed in quadruplicate wells on each plate within a single run of the assay (a) and over 16 days (b) of an EEV antibody specific C-ELISA. The error bars represent two standard deviations from the mean.

Although there was some variation between plates within an assay and between different assays, the absorbance values on each plate were very similar. There were, however, four plates in which the absorbance value in a single well deviated significantly from the rest. It is therefore recommended to use the median instead of the mean absorbance value for the control wells.

Discussion

Competitive ELISAs are routinely and successfully used for the detection of antibodies to several orbiviruses including AHSV, BTV and EHDV. The major objective of this study was to develop a comparative assay that would be suitable for the study of the seroepidemiology of EE. This assay measures the competition between a specific guinea pig antiserum and a test serum for a pre-titrated EEV antigen. The amount of antibody in the guinea pig antiserum that reacts with the EEV antigen, in the presence of test serum, is then measured enzymatically with HRPO conjugated to rabbit anti-guinea pig immunoglobulin. The degree of competition (inhibition of the colour reaction) when compared to control values in the absence of test serum is used to determine positivity or negativity. Selecting a guinea pig serum at a concentration that will just saturate the antigen on the plate will result in optimal conditions for competition. If however, there is slight antigen excess on the plate, it will lead to a slightly less sensitive but more specific assay. This method permits the testing of sera from all vertebrate species except guinea pigs and their close relatives with a single conjugate.

The expression of C-ELISA results as a PI of the competing antibody, results in a continuous scale from 0 to 100 PI, which is directly proportional to antibody activity. This is in agreement with the recommendations made at a joint meeting of the United Nations Food and Agriculture Organisation and the International Atomic Energy Agency, Vienna, 1992[36].

The colour that developed in wells containing negative equine sera was frequently higher than that observed in the control wells in the absence of equine sera. Thus, negative inhibition values were often recorded for negative sera. This phenomenon cannot be explained but does not appear to have any influence on the validity of the test parameters.

Horses vaccinated with cell culture derived vaccine may develop specific antibody against cell culture proteins as well as the vaccine virus. Such animals could be falsely identified as positive in an indirect ELISA that utilises a crude EEV cell culture extract as an antigen where antibody in the test serum is measured directly[18]. To avoid interference from these and any other potential cross-reacting proteins the guinea pig antiserum used in this C-ELISA was prepared using highly purified EEV ISVP.

The C-ELISA detected antibodies to the seven current EEV serotypes. No significant cross-reactions were observed with any of the other 49 reference antisera to arboviruses tested.

Sera from horses resident in the UK, where there have been no reports of EEV, were tested to establish a baseline for a negative population. These results were compared with those obtained for a VNT negative group of sera from South African yearlings. These horses were older than six months, when maternal antibody would have declined and would have been exposed to insect vectors for only one summer. The PI values obtained for the UK sera were distributed slightly to the left of the VNT negative sera from South Africa. This was also reflected by the conventional cut-off values (mean plus two standard deviations) of 23.4% and 33.6% calculated for the respective populations. This suggests a difference between the two sets of data. The most obvious explanation is that one population comes from a naïve area and the other comes from an endemic area. It is perceivable in the latter case, even in the absence of positive VNT and ELISA reactions, that some of these animals had either waning or early antibody below ELISA and VNT detection.

The TG-ROC analysis used to examine the data obtained from testing South African yearlings was developed as a Microsoft-EXCEL spreadsheet by Greiner (1995)[13,14]. The software uses data from positive and negative reference specimens and plots the Se and Sp against the cut-off value, assumed as an independent variable. Thereby, estimates of cut-off values can be read directly from the TG-ROC plots for any valid combination of Se and Sp. This is an improvement over the conventional ROC plot where cut-off values cannot be inferred directly from the plotted curves. A cut-off that results in equal test parameters, i.e. where Se = Sp, can be obtained at the intersection point of the two

graphs. For example, any cut-off value selected in the range between 23.3% and 57.0% (intersection with dotted line, Figure 4.6) would result in both Se and Sp equal to or above 95%. A cut-off value can therefore be selected depending on the application of the test for example when screening horse serum for import/export purposes a cut-off (29.5%) that would give a high Se and Sp would be preferred. A cut-off of 57.0% would give a less sensitive but more specific test for the screening of horse sera in countries that are supposedly free of EEV.

