Comparison of biotinylated monoclonal and polyclonal antibodies in an evaluation of a direct rapid immunohistochemical test for the routine diagnosis of rabies in southern Africa

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I hereby declare that the dissertation/thesis, which I hereby submit for the degree Magister Scientiae (Microbiology) at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

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Summary

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The etiological agent of rabies, rabies virus, is a member of the fatal *Lyssavirus* genus that accounts for the death of more than 55 000 humans per annum, with the number of infected animals far exceeding that number. The process of post-mortem diagnosis of rabies plays a crucial role in general disease surveillance as well as in the implementing and monitoring of disease control programs in animal populations. Although post mortem diagnostic techniques play a crucial role in impeding disease spread, the routine diagnosis of rabies in resource-limited developing countries remains limited due to a lack of stable infrastructures, power supplies, technical expertise and general resources required to perform the routine gold standard fluorescent antibody test (FAT) diagnosis. Based on the aforementioned facts, the development of diagnostic assays that are suitable for application in the resource-limited developing countries has recently gained a lot of consideration, with numerous novel assays being developed and applied in small-scale investigations. Of all the novel diagnostic assays, the direct rapid immunohistochemical test (dRIT) has, to date, shown the most promise in terms of applicability because of its diagnostic sensitivity and specificity, which has been

shown to be equal to that of the FAT in five pilot studies. The main drawback with the current application of the dRIT diagnostic assay is that the Centres for Disease Control and Prevention (CDC) are the only supplier of the required cocktail of two-biotinylated monoclonal antibodies. The singular source of biotinylated antibody thus limits the widespread application of the dRIT diagnostic assay because of the limited availability of the cocktail of biotinylated antibodies.

This study endeavoured to ascertain whether an alternative antibody preparation could be biotinylated and applied to the dRIT diagnostic assay in order to act as a routine replacement for the cocktail of biotinylated monoclonal antibodies supplied by the CDC. In order to gain comparative data pertaining to the diagnostic efficacy and versatility of the dRIT diagnostic test relying on the locally produced biotinylated polyclonal antibody, the research involved a multi-faceted investigation. The investigated facets included the comparison of the dRIT test relying on the locally produced biotinylated polyclonal antibody to the FAT test. Apart from the comparison to the gold standard FAT, the three versions of the dRIT test, each relying on one of three-biotinylated antibodies used in the study was performed. The antibodies involved in the comparison included the locally produced biotinylated polyclonal antibody preparation as well as the two-biotinylated monoclonal antibodies (monoclonal antibody 1 and monoclonal antibody 2) that make up the antibody cocktail supplied by the CDC. Apart from the said investigation into the diagnostic efficacy of the dRIT diagnostic assay, the versatility of the given assay was also investigated by adapting the standard operating procedure to accommodate an acetone fixation step. The sample set used for the study included a significant number of central nervous system (CNS) tissues samples (n=250) derived from five of the major mammalian reservoir species in southern Africa as well as a subset of CNS tissue samples derived from mice inoculated with seven representative African rabies-related lyssavirus isolates. The results indicated that the dRIT diagnostic assay, relying on the biotinylated polyclonal antibody preparation, had a diagnostic sensitivity (100%) and specificity (100%) that was marginally higher than that of the widely recognised gold standard FAT diagnostic test that had produced a single false negative result (diagnostic sensitivity of 99,5%) once applied to the known true positive and negative samples included in the study. The dRIT diagnostic test, relying on either of the two-biotinylated monoclonal antibodies, had reduced levels of diagnostic efficacy compared to the FAT assay in terms of the sensitivity of the given assays (monoclonal antibody 1: 83,08% and monoclonal antibody 2: 90,55%) once applied to the known true positive and negative samples included in the study. Monoclonal antibody 2, once applied to the dRIT assay in this study, was the only antibody to produce a single false positive result (diagnostic specificity of 97,96%). The adaptation of the dRIT protocol to include the acetone fixation step had no influence on

the diagnostic efficacy of the dRIT test, while the results of the study were indicative of the fact that the dRIT diagnostic assay could be used to detect the viral antigen of all the representative rabies-related viruses, irrespective of the biotinylated antibody used.

While the data obtained from the study was used to interpret the diagnostic efficacy of the various biotinylated antibodies applied to the dRIT diagnostic assay, a simulation framework was also developed to analyse the costs involved in performing routine rabies diagnosis with either the FAT or dRIT diagnostic tests in order to broaden the scope of the research. The only cost in the simulation framework that resulted in a significant difference between the two assays was the capital expenditure required to set up a new diagnostic facility, with the costs indicating that five dRIT diagnostic facilities could be established for the price of one FAT diagnostic facility.

In summary, the work presented in this study has shown that not only is it indeed possible to apply alternative biotinylated antibody preparations to the dRIT diagnostic assay, but that it is also necessary to optimize the concentration of the biotinylated antibody preparation of the dRIT diagnostic assay before routine application can occur. In the case of this specific study, the dRIT diagnostic assay relying on the biotinylated polyclonal antibody preparation was shown to be an ideal complimentary diagnostic assay to the FAT due to its high diagnostic efficacy, adaptability and calculated costs.

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Abbreviation list

% - percentage

µl – microliter

ABC - avidin-biotin-complex technique

ABLV – Australian bat lyssavirus

AEC - 3-amino-9-ethylcarbazole

AMV-RT – avian myeloblastosis virus reverse transcriptase

ARC – Agricultural Research Council

AU – absorbance unit

BLAST – Basic Local Alignment Search Tool

BOV – *Bos taurus* (bovine)

BSL – bio-safety level

CAN - *Canis familiaris* (domestic dog)

CDC – Centres for Disease Control and Prevention

cDNA – complimentary deoxyribonucleic acid

CI – confidence interval

CMES - *Canis mesomelas* (Black-backed jackal)

CNS – central nervous system

CO_2 – carbon dioxide

CPEN - *Cynictis penicillata* (Yellow mongoose)

CVS – challenge virus strain

DALY – Disability Adjusted Life Years

DNA – deoxyribonucleic acid

dNTP – dioxirybonucleotide triphosphate

dRIT – direct, Rapid Immunohistochemical Test

DUVV – Duvenhage virus

EtBr – ethidium bromide

EtOH - ethanol

EDTA – ethylene diamine tetraacetic acid

FAT – Fluorescent Antibody Test

FAVN – Fluorescent Antibody Virus Neutralizing test

FEL - *Felis domesticus* (domestic cats)

FITC – fluorescein isothiocyanate

g – gravitational force

hn-PCR – hemi-nested polymerase chain reaction

HRP – horseradish peroxidase

ICTV – International Committee for the Taxonomy of Viruses

IHC – immunohistochemistry

IKOV – Ikoma lyssavirus

LAMP – Loop mediated isothermal amplification of DNA

LBV – Lagos bat virus

MEGA – Molecular Evolutionary Genetic Analysis

mg – milligram

MgCl – Magnesium chloride

MIT – mouse inoculation test

ml – millilitre

mmol - millimole

MOKV – Mokola virus

mRNA – messenger RNA

MW – molecular weight

nm – nanometer

NaOAc – Sodium acetate

NJ – neighbour joining

Nt – nucleotide

OIE – World Organization for Animal Health

OMEG - *Otocyon megalotis* (Bat-eared fox)

OVI – Onderstepoort Veterinary Institute

PAP - peroxidase-anti-peroxidase technique

PBS – phosphate buffered saline

PCR – polymerase chain reaction

PV – Pasteur virus

RABV – rabies virus

RREID – Rapid Rabies Enzyme Immuno-diagnostic assay

RFFIT – Rapid Fluorescent Focus Inhibition Test

RNA - ribonucleic acid

RNP - ribonucleoprotein

RT-PCR – reverse transcription polymerase chain reaction

RTCIT – Rabies Tissue Culture Infection Test

SADC – Southern African Development Community

SOP – standard operating procedure

SHIBV – Shimoni bat virus

VNA – Virus Neutralising Antibodies

WCBV – West Caucasian bat virus

WHO – World Health Organization

Chapter I

Literature Review

Section A: Lyssaviruses

1.1. Lyssavirus classification

The *Lyssavirus* genus is one of the six genera in the *Rhabdoviridae* family*,* with the genus being characterized by members with elongated and bullet shaped viral particles. The rhabdoviruses [Greek: *rhabdos* (rod)] belong to the order *Mononegavirales,* which contains the single stranded, (-) sense, non-segmented, viruses (Tordo, Poch, Ermine, Keith, & Rougeon, 1988). The *Lyssavirus* genus consists of twelve unique viral species, namely rabies virus, Lagos bat virus, Mokola virus, Duvenhage virus, European bat lyssavirus 1, European bat lyssavirus 2, Australian bat lyssavirus, Khujand virus, Irkut virus, Aravan virus, West Caucasian bat virus and Shimoni bat virus (Dietzgen et al., 2011). Apart from the twelve unique lyssavirus species, the genus also contains three putative species namely Ikoma lyssavirus, Bokeloh virus and Lleida bat lyssavirus (Ceballos et al., 2013; Freuling et al., 2011; Marston, Ellis, et al., 2012; Marston, Horton, et al., 2012). The International Committee on Taxonomy of Viruses (ICTV) is currently investigating whether the three putative species are possibly the 13th - 15th species of the *Lyssavirus* genus.

1.2. Lyssaviruses present on the African Continent

1.2.1. Rabies virus (RABV)

The prototype lyssavirus, rabies virus [Latin: *rabere* (to rage)], is the most well known member of the lyssavirus genus that causes rabies. The RABV is known to have a worldwide distribution (with the exception of several islands), but the disease remains endemic in developing countries across the globe. Molecular and immunogenicity studies, performed in southern Africa, have shown that there is not a single variant of the RABV circulating among various host species, but rather that the process of genetic adaptation has resulted in the formation of two predominant variants (von Teichman, Thomson, Meredith, & Nel, 1995). The main variants that circulate predominantly in southern Africa are the canid- and mongoose variants of the RABV (Davis, Rambaut, Bourhy, & Holmes, 2007).

The canid variant is known to predominantly infect carnivores belonging to the *Canidae* family, with the wide range of reservoir hosts consisting of domestic dogs (*Canis familiaris*), black-backed jackals (*Canis mesomelas*), side-striped jackals (*Canis adustus*) and the bat-eared foxes (*Otocyon megalotis*) to mention but a few (Davis et

al., 2007). The reservoir host of the canid variant of the RABV is determined by the geographical location of the specific reservoir host as illustrated in earlier research (C T Sabeta, Bingham, & Nel, 2003). The mongoose variant was initially referred to the as the “viverrid biotype”, but it was noted that the mongoose variant was specifically adapted to the *Herpestidae* family of mammals consisting particularly of the yellow mongoose (*Cynictis penicillata*) and the slender mongoose (*Galarella sanguinea*) (Nel et al., 2005). The two variants were initially separated purely based on their different host reservoir ranges but more recent research highlighted distinct differences between the two variants in terms of their genetic and antigenic characteristics (Nel et al., 2005; Swanepoel et al., 1993; van Zyl, Markotter, & Nel, 2010; von Teichman et al., 1995). Although both the canid and mongoose variants of the RABV have been circulating in their respective hosts for more than a hundred years, a recent study based on molecular clocking has shown that the mongoose variant had been present in South Africa before the canid variant was introduced (van Zyl et al., 2010).

In the case of the RABV it should be noted that although some developing countries have effective and economical control measures in place (Bogel & Meslin, 1990; Cleaveland, Kaare, Tiringa, Mlengeya, & Barrat, 2003), most lack the basic diagnostic facilities required to accurately diagnose whether a specimen has been infected with the RABV. The number of reported deaths from rabies virus infections is greatly understated due to a lack of available facilities and diagnostic techniques, and this results in the major underestimation of the true incidence of the disease (Knobel et al., 2005). The World Health Organization (WHO) initiated an investigation into the mortality rates due to rabies infections in developing countries by utilizing a series of probability steps in order to determine the deaths attributed to rabies infections annually (Knobel et al., 2005). The probability steps were applied to a small region within the African continent and the results were then extrapolated. Based on the results obtained, it was concluded that in developing countries human deaths as a result of rabies number far into the tens of thousands annually. In addition the number of infected animals in these developing countries far exceeds this value even though vaccine distribution and virus eradication programs have been implemented in only a small number of the countries investigated (Knobel et al., 2005). The numbers may not seem significant in comparison to other infectious diseases present in developing countries, but if the metric of the DALY (Disability Adjusted Life Years) score is considered, the severity of the disease becomes apparent (Lembo et al., 2010). The DALY score takes into account both the disability and death caused by a disease, where the morbidity/disability is given different weightings based on a standardized scale of ability to perform certain functions and the duration of the illness. It can thus be stated that the

standard scale enumerates different degrees of disability and death caused by a disease (Murray, 1994). Most of the rabies DALY burden is attributed to death, rather than morbidity, as there is a short duration in terms of clinical disease (Lembo et al., 2010). Decision-tree models applied to the data from East Africa and globally indicate that the DALY burden for rabies exceeds that of neglected zoonotic diseases such as Onchocerciasis, Chagas disease, Dengue fever and leprosy (Coleman, Fevre, & Cleaveland, 2004; Fevre et al., 2005; Knobel et al., 2005; Lembo et al., 2010).

1.2.2. Lagos bat virus (LBV)

The rabies-related lyssavirus, LBV, was first isolated from a straw-coloured fruit bat (*Eidolon helvum*) on the Nigerian island of Lagos (Boulger & Porterfield, 1958), but it wasn't until 1970's that LBV was classified as a member of the *Lyssavirus* genus (Shope et al., 1970). Since then, seropositivity towards the virus has been demonstrated in various bat species such as the straw-coloured fruit bat (*Eidolon helvum*), Gambian epauletted fruit bat (*Epomophorus gambianus*) and Dobson's epauletted fruits bats (*Epomops dobsoni*) (Dzikwi et al., 2010; Johnson et al., 2010). Even though the first LBV isolation was made in Nigeria, the virus has been isolated from numerous countries on the African continent such as; Nigeria, Central African Republic, South Africa, Senegal, Zimbabwe, Ethiopia and Egypt (Dzikwi et al., 2010; Markotter, Randles, et al., 2006; Markotter, Kuzmin, Rupprecht, & Nel, 2008). Although LBV is known to utilize chiropteran hosts as the main reservoir host, the virus has also been isolated directly from various host species such as cats, dogs and mongoose. While numerous spill over events have been documented, LBV remains to be implicated in a zoonotic event (Markotter, Randles, et al., 2006).

1.2.3. Mokola virus (MOKV)

Another lyssavirus species, MOKV, was first isolated in the Mokola forest situated in the district of Ibadan, Nigeria. The virus was initially isolated from an organ pool of a white-toothed shrew (*Crocidura spp*) in the late 1960's (Kemp, Causey, Moore, & Fabiyi, 1971; Shope et al., 1970), after which the virus was isolated from shrews (Kemp et al., 1971), domestic cats (Foggin, 1988; Meredith, Nel, & von Teichman, 1996; C T Sabeta et al., 2007) dogs (Foggin, 1988; Sabeta et al., 2007) and a rodent (Saluzzo et al., 1984). Since the initial discovery, no less than seventeen different isolates of MOKV has been discovered, with ten occurring in South Africa and seven in Zimbabwe (C T Sabeta et al., 2010). Although MOKV remains to be isolated from bats

(C T Sabeta et al., 2007), the reservoir host of the specific virus is still not known as all hosts from which the virus have been isolated have been dead-end hosts (Nel & Markotter, 2007). Despite the relatively high amount of MOKV isolations that have been made, only one confirmed human fatality has been associated with the specific member of the lyssavirus genus (C T Sabeta et al., 2010).

1.2.4. Duvenhage Virus (DUVV)

The rabies-related lyssavirus, DUVV, was first isolated in the 1970's from a fatal human case after an elderly man was bitten by an unidentified species of bat in South Africa (Meredith, Prossouw, & Koch, 1971). Post-mortem diagnosis with the fluorescent antibody test had no positive fluorescent reaction, but histological staining indicated the presence of Negri bodies in the brain material (Tignor et al., 1977). To date, six isolates of DUVV exist, with three isolates originating from bats (Foggin, 1988; van der Merwe, 1982) and three isolates originating from fatal human cases (Meredith et al., 1971; van Thiel et al., 2008). Of the available isolates, only two had been found outside the borders of South Africa. The first case occurred in Zimbabwe when the virus was isolated from an Egyptian slit-faced bat (*Nycteris thebaica*) (Foggin, 1988), while the second isolate identified outside the borders of South Africa occurred after a woman came into contact with an unknown bat in Kenya (van Thiel et al., 2008).

1.2.5. West Caucasian bat virus (WCBV)

The WCBV was initially isolated from the western Caucasus Mountains, Russia, in 2002 from an apparently healthy bat (*Miniopterus schreibersii*). Although never isolated on the African continent, positive seropositivity for WCBV was found during a survey of *Miniopterus spp.* present in Kenya. The presence of neutralizing antibodies towards WCBV was found in 17-26% of the bats samples (I V Kuzmin et al., 2008).

1.2.6. Shimoni bat virus (SHIBV)

In 2009, a new lyssavirus species was discovered during a survey of bats in Kenya. The single SHIBV isolate was identified during a process of screening of samples obtained from striped leaf-nosed bat (*Hipposideros vittatus*) from a coastal region of the Kenyan country, and phylogenetic analysis revealed that the virus is placed between LBV and MOKV (Kuzmin et al., 2010). Subsequent studies provided

serological evidence that *Hipposideros vittatus* (previously reported as *Hipposideros commersoni*) was most likely the reservoir for SHIBV (Ivan V Kuzmin et al., 2011).

1.2.7. Ikoma lyssavirus (IKOV) (putative species)

In 2009, an African civet (*Civettictis civetta*), showing symptoms consistent with rabies, was killed in the Ikoma ward within the Serengeti National Park in Tanzania. The central nervous system tissue was collected and used repeatedly in a training course at the Central Veterinary Laboratory. The fluorescent antibody test was used to detect the presence of lyssavirus antigen in the brain material and a subsequent phylogenetic analysis of the nucleoprotein gene region was performed based on Bayesian reconstruction. The phylogenetic analysis indicated that the sequence was unique and most closely related to WCBV (Marston, Ellis, et al., 2012; Marston, Horton, et al., 2012).

1.3. Antigenic sites associated with lyssaviruses

The rabies virus particle is known to have an average diameter of 75 nm with a varying length of 100-300 nm (Tordo et al., 1988). The viral genomic material consists of approximately 12 000 nucleotides (nt), that encode 5 viral proteins, namely the nucleoprotein (N-gene), phosphoprotein (P-gene), matrix protein (M-gene), glycoprotein (G-gene) and the RNA-dependent RNA polymerase (L-gene). Although all the viral proteins are antigenic (capable of eliciting an immune response), only certain components play a crucial role in this regard (Celis, Rupprecht, & Plotkin, 1990). The basic function and antigenic properties of each viral protein will be discussed briefly.

1.3.1. Nucleoprotein

The nucleoprotein (N protein) is responsible for the formation of the viral nucleocapsid (consisting of the nucleoprotein, phosphoprotein and RNA-dependent RNA polymerase), which is directly involved in the encapsidation of the negative sense, single stranded RNA genome. Based on the fact that the nucleocapsid is a major internal component of the rabies virus, it has been shown that the nucleoproteins making up the capsid layer has group-specific antigenic determinants that are shared among all the rabies and rabies-related viruses (Flamand, Wiktor, & Koprowski, 1980; Goto et al., 2000). The initial investigation of the nucleoprotein has led to the discrimination of four conserved antigenic sites on the viral protein (Lafon & Wiktor,

1985; Minamoto et al., 1994). The antigenic sites I and IV have been shown to be composed of linear epitopes, while the antigenic sites II and III have been shown to be composed of conformation-dependent epitopes (Minamoto et al., 1994). Although the glycoprotein is classified as the most important antigen of the rabies virus, the nucleoprotein has the advantage of being less variable than other antigens making it an ideal target for diagnostic techniques relying on the detection of antigen (Dietzschold et al., 1987).

1.3.2. Phosphoprotein

The phosphoprotein of the rabies virus plays two functional roles in terms of the rabies virus replication cycle. The first function of the protein is to act as a co-factor of the RNA-dependent RNA polymerase, and the second is to form a link with the nucleoprotein complex, preventing the nucleoprotein from self-aggregating and binding to cellular RNA (Dietzgen et al., 2011). The phosphoprotein is a relatively non-conserved protein, which has historically been poorly characterized in terms of the antigenicity associated with the protein. Previous studies have shown that the phosphoprotein has at least four linear and conformation-dependent epitopes. The first antigenic site (site I) was shown to broadly cross-react between all the lyssavirus species involved in their study. The third and fourth antigenic site (site III and IV) exhibited highly variable levels of immunoreactivity, indicating a lower level of conserved epitopes at the specific locations. The second antigenic site (site II), located in a highly conserved central region of the phosphoprotein has been shown to be lyssavirus strain specific, which in turn highlighted the potential of the antigenic site as a tool for lyssavirus identification and discrimination (S. A. Nadin-Davis et al., 2000).

1.3.3. RNA-dependent RNA polymerase

The transcription of the negative sense lyssaviruses is facilitated by the RNA-dependent RNA polymerase enzyme, which is incorporated in the ribonucleoprotein (RNP) complex. The production of complementary mRNA enables the translation process of the viral proteins to begin in earnest, which in turn ensures the continued proliferation of the virus (Tordo, 1996). Although the different components of the ribonucleoprotein complex (nucleoprotein, phosphoprotein and the RNA-dependent RNA polymerase) each have their own unique antigenic sites, with varying degrees of conservation, the ribonucleoprotein complex (aka nucleocapsid) has been shown to be a highly effective elicitor of the host immune response. This is largely due to the fact

that the antigenic sites associated with the ribonucleoprotein complex are not only the most abundant antigen in infected cells, but they are also known to cross-react with all members of the *Lyssavirus* genus that are presently known (Dietzgen et al., 2011).

1.3.4. Matrix protein

The matrix protein is a major structural component of the rabies virus believed to be directly involved in the assembly and budding of the virus during the infection process (Mebatsion, Weiland, & Conzelmann, 1999). The role of the matrix protein in the assembly of new viral particles has been shown to be due to the proteins ability to bind to and condense the preformed ribonucleoprotein complex (Lenard, 1996). Apart from binding to the ribonucleoprotein complex, the matrix protein has also been shown to bind to the host cell plasma membrane (Odenwald, Arnheiter, Dubois-Dalcq, & Lazzarini, 1986), which enables the matrix protein to play a functional role in the viral assembly and budding of the virus (Mebatsion et al., 1999).