The cut-off as calculated by the TG-ROC (Se ≅ Sp ≅ 100%) was 29.5% for the limited number of animals tested. If calculated as mean plus two-fold standard deviation of the negative population, 33.6% would be obtained as a cut-off value. This compares favourably with the cut-off values that have been suggested for similar C-ELISAs: i.e. 45% for a EHD C-ELISA [30], 40% for a BT C-ELISA[2]and 50% for a AHS C-ELISA[16].

The data generated in this study was highly repeatable and suggests that the assay will be valuable for epidemiological studies of EEV by providing an effective and efficient method for the detection of antibodies to EEV. Because all equids, particular zebras, are potential hosts of AHSV and EEV in southern Africa, further studies are required to determine the geographical distribution and prevalence of these viruses. This is extremely difficult with AHSV because horses are routinely vaccinated. It can however, be achieved by testing foals older than 6 months and before they receive their first

vaccination. Since there are currently no vaccines available against EEV, the antibody that is detected can be ascribed to natural infection. Therefore, sero-surveillance and epidemiological studies of this disease will be simplified.

Chapter 5

GENERAL CONCLUSIONS

Serogroup-specific enzyme linked immunosorbent assays (ELISA) for the detection of equine encephalosis virus (EEV) antigen and antibody were designed, developed and validated. These assays were shown to be reliable, sensitive and specific and will prove invaluable in epidemiological studies of EEV infections, including the study of vertebrate and invertebrate host interactions, vector susceptibility and competence, sero-surveillance, virus transmission, and geographic and species distribution. Wildlife and domestic animal interfaces are currently an area of concern and extensive research, especially in Africa. These assays will make significant contributions to studies relating to the reservoir status of zebra and possible transmission to horses.

Unlike African horsesickness (AHS), which is the most lethal virus infection of horses, equine encephalosis (EE) is considered a sub-clinical or mild infection that seldom causes significant disease and death. However, some of the clinical signs and pathological changes observed following EEV infections are similar to those reported for AHSV. Thus, in the event of a suspected outbreak of disease in horses, particularly in countries where AHSV is a consideration, the main concern of the veterinary regulatory authorities, practitioners, owners and vaccine manufacturers is to confirm or

rule out the presence of AHSV so that appropriate control strategies can be implemented immediately.

Both EEV and AHSV are endemic in southern Africa and both appear to be transmitted by the same *Culicoides* vectors. The polyclonal antibody-based, S-ELISA described herein is ideally suited for the rapid identification of EEVs isolated in cell culture and suckling mouse brain and for the laboratory differential diagnosis of EEV and AHSV.

Dual infection with EEV and AHSV is also a distinct possibility. The serogroup differentiation of the causal agent in such a case would be difficult with the traditional VNT because the non-neutralised virus in the respective assay systems would infect the indicator cells. This would result in a unsuccessful attempt to type the virus and might even incorrectly suggest the presence of a new virus serotype. The EEV S-ELISA in conjunction with the similarly designed AHSV S-ELISA offers significant advantages for the rapid, simultaneous identification and confirmation of mixed populations of these viruses.

Homogenated horse spleens are not suitable for virus detection with the S-ELISA because of an unacceptable proportion of non-specific reactions. Fortunately, this is not considered a problem because in practice spleen tissue need only be tested to provide a rapid confirmation of AHSV. If the result is negative, virus isolation attempts using cell culture or intracerebral inoculation of day-old mice will be made notwithsatanding. Isolated viruses are then identified by standard procedures.

Another consideration is the a large proportion of equids in endemic areas that have circulating antibody to either EEV or AHSV or both. These antibodies would form immune complexes with virus present in organ suspensions and could potentially interfere with the binding of virus in the respective S-ELISAs. This is also true in countries where multiple AHSV serotype vaccines are used.

A polyclonal antibody-based C-ELISA for the detection of group-specific antibodies to EEV was also developed. The data generated with the C-ELISA was highly repeatable and confirmed the assay to be sensitive and specific. The C-ELISA will therefore be valuable for seroepidemiological studies of EEV by providing an effective and efficient method for the detection of group-specific antibodies to EEV.

The C-ELISA has distinct advantages because it is rapid and can be used for detection of group-specific antibody to EEV in any animal species except guinea pigs. This would be valuable when studying the seroepidemiology of EEV, especially amongst zebra populations and other yet unknown host species.

The routine use of the C-ELISA in conjunction with the VNT for the screening of horse sera would give the first indication whether an unidentified serotype is circulating in a population. This would become apparent when antibody is detected in a serum with the group-specific C-ELISA but does not react to any of the known serotypes in the VNT.