1.3.5. Glycoprotein

The glycoprotein is the only surface spike protein of the rabies virus, which is known to play a crucial role in the binding of the virus to the host cell receptors in order to facilitate the infection process (Dietzgen et al., 2011). The glycoprotein is considered the most important antigenic site of the rabies virus due to the fact that it is responsible for the induction of the host antibody response while being the target for the hosts neutralising antibodies (Gaudin, Ruigrok, Tuffereau, Knossow, & Flamand, 1992). The glycoprotein contains four known major and one minor antigenic sites (Lafon, Wiktor, & Macfarlan, 1983). The first antigenic site (site I) contains both conformation-dependent and linear epitopes. The second antigenic site (site II) consists of two domains (IIa and IIb), which form a discontinuous conformation-dependent epitope. The third antigenic site (site III) is a continuous antigenic site, but no site-specific monoclonal antibodies have been found to bind to the unfolded protein. The fourth antigenic site (site IV) is known to consist of only two amino acids containing two overlapping linear epitopes. The minor antigenic site (site ‘a’) is located in a close proximity to antigenic site III, but does not contain any overlapping epitopes (Benmansour et al., 1991). Although the glycoprotein contains numerous antigenic sites, it is not an ideal antigenic site for diagnostic techniques relying on the detection of antigens. This is due to the fact that the proteins are relatively conserved within, but not between the different viral species of the lyssavirus genus (Dietzgen et al., 2011).

1.4. Diagnosis of lyssaviruses

The process of lyssavirus diagnosis is predominantly performed once disease symptoms are present, at which stage there is no effective and proven treatment that can prevent death in the infected host. Despite this fact, the post-mortem diagnosis of rabies plays a crucial role in the general surveillance of the disease spread across a geographical area, and in the formulation of RABV control programs in animal populations, whereby vaccine is distributed among animal populations that run a risk of being infected by the disease. The constant monitoring of disease control programs in animal populations, by means of accurate diagnosis, is thus involved in the prevention of human exposure by ensuring that post-exposure prophylaxis is efficiently distributed to specific humans that have come into contact with infected animal species.

In humans, ante-mortem diagnosis is usually undertaken in the form of a PCR assay relying on samples obtained from various sources such as the saliva, cerebrospinal fluid, nuchal biopsies and in rare occasions excreta such as urine and tears (Wacharapluesadee & Hemachudha, 2010). Post-mortem diagnostic methods are most often based on collected brain samples (and/or nuchal biopsies in the case of humans) believed to be infected with the virus, and is performed predominantly in the case of animals animals (WHO, 2005).

1.5. Post-mortem diagnosis in humans and animals

The RABV is a highly neurotropic virus that causes fatal encephalitis due to the fact that the clinical signs of a rabies infection only manifest once the virus has reached the central nervous system and started the proliferation process (WHO, 2005). As a consequence, a central nervous system (CNS) tissue sample is used for the process of post-mortem diagnosis where the presence of antigenic or molecular evidence is needed as proof of viral infection.

1.5.1. Antigen detection

Prior to 1804, the diagnosis of rabies was based solely on the observation of clinical signs, with the first crude diagnostic test being developed thereafter. Georg Gottfried Zinke experimentally transferred the disease to dogs and rabbits by using the saliva of rabid animals to infect cutaneous wounds of healthy animals (Baer, 1991). Numerous refinements were made to this diagnostic test over the following years by the work of scientists such as Victor Galtier (1881) and Louis Pasteur (1884) who

transmitted the rabies virus via intramuscular and intracerebral routes respectively. These tests were key in proving that rabies was caused by a microscopic pathogen, but it was not until 1903 that the first specific rabies diagnostic test was developed (Baer, 1991; Negri, 1900).

1.5.1.1. Histopathological diagnosis

Adelchi Negri (1903) developed the diagnostic method based on the detection of inclusion bodies within the cytoplasm of large neurons of the CNS. The RABV is known to cause the appearance of specific inclusion bodies that are more commonly known as Negri bodies in the cytoplasm of infected nerve cells. Based on the presence of the Negri bodies in RABV infected CNS tissues, a histopathological test more commonly known as the Seller's Stain was developed in order to facilitate the diagnosis of rabies (Negri, 1900; Tierkel, 1973). The diagnosis of rabies based on the presence/absence of Negri bodies was the sole confirmatory test for the antigen until 1958 when Goldwaser and Kissling developed a direct immunofluorescent method for the detection of rabies antigen distributed in squash preparations of infected brain material (Goldwasser & Kissling, 1958). The development of the fluorescent antibody test indicated that the Seller's stain was inadequate in terms of rabies diagnosis due to the poor diagnostic efficacy associated with the high amount of false negative results produced by the assay (WHO, 2005). A retrospective study of 25 292 Seller's stains, confirmed by FAT diagnosis, found that the diagnostic sensitivity and specificity of the Seller's stain was 80,26% and 94,54% respectively (Tepsumethanon, Lumlertdacha, & Wilde, 2004). Even though the Seller's stain has been discontinued by the World Health Organization (WHO) (WHO, 2005), some developing countries still rely on the histopathological diagnosis due to the lack of resources (such as facilities/reagents) required to implement more advanced diagnostic techniques (Tepsumethanon et al., 2004). The reduced diagnostic sensitivity (originating from a high number of false negative results) is a worrying finding because approximately 90% of all human fatalities due to rabies occur in developing countries.

1.5.1.2. Fluorescent Antibody Test (FAT)

The FAT diagnostic assay was developed by Goldwasser and Kissling in 1958 (Goldwasser, Kissling, & Carski, 1959; Goldwasser & Kissling, 1958), and has since then developed to be the only diagnostic technique that is recommended by both the WHO (WHO, 2005) and the World Organization for Animal Health (OIE) (OIE, 2008).

The FAT is routinely performed on either fresh or glycerolized brain tissues derived from multiple segments of the CNS. The brain tissues that are used to create composite impressions include the cerebellum, medulla, hippocampus and thalamus (Bingham & van der Merwe, 2002). The FAT relies on the treatment of CNS tissues with anti-lyssavirus antibodies that interact with the viral antigen present in the CNS tissue. The anti-lyssavirus antibodies are labelled with fluorescein isothiocyanate (FITC), which is more commonly acknowledged as the conjugate that enables the RABV antigens in brain tissues to be viewed under a fluorescent microscope once the anti-lyssavirus antibodies interact with the antigen (Dean, Abelseth, & Atanasiu, 1996; Robardet et al., 2013).

The FAT diagnostic test is undertaken in many laboratories globally but is particularly suited to developed countries due to a stable infrastructure (consisting of a stable power supply, easy access to running water, good quality waste disposal), efficient sample transportation (consisting of efficient cold chains) and diagnosis in well-equipped laboratories. In contrast, most laboratories in developing countries lack stable power supplies, equipment and competent operators required to facilitate efficient rabies diagnosis. The lack of infrastructure in most developing countries has resulted in certain drawbacks associated with performing the FAT, such as power spikes as a result of an unstable power supply. These electrical surges can interfere with the efficient operation of microscopes (Durr et al., 2008; Lembo et al., 2006). Another drawback associated with the FAT technique is the need for a cold chain to transport the samples from the field laboratory to the established laboratories. The lack of an adequate cold chain in most developing countries makes sample transportation, as well as ensuring quality of the reagents, extremely difficult. As such, numerous chemical preservatives are employed to maintain sample integrity such as glycerol and formalin. Although numerous other diagnostic techniques have been developed, they are considered to be either confirmatory test used to confirm the FAT results, or techniques that are still being developed and evaluated to their fullest potential as diagnostic techniques.

1.5.1.3. Immunohistochemical test (IHC)

IHC diagnosis relies on the staining of tissue samples with either alkaline phosphatase- or peroxidase-conjugated lyssavirus antibodies in order to allow the stained antigens to be viewed under a compound light microscope. The two techniques that are used most often are the Peroxidase-Anti-Peroxidase (PAP) technique (Bourgon & Charlton, 1987) and the Avidin-Biotin-Complex (ABC) technique (Fekadu, Greer, Chandler, & Sanderlin, 1988). Although both the PAP and ABC techniques have been

applied to a wide range of samples from across the globe, the ABC technique has been used most often to confirm the presence of RABV antigen after the FAT had been performed (Fekadu et al., 1988; Hamir & Moser, 1994; Sinchaisri, Nagata, Yoshikawa, Kai, & Yamanouchi, 1992; Stein, Rech, Harrison, & Brown, 2010). Through various adaptive processes, the ABC diagnostic test has been adapted and developed into the technique known as the direct, rapid immunohistochemical test (Niezgoda & Rupprecht, 2006).

1.5.1.4. Direct, rapid immunohistochemical test (dRIT)

The standard ABC technique has been adapted in order to develop the relatively new diagnostic technique known as dRIT. Although the dRIT diagnostic test still detects the RABV antigen based on an immunoperoxidase product (Durr et al., 2008; Lembo et al., 2006; Madhusudana, Subha, Thankappan, & Ashwin, 2012; Saturday, King, & Fuhrmann, 2009), there are slight differences between the original IHC tests and the dRIT. The main difference is that the original IHC test requires a linker-antibody conjugated to the biotin moiety, while the dRIT utilizes an anti-rabies antibody that is directly labelled with a biotin-moiety. The biotinylated antibody is then treated with a streptavidin protein that has an extremely high affinity for biotin. The streptavidin protein contains a reporter enzyme that is used to initiate an insoluble colour precipitation once the antibodies are bound to the rabies virus antigens in the presence of an appropriate substrate.

The main advantage of the dRIT test is that the diagnostic process relies on a compound light microscope rather than an expensive fluorescent microscope. The conjugate required for each of the two diagnostic techniques also influences the quality of the two tests. This is because the fluorescein-labelled antibodies lose their fluorescent capabilities as time passes, while dRIT samples can be kept for retrospective analysis due to the fact that the staining does not fade (Last, Jardine, Smit, & van der Lugt, 1994).

1.5.1.5. Lateral flow immunochromatography

Several rapid test kits relying on the principle of lateral flow immunochromatography have been developed and utilised with varying degrees of success in terms of both the diagnostic sensitivity and specificity. The lateral flow immunochromatography (also known as the “rapid test kit”) relies on the premises of gold conjugated detector antibodies that interact with the RABV antigenic sites present

in the sample being tested. The main advantage of the immunochromatography tests is that they can be used to obtain quick results while being performed by a technician at field laboratory with only the bare minimal equipment present. Although the use of the rapid diagnostic test kit under field conditions is a clear advantage, the WHO does not recommend the application of the rapid test kit for the routine diagnosis of rabies. Preliminary results have shown that although the rapid test kit has a relatively diagnostic sensitivity in certain instances, 74%-99% (Ahmed, Wimalaratne, Dahal, Khawplod, & Nanayakkara, 2012); 91,7% (Kang et al., 2007); 93,2% (Nishizono et al., 2008); 88,3% (Servat et al., 2012), it is not as high as the sensitivity found in the FAT (98-100%) (Ahmed et al., 2012; Kang et al., 2007; Markotter et al., 2009; Nishizono et al., 2008; Servat et al., 2012; WHO, 2005). The lowered diagnostic sensitivity, associated with an increase in false negative results, makes the use of lateral flow immunochromatography for post-exposure confirmation in human populations ill advised, as the results are not conclusive. Although controversial, the rapid test kits might be considered ideal for some developing African countries because a much lower level of training is required to enable a technician to perform the diagnostic test. The rapid tests kits can thus allow facilities that do not possess FAT capabilities the opportunity to perform active rabies surveillance in animals by either performing routine diagnosis in a designated diagnostic facility or at a basic in field laboratory that is situated closer to regions where regular rabies outbreaks occur.

1.5.1.6. Antigen capture ELISA

One of the most widely used antigen capture ELISA assay, better known as the rapid rabies enzyme immune-diagnosis (RREID), is an antigen capture ELISA that relies on polyclonal sera of rabbits that have been hyperimmunized against the nucleocapsid of the Pasteur virus (PV) strain of rabies (Perrin, Rollin, & Sureau, 1986). The diagnostic assay was shown to have a lower limit of detection of 2 ng/ml of RNP, but the assay had a reduced sensitivity pertaining to the antigen from the rabies-related lyssaviruses (Bourhy, Rollin, Vincent, & Sureau, 1989; Jayakumar, Ramadass, & Raghavan, 1989; Oelofsen & Smith, 1993).

A more recent assay, called the WELYSSA, is based on the same principle as the RREID assay but uses a cocktail of four mouse monoclonal antibodies instead of the rabbit polyclonal serum. The pilot study relying on the WELYSSA assay indicated that the assay had a lower limit of detection of 0,8 ng/ml, while the assay had a diagnostic sensitivity and specificity of 97% and 99% respectively (Xu et al., 2007). Apart from the panel of 1030 samples (67 positive and 963 negative) the assay also accurately detected representative rabies-related viruses such as LBV, MOKV, DUVV,

EBLV-1, EBLV-2 and ABLV, which was a marked improvement on the RREID assay (Xu et al., 2007).

1.5.2. Virus isolation

1.5.2.1. Mouse Inoculation Test (MIT)

In order to confirm doubtful diagnostic results, whereby the presence or absence of viral inclusion could not be confirmed by the FAT, the MIT is recommended during which suckling mice are injected intracerebrally with a suspected rabies specimen (W A Webster, Casey, & Charlton, 1976). After an observation period of 21-28 days, the mice that survived are euthanized and the brain tissue subjected to the FAT diagnostic test. In the case of a potential rabies infection, where time is a crucial factor, the MIT test is not a sufficient diagnostic technique on its own and as such it is used as a backup diagnostic technique.

1.5.2.2. Rabies tissue culture isolation test (RTCIT)

Briefly, RTCIT relies on cultivating the viral isolates on murine neuroblastoma cells due to the fact that the specific cell line is the most susceptible cell line for field samples (W.A. Webster & Casey, 1973). After a predetermined time period has elapsed (normally 3 - 4 days), the neuroblastoma cells are tested for the presence of viral infection by FAT. The RTCIT method of cultivating live virus has the advantage of being able to deliver diagnostic results much quicker than the MIT (MIT test: 21 - 28 days vs. RTCIT: 3 - 4 days), while the use of cell lines to cultivate viral isolates have been shown to be equally effective (WHO, 2005).

1.5.3. Molecular Techniques

Molecular techniques based on the amplification of the viral nucleic acid are important diagnostic tools that can be used for rabies diagnosis in animals. The most important and widely used method is the polymerase chain reaction (PCR) (S A Nadin-Davis, Huang, & Wandeler, 1996; Nel, Bingham, Jacobs, & Jaftha, 1998). Various adapted amplification techniques have been developed for diagnostic purposes. These techniques include: Real-time PCR (Coertse, Weyer, Nel, & Markotter, 2010), nested PCR, hemi-nested PCR (Heaton et al., 1997; Wacharapluesadee & Hemachudha, 2010) and loop mediated isothermal amplification of DNA (LAMP) (Boldbaatar et al.,

2009; Saitou et al., 2010). The main drawback associated with the amplification of nucleic acid is the fact that they are all reactions that require a complicated mixture of reagents, which make them more prone to contamination when setting up the required reactions. A crucial pre-requisite of molecular techniques is that the integrity of all the reagents must be maintained. This factor is complicated in developing countries where effective cold chains are lacking or unavailable. Due to the fact that molecular amplification is virus sequence specific, the molecular techniques also require standardization and stringent quality control in order to maintain the high levels of sensitivity (WHO, 2005). Because of the requirements for trained personnel and expensive equipment, molecular techniques are not ideal in many African settings. Despite the aforementioned drawbacks associated with the molecular amplification techniques, they do have certain advantages such as the ability to distinguish the viral species of the lyssavirus present in the given CNS tissue sample. Apart from the diagnostic aspect, the molecular techniques also allow subsequent phylogenetic analysis to be performed, which plays a direct role in epidemiological studies.

1.6. Ante-mortem diagnosis of rabies in humans

1.6.1. Clinical diagnosis

The clinical features of rabies are easily classified into four separate stages before death occurs in the victim. The first step, known as the incubation phase, is generally no longer than 90 days, while the general consensus is that the incubation period is between two and eight weeks in length. The next phase is known as the prodrome phase, which is generally an uneventful phase characterized by a distinct sensation (tingling, itching, pain, etc.) at the actual bite site in about one third of all cases (Hemachudha, 1994). The following phase, known as the acute phase, can be characterized by one of the two distinct forms of rabies. During this phase, the rabies virus will present itself in either the furious (encephalitic) or the dumb (paralytic) form of the disease. The encephalitic form is characterized by certain key symptoms such as the fluctuation of consciousness, phobic/inspiratory spasms and autonomic stimulation signs (Hemachudha, Laothamatas, & Rupprecht, 2002). The ratio of furious to paralytic forms in humans has been shown to be 3:1 (Hemachudha et al., 2002), while a recent study performed on 957 dogs showed a ratio of 5:2 (Shuangshoti et al., 2013). The acute phase only lasts about a week in the encephalitic cases and about two weeks in the paralytic cases before the last phase of the disease progress, known as the coma phase, is initiated (Weyer & Blumberg, 2007). The diagnosis of the rabies virus on a

clinical basis is unreliable due to the fact that the symptoms that manifest in patients showing signs of a paralytic rabies infection could resemble symptoms to those observed in patients with encephalitis and resulting CNS deterioration (e.g. cerebral malaria), which complicates the diagnostic processes (J S Smith, 1996; WHO, 2005). Based on the aforementioned results, ante-mortem diagnosis of rabies in humans remains important because a timely diagnosis allows effective medical management, involving the application of the correct medical treatment of a specific disease, to occur.

1.6.2. Antigen detection

1.6.2.1. Fluorescent Antibody Test (FAT)

The application of the FAT is used most commonly to detect the presence of rabies antigen in various ante-mortem samples. The most commonly used samples are obtained from nuchal (skin from the nape of the neck) biopsies (Dean et al., 1996). Although the FAT diagnostic technique applied to skin samples have been shown to possess a sensitivity of approximately 100% (Blenden, Creech, & Torres-Anjel, 1986), the use of nuchal biopsies as intra-vitam samples are not ideal. This is due to the fact that at least twenty sections of nuchal skin samples are required for effective diagnosis, while the diagnostic facility needs to possess an extremely specialized cryostat machine in order to prepare the samples (Weyer & Blumberg, 2007). The application of the FAT on corneal impressions has been investigated previously (Mathuranayagan & Rao, 1984), but is not recommended by due to the fact that the results obtained are unreliable (WHO, 2005).

1.6.2.2. Immunohistochemical Test (IHC)

In 2004, an Austrian tourist was believed to be infected with the rabies virus after receiving multiple bite wounds from an infected puppy. Multiple samples (skin biopsy of the neck, CSF, serum and pharyngeal-, nasal- and conjunctival swabs) were taken in order to perform a whole barrage of diagnostic tests. The nuchal sample was subjected to both a FAT and IHC test and it was found that the results obtained from the FAT test revealed only a few specific signals, while the IHC test could be used to clearly distinguish positive epidermal cells at the border of the stratum granulosum and stratum corneum. The researchers found that the immunohistochemical test followed by a complementary RT-PCR should be considered for a potential ante-mortem diagnostic test for the rabies virus (Bago, Revilla-Fernandez, Allerberger, & Krause, 2005).

1.6.3. Virus Isolation

Virus isolation for ante-mortem diagnosis is routinely performed from the saliva of potentially infected patients, but both the antibody status of the specific patient, as well as the intermittent shedding of the RABV particles hamper the sensitivity of the diagnostic process. These factors could potentially deliver false negative results if the sampling is done during the acute phase of the disease progress, and thus virus isolation is not recommended as a routine ante-mortem diagnostic technique by the WHO (WHO, 2005).

1.6.4. Antibody detection

The detection of Virus Neutralizing Antibodies (VNA) is routinely based on either the Rapid Fluorescent Focus Inhibition Test (RFFIT) (J. Smith, Yager, & Baer, 1996) or the Fluorescent Antibody Virus Neutralizing Test (FAVN) (Cliquet, Aubert, & Sagne, 1998). Both the VNA tests rely on the fact that neutralizing antibodies can be found in the serum and cerebrospinal fluid of unvaccinated specimens. As such, the neutralizing antibody levels can be measured using either of the two tests in order to determine whether seroconversion has occurred (WHO, 2005).

1.6.5. Molecular Techniques

Of all the molecular techniques discussed previously, the RT-PCR has been tested in numerous studies whereby the viral genomic material was amplified from samples such as saliva, cerebrospinal fluid or nuchal biopsy specimens. Of all the available sample sources, saliva is the preferred specimen with serial testing an important factor due to the intermittent shedding of viral particles (Crepin et al., 1998). In more recent comparative studies, real-time PCR amplification of viral genomic material has been shown to detect the presence of virus in various ante-mortem samples, resulting in a diagnostic assay that has a diagnostic efficacy equal or better than that of the conventional RT-PCR or nested-PCR (Wacharapluesadee & Hemachudha, 2010)

Section B: Direct, rapid immunohistochemical diagnosis of lyssaviruses

1.7. Direct, rapid immunohistochemical test principle

The dRIT diagnostic assay relies on a series of component and reagents that are applied to a tissue sample in a chronological order in order to ensure that effective diagnostic results are obtained.

1.7.1. Tissue fixation

The fixation process is a crucial component of the dRIT diagnostic test due to the fact that proper fixation plays a role in the prevention of antigen degradation while maintaining and preserving the secondary and tertiary structures of the given antigens. This antigenic preservation in turn allows the maximum amount of interaction between the applied antibodies and the antigens present in the tissue sample (Farmilo & Stead, 2009).

1.7.1.1. Formalin

For IHC diagnostic techniques, the most widely used fixative is 10% neutral buffered formalin, which is a good fixative due to its ability to form cross-linking between proteins in the sample while preventing the breakdown of the tissue due to processes such as autolysis and putrification (Mies, 1994). Although formalin fixation is widely used for dRIT diagnosis, there are certain drawback associated with the fixation method due to the fact that excessive formalin fixation has been shown to destroy antigenic epitopes leading to a general loss in antigenicity (Farmilo & Stead, 2009). The application of the FAT on formalin fixed tissue samples showed signs of a decreased diagnostic sensitivity leading to diagnostic results that were inconclusive in terms of negative results. As such, the application of the FAT diagnostic test on formalin fixed tissue samples requires subsequent digestion with trypsin and pepsin in order to achieve an acceptable level of diagnostic efficacy (Reid, Hall, Smith, & Baer, 1983).

1.7.1.2. Cold acetone

Another fixative that is widely used for the investigation of tissues presumed to contain lyssavirus antigen is cold acetone. Acetone fixation is the recommended fixation method for the FAT diagnostic technique because it is a strong dehydrating agent that is known to irreversibly precipitate the tissue proteins. The precipitation of proteins is

known to enhance the antigen-antibody interaction in the diagnostic test leading to a reaction with a high diagnostic sensitivity (Grizzle, Fredenburgh, & Myers, 2008). The main disadvantage of using cold acetone as a fixative is that the acetone is not a good penetrator of tissue samples while excessive fixation could lead to either the loss or shrinkage of antigenic sites in the tissue sample (Grizzle et al., 2008).