The C-ELISA, developed as part of this study, has already been applied successfully in a survey of South African horses by Howell *et al* (2001)[19] and is currently being used by Hamblin (unpublished data) in a serological survey of equine athletes and indigenous equids from many countries around the world.

Reference List

1. ANDERSON,E.C., MELLOR,P. & HAMBLIN,C. 1989 African horse sickness in Saudi Arabia. *Veterinary Record*, 125:489-489.

2. ANDERSON,J. 1984 Use of monoclonal antibody in a blocking ELISA to detect group specific antibodies to bluetongue virus. *Journal of Immunological Methods*, 74:139-149.

3. ANON. 1996 Principles of validation of diagnostic assays for infectious diseases, in *Manual of Standards for Diagnostic Tests and Vaccines,* edited by OIE Standards Commission. Paris: Office International des Epizooties: 8-15.

4. ANON. 1997 Commission Decision of 12 December 1996 amending Council Decision 79/542/EEC and Commission Decisions 92/160/EEC, 92/260/EEC and 93/197/EEC in relation to the temporary admission and imports into the Community of registered horses from South Africa. Official Journal of the European Communities L, 3-9-3/10.

5. BARNARD,B.J.H. 1997 Antibodies against some viruses of domestic animals in southern African wild animals. *Onderstepoort Journal of Veterinary Research*, 64:95-110.

6. BARNARD,B.J.H. & PAWESKA,J.T. 1993 Prevalence of antibodies against some equine viruses in zebra (*Zebra burchelli*) in the Kruger National Park, 1991-1992. *Onderstepoort Journal of Veterinary Research*, 60:175-179.

7. BURROUGHS,J.N., O'HARA,R.S., SMALE,C.J., HAMBLIN,C., WALTON,A., ARMSTRONG,R. & MERTENS,P.P.C. 1994 Purification and properties of virus particles, infectious subviral particles, cores and VP7 crystals of African horsesickness virus serotype 9. *Journal of General Virology*, 75:1849-1857.

8. COETZER,J.A.W. & ERASMUS,B.J. 1994 Equine encephalosis, in *Infectious diseases of livestock with special reference to southern Africa,* edited by J.A.W.Coetzer , G.R.Thomson & R.C.Tustin. Cape Town: Oxford University Press: 476-479.

9. ERASMUS,B.J., ADELAAR,T.F., SMIT,J.D., LECATSAS,G. & TOMS,T. 1970 The isolation and characterization of equine encephalosis virus. *Bulletin del'Office International des Epizooties*, 74:781-789.

10. ERASMUS,B.J., BOSHOFF,S.T. & PIETERSE,L.M. 1978 The isolation and characterization of equine encephalosis and serologically related orbiviruses from horses, in *Proceedings of the Fourth International Conference on*

Equine Infectious Diseases, edited by J.T.Bryans & H.Gerber. Lyon: Princeton, New Jersey: Veterinary Publications Incorporated: 447-450.

11. GERDES,G.H. & PIETERSE,L.M. 1993 The isolation and identification of Potchefstroom virus: A new member of the equine encephalosis group of orbiviruses. *Journal of the South African Veterinary Association*, 64:131-132.

12. GORMAN,B.M. 1992. An overview of the orbiviruses, in *Bluetongue, African horse sickness and related orbiviruses,* edited by T.E.Walton & B.I.Osburn. Boca Raton, Florida: CRC Press, Inc.: 335-347.

13. GREINER,M. 1995 Two-graph receiver operating characteristic (TG-ROC): a Microsoft-EXCEL template for the selection of cut-off values in diagnostic tests. *Journal of Immunological Methods*, 185:145-146.

14. GREINER,M., SOHR,D. & GÖRLICH,P. 1995 A modified ROC analysis for the selection of cut-off values and the definition of intermediate results of serodiagnostic tests. *Journal of Immunological Methods*, 185:123-132.

15. HAMBLIN,C., ANDERSON,E.C., MELLOR,P.S., GRAHAM,S.D., MERTENS,P.P.C. & BURROUGHS,J.N. 1992 The detection of African

horse sickness virus antigens and antibodies in young equidae. *Epidemiology and Infection*, 108:193-201.