1.7.2. Flooding of tissue impressions with hydrogen peroxide

The presence of non-specific background staining is a phenomenon that occurs regularly in IHC diagnostic techniques. The cause of the observed background staining could be due to numerous factors, but the most common cause is the presence of endogenous peroxidase activity in the tissue sections. Endogenous peroxidase activity is generally defined as “any activity in the tissue that results in the decomposition of the hydrogen peroxide (H_2O_2) once it has been applied to the tissue sections (Wendelboe & Bisgaard, 2009)”. Although the effects of endogenous peroxidase could influence the results of the dRIT test by producing false positive results, submerging the slides in 3% hydrogen peroxide prevents the endogenous peroxidase activity by quenching the endogenous enzymes present in the tissue sections (Wendelboe & Bisgaard, 2009).

1.7.3. Biotinylated antibodies

1.7.3.1. Monoclonal and polyclonal antibodies

The main component that is essential to the dRIT reaction is the antibody being applied to the reaction because the antibody is directly involved with the interaction with the antigenic sites present in the sample being investigated. In order to obtain a better understanding of the role that antibodies play in the dRIT diagnostic test it is essential to discuss the nature of both monoclonal and polyclonal antibodies (M. Boenisch, 2009).

1.7.3.1.1. Monoclonal antibodies

Monoclonal antibodies are defined as “a homogenous population of immunoglobulin that is directed towards interacting with a single epitope on a specific antigen (M. Boenisch, 2009)” (Figure 1.1). The process of creating monoclonal antibodies is more labour intensive than that of creating the polyclonal antibodies, but the end result is antibodies that are generated by a single B-cell clone from one animal. Experimental animals, most commonly mice and rabbits, are immunized with an antigenic molecule and subsequently boosted every two weeks for a period of two-four

months. As soon as the experimental animal has achieved an acceptable level of immune response it is sacrificed and the B-lymphocytes are harvested from the spleen cells. The isolated B-lymphocytes are fused with an immortal cell line to create what is known as a hybridoma. The hybridoma cell lines are cultured and a stable clone with a high antibody production is selected by means of sub-culturing (M. Boenisch, 2009).

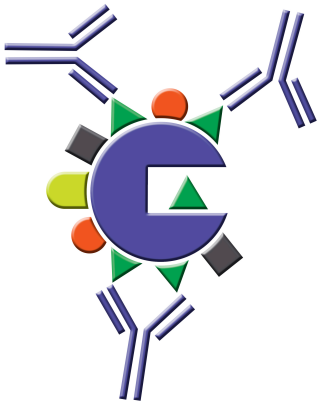


Figure 1.1. Representation of monoclonal antibodies binding to a single type of epitope associated with an antigen (modified from M. Boenisch, 2009)

The main advantage of using monoclonal antibodies is that their production ensures a lot-to-lot consistency while the hybridoma cell lines ensures a sustainable production of the antibodies. The fact that the monoclonal antibodies target a single epitope on the antigen ensures a higher specificity while possible limiting the application if secondary or tertiary changes of the epitope occur (M. Boenisch, 2009).

1.7.3.1.2. Polyclonal antibodies

Polyclonal antibodies are produced by inducing a natural immune response by immunizing experimental animals with the specific antigen of interest. The immunization period usually ranges from three to eight months during which a series of boosters are applied in order to achieve the maximum immune response in the host. The polyclonal antibodies are routinely produced in animals such as goats, swine, guinea pigs and cows, but rabbits are used most often due to the general ease of maintenance of the animal. Based on the nature of production of the polyclonal antibodies, the antibodies consist of a mixture of antibodies directed towards various epitopes of the same antigenic site (Figure 1.2). Because polyclonal antibodies are generated by different B-cell clones of an experimentally infected animal host, the antibodies are known to immunohistochemically dissimilar. This is as a result of the antibodies produced being

characterized by slight differences in their specificities and affinities (M. Boenisch, 2009).

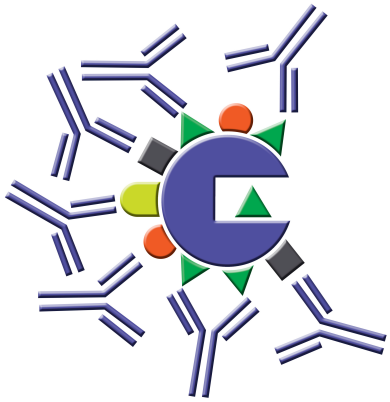


Figure 1.2. Representation of polyclonal antibodies binding to various epitopes associated with an antigen (modified from (M. Boenisch, 2009)

The main advantage associated with the use of polyclonal antibodies originates from the fact that multiple clones of the antibody producing B-cells gives rise to high levels of labelling for a single antigen (M. Boenisch, 2009).

1.7.3.2. Biotin (antibody conjugate)

The biotin moiety acts as a small hydrophobic molecule that functions as a coenzyme of enzymatic carboxylases and is present as a naturally occurring vitamin (vitamin H) in all living cells to a certain degree (Knappe, 1970). The biotin moiety is regularly conjugated to proteins and antibodies, not only, due to its high affinity for avidin and streptavidin (section 1.7.4) but also due to the fact that biotinylation is rapid, effective and unlikely to disturb the natural function of the molecule to which the biotin compound is conjugated (Knappe, 1970). The process of antibody conjugation (biotinylation) is highly effective because the low molecular weight (244,3 MW) of the biotin moiety does not alter the secondary or tertiary structures of the conjugated antibody. This effective conjugation allows the antibody to be labelled without affecting its affinity for antigens and their associated epitopes.

The main disadvantage associated with the use of biotinylated antibodies that interact with streptavidin is the presence of non-specific background staining. The background staining originates as a result of the presence of endogenous avidin-binding activity (EABA) in a wide variety of tissues. The most common cause of EABA is biotin that is naturally bound to enzymes and other proteins in tissues found most commonly in the liver, kidney and lymphoid system. The presence of endogenous biotin is known to bind with streptavidin-peroxidase during the dRIT reaction (section 1.7.4), resulting in the subsequent conversion of the colourless chromogen into a coloured end

product (section 1.7.5.). The presence of results in the endogenous avidin-binding activity causes the production of positive results (red inclusions) without the presence of antigens in the tissue sample (Wendelboe & Bisgaard, 2009).

1.7.4. Streptavidin peroxidase

1.7.4.1. Streptavidin

The initial investigations into enzyme-protein conjugates (such as streptavidin peroxidase) came in the form of avidin, a 68,000 molecular weight glycoprotein that was found to occur naturally in egg whites. Upon further investigation, it was found that avidin had an exceptionally high affinity for the biotin moiety, which is used to biotinylate the primary antibody in the dRIT reaction (Guesdon, Ternynck, & Avrameas, 1979). The catalytic enzyme, such as horseradish-peroxidase, could then simply be attached to the avidin protein in order to initiate the appropriate coloured precipitate. Although the avidin conjugation provided a marked improvement in the sensitivity of the immunohistochemical reactions, it was found that the avidin component could be replaced by homologous protein known as streptavidin in order to obtain a protein ligand with an even higher natural affinity for the biotin moiety (Weber, Ohlendorf, Wendoloski, & Salemme, 1989). The streptavidin protein, initially isolated from the *Streptomyces avidinii* bacteria (Chaiet & Wolf, n.d.), has been applied to numerous diagnostic reactions due to their extremely high affinity for the biotin vitamin. The high affinity thus made them ideal for the use in the dRIT diagnostic test, which relies on the interaction of biotinylated antibodies and antigenic sites (Chaiet & Wolf, n.d.; Green, 1965a).

1.7.4.2. Horseradish peroxidase

Although the strong association between the biotin moiety and the streptavidin protein is a crucial component of the dRIT reaction, the reaction would come to a standstill without the presence of the catalytic enzyme which is paired with the streptavidin protein in the reaction. The general enzymatic reaction observed in the presence of a catalytic enzyme could be interpreted as follows (T. Boenisch, 2009):

$$Enzyme\ (E) + Substrate\ (S) = Enzyme\text{-}Substrate\ (ES)\ complex$$

$$ES\ complex \rightarrow Enzyme\ (E) + Products\ (P)$$

Based on the explanation of the enzymatic reaction, it becomes evident that before the product can be formed, a transient enzyme-substrate complex is formed at the active site of the catalytic enzyme. In the case of immunohistochemical tests, the enzyme-substrate complex drives the reaction resulting in a colourless chromogen being converted into coloured end products observed as insoluble precipitates among the mounted tissue samples (T. Boenisch, 2009). For the purpose of the dRIT diagnostic reaction, the streptavidin protein is routinely conjugated with an oxidative horseradish peroxidase enzyme. The horseradish peroxidase enzyme is routinely isolated from the root of the horseradish plant (*Cochlearia armoracia*). The oxidative enzyme contains a heme group as its active site, which in turn allows the enzyme to form a complex with hydrogen peroxide (H_2O_2) resulting in its subsequent decomposition into water and atomic oxygen (Haines & Chelack, 1991; Rodriguez-Lopez et al., 2001).

1.7.5. Chromogen

The catalytic effect of the horseradish peroxidase, leading to the catalysis of the hydrogen peroxide substrate, would however come to a standstill if an electron donor was not present in the dRIT reaction. These electron donors become oxidized during the enzymatic reaction and change from a colourless compound to a coloured end product. The electron donors in the dRIT reaction are referred to as chromogens (T. Boenisch, 2009). The chromogen utilized in the dRIT diagnostic reaction is 3-amino-9-ethylcarbazole (AEC), which upon oxidation forms a rose red end product that is soluble in alcohol. The main disadvantage associated with the use of 3-amino-9-ethylcarbazole in the dRIT reaction is that the chromogen is susceptible to further oxidation in the presence of excessive light leading to a loss of staining intensity (T. Boenisch, 2009).

1.7.6. Counterstaining

The last step in the dRIT reaction is the immersion of the appropriately stained tissue in a counterstain in order to increase the contrast of the tissue sample and the accompanying red inclusions (Happel, 2009). Gill's hematoxylin is a biological counterstain that is routinely used to stain the chromatin of both normal and abnormal cells that have been fixed beforehand (Gill, Frost, & Miller, 1974). The advantage of Gill's Hematoxylin is that the chromatin is stained at a controlled rate with a narrow range of optical densities. This allows a tissue sample to undergo a delicate staining

procedure while minimizing the chances of over staining the tissue sections (Baker & Jordan, 1953; Baker, 1962).

1.8. Direct, rapid immunohistochemical test of lyssaviruses

1.8.1. Previous studies evaluating the dRIT diagnostic assay

Since the development of the dRIT diagnostic assay in 2006 (Niezgoda & Rupprecht, 2006), the dRIT diagnostic technique has been applied in five separate studies, during which time the newly developed diagnostic test was compared to the FAT diagnostic assay.

Evaluation of a direct, Rapid Immunohistochemical Test for rabies diagnosis

In the first study, performed in 2006 in the Northwestern parts of Tanzania (Mara, Mwanza and Shinyanga regions), the brain stems of various animals were collected over a period of two years. After collecting central nervous system tissues from the various animal species (159 samples in total), the samples were preserved via four different methods for up to four months before freezing. The methods included the following: freezing at -20ºC, preserving in phosphate buffered glycerol and storing at 4ºC, preserving in phosphate buffered glycerol and storing at -20ºC and preserving in phosphate buffered glycerol and storing at room temperature. After the required time periods had elapsed, the samples were tested with both FAT and dRIT at both the local veterinary diagnostic facility in Tanzania as well as at the CDC in Atlanta, USA. The results obtained from the study indicated that the dRIT had a diagnostic sensitivity and specificity equivalent to the FAT with a diagnostic sensitivity and specificity of 100% respectively (WHO, 2005).

Rabies diagnosis for developing countries

The second study was performed in 2008 in N'Djamena, the capital city of Chad. In the specific study, 48 brain samples were collected during the period of a single year. All collected brain samples were submitted for diagnosis by both the FAT and dRIT diagnostic tests. The results obtained from this study indicated that the dRIT diagnosis performed on fresh samples had a diagnostic sensitivity of nearly 100% when compared to the results obtained for FAT diagnosis. Another factor that was found to differentiate between the FAT and dRIT was the conditions under which samples were stored. Glycerol saline, a preservative widely used on the African continent, is used to preserve samples in situations where the samples cannot be frozen. The results

obtained from this specific study showed that the storing of samples in glycerol saline reduced the performance of the FAT to a much larger extent than in the case of the dRIT.

*The primary application of direct rapid immunohistochemical test to rabies diagnosis in China**

During the study, 72 samples derived from both domestic dogs and human patients from the Guizhou, Guangxi, Hunan, Anhui, Jiangsu and Yunnan provinces of mainland China was diagnosed. All the samples used in the study were diagnosed with the FAT, dRIT and the RT-PCR assay. The results of the research indicated that the dRIT would be more applicable for laboratories with limited funding and/or reduced diagnostic capabilities due to the fact that assay had a diagnostic sensitivity and specificity that equalled that of the FAT and RT-PCR, while being less expensive to perform routinely.

*Although the research was published in Chinese, the abstract was translated into English in order to provide the basic results derived from the study.

Validation and operational application of a rapid method for rabies antigen detection

During March and June 2008, the Veterinary Laboratory Europe (VLE) deployed a veterinary pathologist to train US military units stationed in Iraq and Afghanistan to initiate the diagnosis of endemic rabies using the dRIT diagnostic assay. Subsequent to the training program, the veterinarians enlisted in the US military performed the dRIT on samples collected from February to June of the same year. The study involved 268 samples collected in Iraq and 112 samples collected in Afghanistan. All the samples that were diagnosed by means of the dRIT diagnostic test were sent to the VLE or CDC in order to have the samples diagnosed by means of FAT. The results of the study showed a 100% agreement between the FAT and dRIT results based on the samples that were tested.

Evaluation of a direct rapid immunohistochemical test (dRIT) for rapid diagnosis of rabies in animals and humans

Over a period of two years, 400 brain samples derived from dogs (320), cats (10), cattle (30), wild foxes (2) and humans (38), originating from the Karnataka and Kerala states of southern India, were tested with both the FAT and dRIT diagnostic tests. The results of the two tests under investigation were found to have a 100% agreement, with the researchers concluding that the dRIT results were easier to interpret due to presence of the red inclusion on the blue neuronal background in

contrast to the green fluorescent inclusions visible during the FAT diagnostic test (Madhusudana et al., 2012).

1.8.2. Limitations of previous studies

All of the aforementioned studies had indicated that the dRIT diagnostic assay had a diagnostic efficacy equal to that of the FAT, but certain limitations were not addressed in the studies.

- All of the studies utilized the cocktail of highly concentrated biotinylated monoclonal antibodies supplied by the CDC. As such, no other sources of biotinylated antibodies were investigated in the studies.
- None of the studies were performed in developing countries in southern Africa, resulting in none of the studies including the mongoose variant (section 1.2.1) of the RABV in the sample sets tested in the studies.
- None of the studies included any of the antigenically distinct rabies-related lyssaviruses.
- The OIE stipulates that the theoretical number of samples required to determine the diagnostic sensitivity and specificity of an assay should be no less than 190 samples (assuming a 95% confidence interval and a 2% allowed error margin) (OIE, 2008a). Three of the previous studies had performed the evaluation of the diagnostic efficacy on an insufficient number of samples, resulting in an inaccurate representation of the true diagnostic sensitivity and specificity.

Section C: Significance of study & specific objectives

1.9. Significance of the study

The lack of diagnostic facilities and resources available to most laboratories situated in developing countries limits the use and applicability of the FAT as a routine diagnostic test for rabies (Weyer & Blumberg, 2007). A more suitable diagnostic test is required to fit the needs of the developing countries and their available resources. The dRIT is one such test due to the fact that it requires an inexpensive light microscope to diagnose samples that are fresh, preserved in glycerol saline or even formalin fixed. The fact that the dRIT relies on immunohistochemistry has the added benefit of allowing retrospective analysis due to the fact that the red coloured precipitate formed by the chromogen in the case of a positive dRIT diagnostic reaction, does not fade or

disappear over a short period of time. Although all the reagents required to perform the dRIT diagnostic assay are available for purchase via various commercial suppliers, the CDC remains the sole supplier of the cocktail of high concentrated biotinylated antibodies. The single supplier of biotinylated antibodies limits the widespread application of the diagnostic technique in developing countries. The cocktail of biotinylated monoclonal antibodies, consisting of two highly concentrated anti-nucleocapsid biotinylated monoclonal antibodies, is supplied as a “ready to use” vial for the purpose of the dRIT diagnostic test. Although this cocktail of highly concentrated antibodies has been shown to be effective in terms of routine rabies diagnosis, the general efficacy of the two individual monoclonal antibodies has not been investigated in terms of their individual diagnostic specificity and sensitivity. Despite the obvious need for the widespread application and validation of the dRIT diagnostic test, the limited supply of the required cocktail of biotinylated monoclonal antibodies does in fact hinder the widespread application in developing countries. As a result of the aforementioned fact, the possibility of alternative biotinylated antibodies that could be applied to the dRIT diagnostic test as substitutes for the cocktail of highly concentrated monoclonal should be investigated.

1.10. Aim of the study

The main aim of the study was to investigate the possible biotinylation of a locally produced (South Africa) biotinylated anti-ribonucleoprotein polyclonal antibody and the testing of the biotinylated preparation in the dRIT diagnostic reaction. In order to truly investigate the potential of the polyclonal antibody, the efficacy of the biotinylated polyclonal antibody was compared to the efficacy of the two individual biotinylated monoclonal antibodies that are routinely used in the dRIT test as the “cocktail of highly concentrated biotinylated monoclonal antibodies”. The specific objectives included the following:

- Labelling the polyclonal anti-ribonucleoprotein polyclonal antibody (produced at the Agricultural Research Council-Onderstepoort Veterinary Institute (ARC-OVI), Rabies unit) with a biotin moiety according to the standard operating procedure stipulated by the CDC.
- Applying all three of the biotinylated antibodies under investigation in three individual dRIT diagnostic reactions to a significant number of samples originating from southern Africa. The application of dRIT diagnostic assays (relying on the three-biotinylated antibodies) concurrent with the FAT enabled the statistical analysis of the dRIT assays to be investigated.

- Applying the biotinylated polyclonal antibody to a dRIT diagnostic reaction adapted to utilise acetone fixation instead of fixing with 10% neutral buffered formalin. The adapted dRIT assay was applied to the same sample set of southern African samples along with the FAT diagnostic assay, which enabled the statistical analysis of the diagnostic efficacy to be performed.
- Applying the dRIT diagnostic assay (relying on the three-biotinylated antibodies) to a panel of seven rabies-related isolates that are known to circulate on the African continent.
- Developing a simulation framework to determine all the costs involved in performing routine FAT or dRIT diagnosis in an established biosafety level 2 laboratory. The simulation framework was used to predict the possible cost per diagnostic reaction based on estimated annual throughput rates and available start-up capital funds.

Chapter II

Comparison of biotinylated monoclonal and polyclonal antibodies in a direct rapid immunohistochemical test

2.1. Introduction

Despite the fact that rabies is a preventable disease and that effective control measures are available to prevent disease spread (Bogel & Meslin, 1990; Knobel, Kaare, Fevre, & Cleaveland, 2007), it is still responsible for the death of tens of thousands of humans per annum (Knobel et al., 2005; WHO, 2005). The main reason why rabies remains a neglected disease in the resource-limited developing countries can directly be attributed to the lack of laboratory diagnosis that subsequently results in the number of annual deaths associated with rabies being greatly underestimated (Weyer & Blumberg, 2007; WHO, 2005). The underestimated severity of the disease, and as such, the limited accurate data based on the public health impact of the disease has led to a lack of political awareness and subsequent intervention (Cleaveland, Fevre, Kaare, & Coleman, 2002; WHO, 2005).

The process of rabies diagnosis does not only play a direct role in disease surveillance, but is also used in the implementation and monitoring of rabies control programs in animal populations that are directly correlated with the prevention of disease spread to human populations that inhabit the same geographical niches. The lack of routine rabies diagnosis in developing countries on the African continent can be attributed to the fact that the FAT cannot be performed routinely due to the high cost involved with the acquisition and maintenance of the required equipment. In order to increase RABV surveillance, novel diagnostic assays catering for the prevailing economical and logistical conditions of developing countries, had been investigated with the dRIT showing the most promise. Due to the singular source of the biotinylated antibody cocktail (supplied by the CDC) the widespread application of the dRIT assay has been restricted, resulting in limited data to support of the efficacy and applicability of the dRIT assay.

Based on the limited availability of the required biotinylated monoclonal antibodies, the main aim of this chapter was to verify whether an alternative biotinylated antibody preparation could be applied to the dRIT diagnostic assay in order to obtain a working diagnostic reaction. In order to validate the efficacy of the biotinylated antibody preparation, the polyclonal anti-ribonucleoprotein antibody was biotinylated to a sufficient level and the antibodies were applied to the dRIT diagnostic reaction. The FAT and dRIT diagnostic reactions were performed concurrently on a panel of RABV isolates circulating in southern Africa in order to achieve comparative results in terms of the diagnostic efficacy between the two diagnostic assays. A further evaluation of the diagnostic efficacy of the biotinylated polyclonal antibody involved the application of the dRIT diagnostic assay (relying on the two individual biotinylated monoclonal antibodies provided by the CDC) to the same set of southern Africa samples in order to obtain

information regarding the diagnostic efficacy of the dRIT diagnostic assay relying on the various biotinylated antibodies.

2.2. Materials and Methods

2.2.1. Production and preparation of the polyclonal antibodies

2.2.1.1. Anti-ribonucleoprotein polyclonal antibody

The regional OIE rabies reference laboratory for the African continent (ARC-OVI, Rabies division) is responsible for the labelling and distribution of the FITC-conjugated polyclonal anti-ribonucleoprotein antibody to Southern African Development Community (SADC) member countries in order to facilitate and promote the application of the FAT diagnostic test for routine rabies diagnosis. For the purpose of this study the unlabeled polyclonal anti-ribonucleoprotein antibody (henceforth referred to as the “polyclonal antibody”), produced at the ARC-OVI (Harlow & Lane, 1988; Perrin, 1973), was used for the purpose of biotinylation and subsequent dRIT reactions.

Briefly, a goat was immunized weekly with the ribonucleoprotein (RNP) complex derived from RABV in order to induce an appropriate immune response. After a month of weekly immunizations, a booster of RNP complex derived from MOKV was used to immunize the animal in order to produce goat polyclonal anti-RNP immunoglobulin that could interact with multiple epitopes on the RNP complex due to the induction of a natural immune response in the host (ARC-OVI Ethical approval, 15/4/P001).

2.2.1.2. Polyclonal antibody clarification

In order to clarify the serum containing the polyclonal antibody, 6 ml of the goat serum (ARC-OVI,rabies division) was centrifuged (Eppendorf Minispin) at 45 *g* for fifteen minutes, after which the supernatant was transferred to sterile eppendorf tubes. Stock solutions were created by transferring 4 ml of the highly concentrated clarified antibodies to cryotubes that were subsequently frozen at -80 ºC in order to maintain the integrity of the immunoglobulin. The remaining 2 ml of clarified antibodies was used for the biotinylation reaction.