16. HAMBLIN,C., GRAHAM,S.D., ANDERSON,E.C. & CROWTHER,J.R. 1990 A competitive ELISA for the detection of group-specific antibodies to African horse sickness virus. *Epidemiology and Infection*, 104:303-312.

17. HAMBLIN,C., MELLOR,P.S. & BONED,J. 1991 The use of ELISA for the detection of African horse sickness viruses in *Culicoides* midges. *Journal of Virological Methods*, 34:221-225.

18. HAMBLIN,C., MERTENS,P.P.C., MELLOR,P.S., BURROUGHS,J.N. & CROWTHER,J.R. 1991 A serogroup specific enzyme-linked immunosorbent assay for the detection and identification of African horse sickness viruses. *Journal of Virological Methods*, 31:285-292.

19. HOWELL,P.G., GROENEWALD,D.M., VISAGE,C.W., BOSMAN,A., COETZER,J.A.W. & GUTHRIE,A.J. 2001 The classification of seven serotypes of equine encephalosis virus and the prevalence of homologous antibody in horses in South Africa. *Onderstepoort Journal of Veterinary Research*, In press:

20. JACOBSON,R.H. 1998 Validation of serological assays for diagnosis of infectious diseases. *Rev.sci.tech.Off.int.Epiz*, 17:469-486.

21. KÄRBER,G. 1931 Beitrag zür kollektiven Behandlung pharmakologischer Reihenversuche. *Arch Exp Pathol Pharmakol*, 162:480-481.

22. LECATSAS,G., ERASMUS,B.J. & ELS,H.J. 1973 Electron microscopic studies on equine encephalosis virus. *Onderstepoort Journal of Veterinary Research*, 40:53-57.

23. MCINTOSH,B.M. 1956 Complement fixation with horsesickness viruses. *Onderstepoort Journal of Veterinary Research*, 27:165-169.

24. MERTENS,P.P.C., BURROUGHS,J.N. & ANDERSON,J. 1987 Purification and properties of virus particles, infectious subviral particles and cores of bluetongue virus serotypes 1 and 4. *Virology*, 157:375-386.

25. NEVILL,E.M., ERASMUS,B.J. & VENTER,G.J. 1992. A six-year survey of viruses associated with *Culicoides* biting midges throughout South Africa (Diptera: Ceratopogonidae), in *Bluetongue, African horse sickness, and related orbiviruses,* edited by T.E.Walton & B.I.Osburn. Boca Raton, Florida: CRC Press, Inc.: 314-319.

26. PAWESKA,J.T., GERDES,G.H., WOODS,P.S.A. & WILLIAMS,R. 1999 Equine encephalosis in southern Africa: current situation, in *Equine infectious diseases VIII: Proceedings of the Eighth International Conference; Dubai; 23rd-26th March 1998,* edited by U.Wernery, J.F.Wade, J.A.Mumford & O.R.Kaaden. Newmarket; UK: R & W Publications Ltd.: 303-305.

27. SÁNCHEZ-VIZCAÍNO,J.M. 1996 African horse sickness, in *Manual of Standards for Diagnostic Tests and Vaccines,* edited by OIE Standards Commission. Paris: Office International des Epizooties: 128-136.

28. THEILER,A. 1921 African horse sickness (pestis equorum). *Science Bulletin*, 19:1-29.

29. THEODORIDIS,A., NEVILL,E.M., ELS,H.J. & BOSHOFF,S.T. 1979 Viruses isolated from *Culicoides* midges in South Africa during unsuccessful attempts to isolate bovine ephemeral fever virus. *Onderstepoort Journal of Veterinary Research*, 46:191-198.

30. THEVASAGAYAM,J.A., MERTENS,P.P.C., BURROUGHS,J.N. & ANDERSON,J. 1995 Competitive ELISA for the detection of antibodies against epizootic haemorrhagic disease of deer virus. *Journal of Virological Methods*, 55:417-425.

31. THEVASAGAYAM,J.A., WELLBY,M.P., MERTENS,P.P.C., BURROUGHS,J.N. & ANDERSON,J. 1996 Detection and differentiation of epizootic haemorrhagic disease of deer and bluetongue viruses by serogroup-specific sandwich ELISA. *Journal of Virological Methods*, 56: 49-57.

32. THEVASAGAYAM,J.A., WOOLHOUSE,T.R., MERTENS,P.P.C., BURROUGHS,J.N. & ANDERSON,J. 1996 Monoclonal antibody based competitive ELISA for the detection of antibodies against epizootic haemorrhagic disease of deer virus. *Journal of Virological Methods*, 57:117-126.