2.2.1.3. Polyclonal antibody dilution

The concentration of the undiluted polyclonal antibody was determined by transferring 1 µl of highly concentrated immunoglobulin to a NanoDrop™ 1000 Spectrophotometer (Thermo Scientific) and the antibody concentration (80 mg/ml) was determined by measuring the absorbance at 280 nm. After the concentration of the polyclonal antibody had been determined, the polyclonal antibody was diluted to the desired 10 mg/ml using phosphate buffered saline (PBS consisting of 0.1 M sodium phosphate, 0.15 M sodium chloride; pH 7.2) (Lonza), which in turn allowed the optimum biotinylation to occur.

2.2.2. Biotinylation of the polyclonal antibody

The polyclonal antibodies were biotinylated with the EZ-Link® Sulfo-NHS-Biotinylation Kit (Thermo Scientific) according to the manufacturer's instructions. Briefly, the following steps were taken in order to ensure that effective and sufficient biotinylation had occurred.

2.2.2.1. Biotinylation

In order to reconstitute the 10 mM Sulfo-NHS biotin compound (sulfosuccinimidyl-6-[biotin-amido]-hexanoate) for further use, 2.2 mg of the lyophilized biotin compound was dissolved in 500 µl of nuclease free water (Promega) as per the supplier's exact instructions.

The quantity of reconstituted Sulfo-NHS biotin (MW: 443mg), required to biotinylate 10 mg/ml of the polyclonal antibodies, was determined according to the formulas supplied with the EZ-link Sulfo-NHS Biotinylation kit (EZ-Link® Sulfo-NHS-Biotinylation Kit, Thermo Scientific):

$$\text{ml protein x } \frac{mg\ protein}{ml\ protein} \text{ x } \frac{mmol\ protein}{mg\ protein} \text{ x } \frac{20\ mmol\ Biotin}{mmol\ protein} = \textit{mmol Biotin}$$

$$\textit{mmol Biotin x } \frac{443mg}{mmol\ Biotin} \text{ x } \frac{500\mu l}{2.2mg} = \textit{µl Biotin Solution}$$

Based on the calculations, 268 µl of the 10mM Sulfo-NHS biotin was added to three individual tubes (labelled as "Batch #1", "Batch #2" and "Batch #3"), each containing 2 ml of 10 mg/ml clarified polyclonal antibodies after which the tubes were incubated on ice for two hours.

2.2.2.2. Buffer exchange

The contents of the three tubes (Batch 1 - 3) containing the biotinylated polyclonal antibodies were desalted using Zeba™ desalt spin columns (Thermo Scientific) supplied with the EZ-link Sulfo-NHS Biotinylation kit. Briefly, the Zeba™ desalt spin columns were centrifuged (Beckman coulter, Avanti® J-E centrifuge) at 1000 *g* for two minutes in order to remove the storage buffer present in each of the spin columns. The storage buffer was discarded and, 2.5 ml of PBS buffer (Lonza) was added on top of the resin bed present inside each of the desalting columns. The Zeba™ desalt spin columns were placed in their respective collection tubes, after which the tubes were centrifuged at 1000 *g* for two minutes. Subsequent to centrifugation, the flow-through was discarded in order to transfer the spin columns back to their respective collection tubes. The process of equilibration was repeated three times before the Zeba™ desalt spin columns were transferred to new collection tubes. The biotinylated antibody was purified by dispensing 2 ml polyclonal antibodies directly on top of the resin bed present in each of the three Zeba™ desalt spin columns, and the columns were centrifuged at 1000 *g* for two minutes. The three tubes of purified biotinylated polyclonal antibodies were collected as the flow through and stored at -80 °C until further use.

2.2.2.3. Quantifying biotinylation

The level of biotinylation of the three individual tubes of biotinylated polyclonal antibodies (Batch 1 - 3) was determined using a HABA/Avidin assay as supplied with the EZ-link Sulfo-NHS Biotinylation kit (EZ-Link® Sulfo-NHS-Biotinylation Kit, Thermo Scientific). Briefly, the HABA/Avidin solution was prepared according to the manufacturer's instruction by mixing 10 mg of lyophilized Avidin and 600 µl of 10 mM HABA (4'-hydroxyazobenzene-2-carboxylic acid) to 19.4 ml of PBS (Lonza). After the preparation of the HABA/Avidin solution, 180 µl was transferred to a microplate well and the absorbance was measured at 500 nm (Multiskan Go, Thermo Scientific). The absorbance value was recorded as "HABA/Avidin solution", and after the initial absorbance measurement, 20 µl of biotinylated polyclonal antibodies derived from each of the three batches were added to the wells containing the HABA/Avidin solution and the plate was briefly shaken using an orbital plate shaker (Multiskan Go, 5 second continuous shake at "medium speed"; Thermo Scientific). After sufficient mixing, the absorbance was measured at 500 nm (Multiskan Go, Thermo Scientific) until the absorbance remained constant for an average of fifteen seconds. The absorbance was recorded as "HABA/Avidin/Biotin solution". The absorbance values of both the "HABA/Avidin solution" and the "HABA/Avidin/Biotin solution" was inputted into the

"Pierce HABA calculator" (http://www.piercenet.com/haba/habacalc.cfm) along with the concentration and molecular weight of the polyclonal antibody (Concentration: 10 mg/ml, MW – IgG: 150 000 mg/mM). The "Pierce HABA Calculator" proceeded to calculate the "ratio of moles of biotin/moles of protein" associated with the respective tubes of biotinylated polyclonal antibodies (Batch 1 - 3) (Green, 1965b).

2.2.3. Sample selection and preparation

2.2.3.1. Sample selection

The sample set used in this study consisted of 250 CNS tissue samples (Table A1) derived from five mammalian species that occur in southern Africa. The mammalian species used in this study included the following: domestic dogs (*Canis familiaris*; n=132*)*, domestic cats (*Felis domesticus*; n=27), Black-backed jackal (*Canis mesomelas*; n=26), Bat-eared fox (*Otocyon megalotis*; n=11), Yellow mongoose (*Cynictis penicillata*; n=27) and bovines (*Bos Taurus* n=27). The species chosen for the purpose of this study was selected based on their importance as reservoirs for the RABV, and are thus routinely subjected to diagnosis in southern Africa.

The samples used in this study (Table A1) had been selected from a much larger set of samples submitted to the ARC-OVI, Rabies division for routine RABV diagnosis over a period of two years (year: 2011-2012), while a sub-set of thirty archival samples (year: 1999) were selected to investigate the effect of long term storage on both the FAT and dRIT diagnostic tests once applied to CNS tissue samples stored at -80 °C. The specific year (1999) was chosen due to the fact that no older and freshly preserved samples were stored at the ARC-OVI, Rabies division.

2.2.3.2. Preparation of CNS tissue

In order to facilitate the efficient antigen spread throughout each of the CNS samples being investigated, all of the CNS samples were homogenised prior to initiating the diagnostic tests. In order to ensure even antigen spread, each of the CNS samples was placed in a sterile petri dish and small pieces of tissue was removed from numerous sites selected from the CNS sample. The removed pieces were homogenised and mixed, using a sterile mortar and pestle, until a consistent texture was observed with no visible solid pieces of tissue. The homogenized tissue was used for all further diagnostic tests in this study.

2.2.4. Fluorescent antibody test

All of the CNS samples selected for the purpose of the research project were subjected to the FAT diagnostic assay in order to confirm the level of positivity of the chosen samples before subjecting the samples to dRIT diagnosis. Briefly, a single touch impression was made by placing a small amount of homogenized CNS material on clean tissue paper, after which a clear microscope slide (Lasec) was depressed on top of the sample. The samples were submerged in 4 °C filtered acetone (Associated Chemical Enterprises) for thirty minutes in a refrigerator (set to 4 °C) in order to enable sufficient tissue fixation. After the fixation process was completed the samples were allowed to air dry for five minutes, and a working dilution (1:1000) of FITC conjugated polyclonal antibodies (ARC-OVI, Rabies division) was added drop-by-drop to the impressions until they were completely covered. The slides were placed in humidity chambers (Sigma-Aldrich) and incubated at 37 °C for thirty minutes in a 5% CO_2 incubator (Labcon). After incubation the slides were subjected to three separate wash steps with PBS buffer (pH 7.2) (ARC-OVI, Rabies division) after which a cover slip (Menzel) was placed over the fixed and stained touch impression. The slides were viewed using a fluorescent microscope (Zeiss Axiovert 25, Axiolab) in order to score the respective immunoreactivity of each sample based on the intensity and amount of observed fluorescence by a single microscopist. A total of 40 fields were viewed at a minimum magnification of 400x (Excitation: 490 nm; Emission: 525 nm) before a sample was scored. All samples originally designated as rabies infection negative were repeated a further two times to confirm the diagnosis.

2.2.5. Direct, rapid immunohistochemical test

All the samples that were subjected to the FAT diagnosis were blindly tested with the dRIT assay by creating three separate slides for each sample of CNS tissue in order to apply the two monoclonal antibodies (CDC) and the polyclonal antibody (ARC-OVI, Rabies division) separately. Briefly, a single touch impression was made from the CNS tissues by placing a small amount of homogenized CNS material on clean tissue paper. After pressing a clear microscope slide (Lasec) down on top of the sample, the touch impressions were allowed to air dry for five minutes before being submerged in 10% neutral buffered formalin (Sigma-Aldrich) for ten minutes. After the fixation period had passed, the touch impression were re-hydrated by dip rinsing the slides in TPBS buffer (PBS containing 1% Tween 80 (Merck chemicals)). After the re-hydration process, the slides were submerged in 3% hydrogen peroxide (Merck chemicals) for ten minutes at

room temperature in order to cease all endogenous peroxidase activity. Subsequent to the hydrogen peroxide flooding, the slides were dip rinsed in fresh TPBS buffer and the excess buffer was shaken from the slides and the area surrounding the smear impressions were blotted dry using fresh paper towel. Since each CNS tissue section was used to create impression slides in triplicate, each of the three slides could be treated with one of the three-biotinylated antibodies being investigated. Monoclonal antibody 1 and monoclonal antibody 2 (CDC) was each applied to one of the three tissue impressions, while the polyclonal antibody (ARC-OVI, Rabies division) was used to treat the third and final tissue impression. Monoclonal antibody 1 and 2 were supplied as ready-to-use reagents with unknown concentrations and the polyclonal antibody was applied as a 1:220 solution (pre-determined by means of a titration series). The respective antibodies were applied drop by drop until the impressions were completely covered. After antibody application, the slides were placed in a humidity chamber (Sigma-Aldrich) and incubated at room temperature for ten minutes. After the incubation period had lapsed, the slides were dip rinsed in fresh TPBS buffer, after which the excess buffer was shaken from the slides and the area surrounding the smear impressions were blotted dry using fresh paper towel. All the touch impressions were covered in a ready-to-use solution of 2 µg/ml streptavidin-peroxidase (Kirkegaard and Perry Laboratories) after which the slides were transferred to a humidity chamber. The humidity chamber was incubated at room temperature for ten minutes, after which the slides were dip rinsed in fresh TPBS buffer. The excess buffer was shaken from the slides and the area surrounding the smear impressions were blotted dry using fresh paper towel. A working solution of 3-amino-9-ethylcarbazole (AEC) chromogen was made according to the instructions provided with the AEC staining kit (AEC Chromogen Kit, Sigma-Aldrich). Briefly, the kit contains all the reagents in a ready to use format in order to ease the process of creating the working solution of AEC chromogen for the dRIT reaction. Two drops of supplied acetate buffer (2.5M, pH 5.0), one drop of supplied AEC chromogen (3-amino-9-ethylcarbazole in N,N-dimethylformamide) and one drop of supplied 3% hydrogen peroxide was added to 4 ml of distilled water. The solution was sufficiently mixed right before application and stored at 4°C until use. The impressions on the slides were covered in the working solution of the 3-amino-9-ethylcarbazole (AEC) chromogen, after which the slides were transferred to a humidity chamber and incubated at room temperature for five minutes. After sufficient staining had occurred the slides were submerged in distilled water. The touch impressions were counterstained with a 1:2 dilution of Gill's formulation #2 (Sigma-Aldrich) for two minutes after which the slides were dip rinsed in distilled water in order to wash away the residual counterstain. Finally, the slides were mounted with a water-soluble mounting medium (PBS/glycerol (Sigma-Aldrich) mixed 1:1) and examined by light

microscopy (Nikon, Alphashot YS) at both 200x and 400x magnification in order to score the respective immunoreactivity based on both the presence and staining intensity of the visible red inclusions present on the blue cellular background. A total of 40 fields were viewed by a single microscopist, and all samples originally designated as rabies infection negative were repeated a further two times to confirm the diagnosis. The immunoreactivity of the various samples was determined in a blind fashion by not relying on the FAT immunoreactivity scores to influence the interpretation of the dRIT results.