33. VENTER,G.J., GROENEWALD,D.M., PAWESKA,J.T., VENTER,E.H. & HOWELL,P.G. 1999 Vector competence of selected South African *Culicoides* species for the Bryanston serotype of equine encephalosis virus. *Medical and Veterinary Entomology*, 13:393-400.

34. VILJOEN,G.J. & HUISMANS,H. 1989 The characterization of equine encephalosis virus and the development of genomic probes. *Journal of General Virology*, 70:2007-2015.

35. WILLIAMS,R., DU PLESSIS,D.H. & VAN WYNGAARDT,W. 1993 Group-reactive ELISAs for detecting antibodies to African horsesickness and equine encephalosis viruses in horses, donkey, and zebra sera. *Journal of Veterinary Diagnostic Investigation*, 5:3-7.

36. WRIGHT,P.F., NILSSON,E., VAN ROOIJ,E.M.A., LELENTA,M. & JEGGO,M.H. 1993 Standardization and validation of enzyme-linked immunosorbant assay techniques for the detection of antibody in infectious disease diagnosis. *Rev.sci.tech.Off.int.Epiz*, 12:435-450.

Appendices

1. ELISA equipment

Cold room/refrigerator	Between 1 and 8°C.
Hot room/incubator	Between 35 and 39°C.
ELISA plates	Nunc Maxisorb flat-bottomed or Dynex flexible U-well plates.
Orbital shaker	Luckhams Rotatest R-100 or equivalent capable of rotating at between 40 and 80 rpm. The shaker should be placed in a walk in hot room/incubator at 35 to 39°C.
Freezer	Any type in the range –30 to -5°C.
Glassware/plastic ware	A selection of flasks (50-1000 mℓ), graduated cylinders (10-2000 mℓ), graduated pipettes 91-10 mℓ) with suitable safety bulbs.
Absorbent paper	Disposable paper towel or cloth, lint-free and non-absorbent.
Digital pipettes	Multi-channel and single-channel pipettes in the range 5-50ul and 50-200ul and suitable pipette tips.
Troughs	Commercially produced, autoclavable polystyrene reagent troughs, which should be washed after the addition of each reagent.
ELISA plate reader system and software	Multichannel spectrophotometer micoplate reader with interference filter of 492nm. Any make linked to a compatible PC.

2. Chemicals

Disinfectant	FAM - Evans Vanodine International - make up fresh at a dilution of 1/240 in tap water.
	Citric acid - about 0.2% in tap water.
Tween – 20	Non-ionic detergent. Any supplier.
Milk powder	Cadburys Marvel or similar any supplier.
Phenol red indicator	Any supplier. Make up a 1.0% solution.
Sulphuric acid	Analar specific gravity 1.84 any supplier.
Hydrogen peroxide	Any supplier 30% v/v.
Orthophenylene diamine	Sigma, 2, 4, 30 mg tablets, make up to a final concentration of 0.4mg/mℓ in distilled water.
Coating buffer	0.5M carbonate/bicarbonate pH 9.6 Sigma C-3041. Dissolve the contents of one capsule in 100 mℓ of distilled water and store at room temperature.

3. Reagents

Positive antigen control	Infectious tissue culture fluid store at -30 to-5°C
Negative antigen control	BHK-21 tissue culture fluid store at -30 to -5°C.
Rabbit antiserum	Supplied freeze-dried, store at +1 to 8°C or -30 to -5°C. Once reconstituted, store at -30 to -5°C.
Diluent	PBS supplemented with 0.05 to 0.1% (v/v) Tween 20
Blocking buffer	PBS supplemented with 0.05 to 0.1% (v/v) Tween 20 and 5% Marvel dried milk powder or similar.
Wash buffer	PBS - Dulbecco's phosphate buffered saline pH 7.3-7.4 diluted 1 in 5 with distilled of milleQ water.
Guinea-pig antiserum	Supplied freeze-dried, store at +1 to 8°C or -30 to -5°C. Once reconstituted, store at -30 to -5°C.
Conjugate	Rabbit anti-guinea-pig immunoglobulins, conjugated to horse radish peroxidase (DAKO) store at +1 to 8°C.
Substrate/chromogen	OPD at 0.4mg/mℓ in sterile distilled/milleQ water plus 0.05% Hydrogen peroxide (30%).
Stop solution	1M sulphuric acid.