2.2.6. Molecular determination of false positive result

Two samples (664/12 and 711/12) characterised as lyssavirus-negative according to the FAT diagnostic assay, produced positive immunoreactivity once the dRIT diagnostic assay had been applied to them. The first sample (664/12) was collected from a canid in the Limpopo province of South Africa, and produced a false positive result once the dRIT diagnostic assay with monoclonal antibody 2 had been applied. The second sample (711/12) was collected from a canid in the Mpumalanga province of South Africa, and produced a false positive result once the dRIT diagnostic assay using any of the three antibodies (monoclonal antibody 1, monoclonal antibody 2 or polyclonal antibody) had been applied.

In order to determine whether the FAT produced a false negative the total RNA was extracted from the CNS tissue of the two specific samples. Real-time and conventional PCR was applied to sample 711/12, and only a conventional PCR was applied to sample 664/12, in order to determine whether any lyssavirus nucleic acid was present in the samples.

2.2.6.1. RNA extraction

The RNA extraction, from sample 664/12 and 711/12, was performed using the Trizol reagent (Sigma-Aldrich) according to the manufacturer's instructions. Briefly, 50-100 mg of brain material was homogenized in 1 ml of Trizol reagent. After homogenization was performed, 200 µl chloroform (Merck Chemicals) was added and mixed well for fifteen seconds. Subsequently, the mixture was centrifuged at 10 000 *g* for fifteen minutes and the aqueous and organic phase separated. The aqueous phase was transferred to a sterile 1.5 ml microcentrifuge tube (Quality scientific plastics) and the RNA was precipitated by adding 500 µl isopropanol (Sigma-Aldrich). After a ten minute incubation period at room temperature, the RNA was recovered by

centrifugation at 10 000 *g* for thirty minutes. After centrifugation, the precipitated RNA was washed with 1 ml of 75% ethanol (Merck Chemicals), followed by centrifugation at 10 000 *g* for five minutes. The supernatant was removed and the pellet was allowed to air dry at room temperature. The RNA was resuspended in 50 µl nuclease free water (Promega).

2.2.6.2. Real-time PCR amplification of viral nucleic acid

The one-step real-time PCR amplification of the viral nucleic acid was applied to the sample that was dRIT positive regardless of the applied biotinylated antibody (711/12) (Coertse et al., 2010). Along with the isolated RNA of the sample, a negative control (nuclease free water) and positive control (RNA with a standard concentration) was included in the reactions in order to judge the authenticity of the real-time results. The probe-based real-time PCR assay was designed to amplify the viral nucleoprotein using a specific fluorescent probe and primer set on a LightCycler® 1.0 Thermocycler (Roche Diagnostics), while the LightCycler® software (Version 4.05) was used for the subsequent fluorescence analsysis. In order to confirm the results obtained from the real-time PCR amplification of the nucleic acid, an established hn-PCR reaction was also performed.

2.2.6.3. hn-PCR amplification of viral nucleic acid

The reverse transcription of the isolated RNA (section 2.2.6.1) and the initial amplification of the nucleic acid were performed using a protocol that was previously described (Markotter, Kuzmin, et al., 2006). The reverse transcription and initial PCR amplification relied on the 001lys and 550B primer set. After the initial PCR reaction had been performed, the hn-PCR reaction relying on an established protocol, (Heaton et al., 1997), was performed. A positive control (CVS, cDNA) and a negative control (nuclease free water) were included in all amplification reactions. Briefly, 1 µl of the primary amplified PCR product (undiluted) and 20 µl of the diluted primary amplified PCR product (diluted 1:1000) was each added to a reaction mixture containing 5 µl 10xDreamTaq™ buffer (Fermentas), 2,2 µl dNTP mixture (10 mM) (Promega), 10 pmol forward primer, SB1, 12,5 pmol reverse primer, 550B, and 0,25 µl DreamTaq™ DNA Polymerase (5 U/µl, Fermentas). The subsequent amplification was performed in a GeneAmp PCR system 2700 (Applied Biosystems) set to the following amplification conditions. After denaturation at 94 °C for one minute, the reaction were cycled 40 times at the following conditions: 94 °C for 30 seconds, 37 °C for 30 seconds and 72 °C

for 90 seconds, with a final extension at 72 °C for 7 minutes. The hn-PCR product associated with one sample (711/12) was visualised on a 1% agarose gel (Lonza) that had been stained with ethidium bromide (0,5 µg/ml; Merck Chemicals), with the agarose gel electrophoresis analysis being performed in a LabNet gel dock (LabNet powerpack set to 100 Volts). The band visualised on the 1% agarose gel was subsequently purified using the Wizard® SV Gel and PCR cleanup system (Promega, USA) according to the manufacturer's protocol.

2.2.6.4. Sequencing of purified hn-PCR product

The ABI Prism® BigDye® Terminator Version 3.1 Cycle sequencing kit (Applied Biosystems) was used for the sequencing reactions according to the manufacturer's predetermined protocol. In order to sequence both the 5'-3' and 3'-5' strands of the amplified and purified PCR product, both the forward (SB1) and the reverse (550B) primers were cycled separately in a Geneamp PCR system 2700 (Applied Biosystems) as follows: 1 cycle of 94 °C for 1 minute, 25 cycles of 94 °C for 10 seconds, 50 °C for 5 seconds and 60 °C for 4minutes. The sequencing reactions were thereafter precipitated according to the BigDye® V3.1 protocol (Applied Biosystems). Briefly, 1 µl EDTA (125mM), 1 µl NaOAc (3M) and 25 µl EtOH (100%) were added to each of the 10 µl sequencing reactions. The samples were subjected to a brief vortex followed by centrifugation at 10 000 *g* for 30 minutes. The supernatant was removed and 100 µl EtOH (70%) was added followed by centrifugation at 10 000 *g* for 15 minutes. The supernatant was removed and the DNA pellet was air dried for 20 minutes at room temperature. The precipitation reactions were sequenced at the University of Pretoria on an ABI 3100 automated capillary sequencer (Applied Biosystems). The sequences obtained from using both the forward (SB1) and reverse (550B) primers were used to create a trimmed consensus sequence using CLC Main Workbench (CLC bio). The consensus sequence was subsequently subjected to a BLAST analysis on the GenBank website.

2.2.6.5. Phylogenetic analysis of RABV isolate

After the assembly of the consensus sequence, an alignment was created using the ClustalW subroutine of the BioEdit software (Hall, 1999). A neighbour joining (NJ) phylogenetic analysis was subsequently performed using the "Kimura-2" parameter

(determined by JModel test Version 2.1.3 software) in MEGA, version 2.1 (Kumar, Tamura, & Nei, 2004). The bootstrap support was estimated for 1000 replicates.

2.2.7. Antigenic typing of false negative results

All the samples that provided false negative results with the dRIT compared to the FAT were subjected to antigenic typing using a panel of sixteen monoclonal antibodies supplied by the Centres of Expertise for Rabies (Canadian food inspection agency, Ottawa, Canada) according to an established protocol (J. S. Smith & King, 1996). Touch impressions of CNS tissue sample was made in sixteen separate wells of polytetrafluoroethylene-coated glass slides (Menzel) and left at room temperature for one hour in order to allow the impressions to dry. After the tissue impressions had dried, the slide was submerged in 4 °C filtered acetone (Associated Chemical Enterprises) for one hour in order to enable tissue fixation. After tissue fixation, the slide was dried at room temperature for ten minutes and each of the sixteen monoclonal antibodies in the panel were added to one of the sixteen tissue impressions, while taking care to avoid cross-contamination. The slide was incubated at 37 °C for thirty minutes in a 5% CO_2 incubator (Labcon). The unbound antibody was removed by washing the slides with PBS buffer (pH 7.2, ARC-OVI, Rabies division), while avoiding the transfer of monoclonal antibodies between the various impressions. After the PBS wash step, pre-titrated 1:300 FITC-conjugated goat anti-mouse immunoglobulin (SigmaAldrich) was added to each of the sixteen impressions on the slide after which the slide was incubated at 37 °C for thirty minutes in a 5% CO_2 incubator (Labcon). The unbound immunoglobulin was washed from the slide by dip rinsing the slide in PBS buffer, after which the immunoreactivity pattern was observed using a fluorescent microscope at 400x magnification (Zeiss Axiovert 25, Axiolab). The immunoreactivity patterns were used to define the strains as either the canid or mongoose variant of the RABV.

2.2.8. Statistical analysis

The statistical analysis of the dRIT diagnostic tests relying on the three respective biotinylated antibodies (monoclonal antibody 1, monoclonal antibody 2 and the polyclonal antibody) was determined by comparing the dRIT results to the results obtained from the FAT diagnosis. The statistical analysis of the diagnostic efficacy was performed by assuming an exact binomial distribution (MedCalc® 12.2.1.0, Ostend Belgium).

2.2.8.1. Diagnostic sensitivity

The diagnostic sensitivity was used to determine the probability of a diagnostic test delivering a "positive" result once applied to a diseased specimen. The diagnostic sensitivity could thus only be calculated from individuals that were truly infected with the disease in question (Zou, O'Malley, & Mauri, 2007).

2.2.8.2. Diagnostic specificity

The diagnostic specificity was used to determine the probability of a diagnostic test delivering a "negative" result once applied to a disease-free specimen. The diagnostic specificity could thus only be calculated from individuals that were known to be free of the disease in question. (Zou et al., 2007).

2.2.8.3. Cohens' kappa measure of agreement

The Cohen's kappa measure of agreement was used to indicate whether the developing diagnostic assay correctly predicted the outcome of the test based on the results obtained from the recommended FAT test applied to the same sample. The measure of agreement could thus be summarized as the quantitative value of the reliability of a diagnostic test. In order to determine the Kappa measure of agreement was thus calculated by assuming a binomial distribution resulting in a specific "agreement category" (Landis & Koch, 2012).

Table 2.1. Formulas applied for statistical analysis of comparative results

Diagnostic sensitivity	*Diagnostic sensitivity* = $\frac{True\ Positive}{(True\ Positive + True\ Negative)}$
Diagnostic specificity	*Diagnostic specificity* = $\frac{True\ Negative}{(True\ Negative + False\ Positive)}$
Cohens' kappa measure of agreement*	*Kappa value* = $\frac{(Observed\ agreement - Expected\ agreement)}{(1 - Expected\ agreement)}$

* The levels of agreement were interpreted as follows: poor agreement (0), slight agreement (0-0.2), fair agreement (0.21-0.4), moderate agreement (0.41-0.6), substantial agreement (0.61-0.8) and almost perfect agreement (>0.81) (Landis & Koch, 2012)

2.3. Results

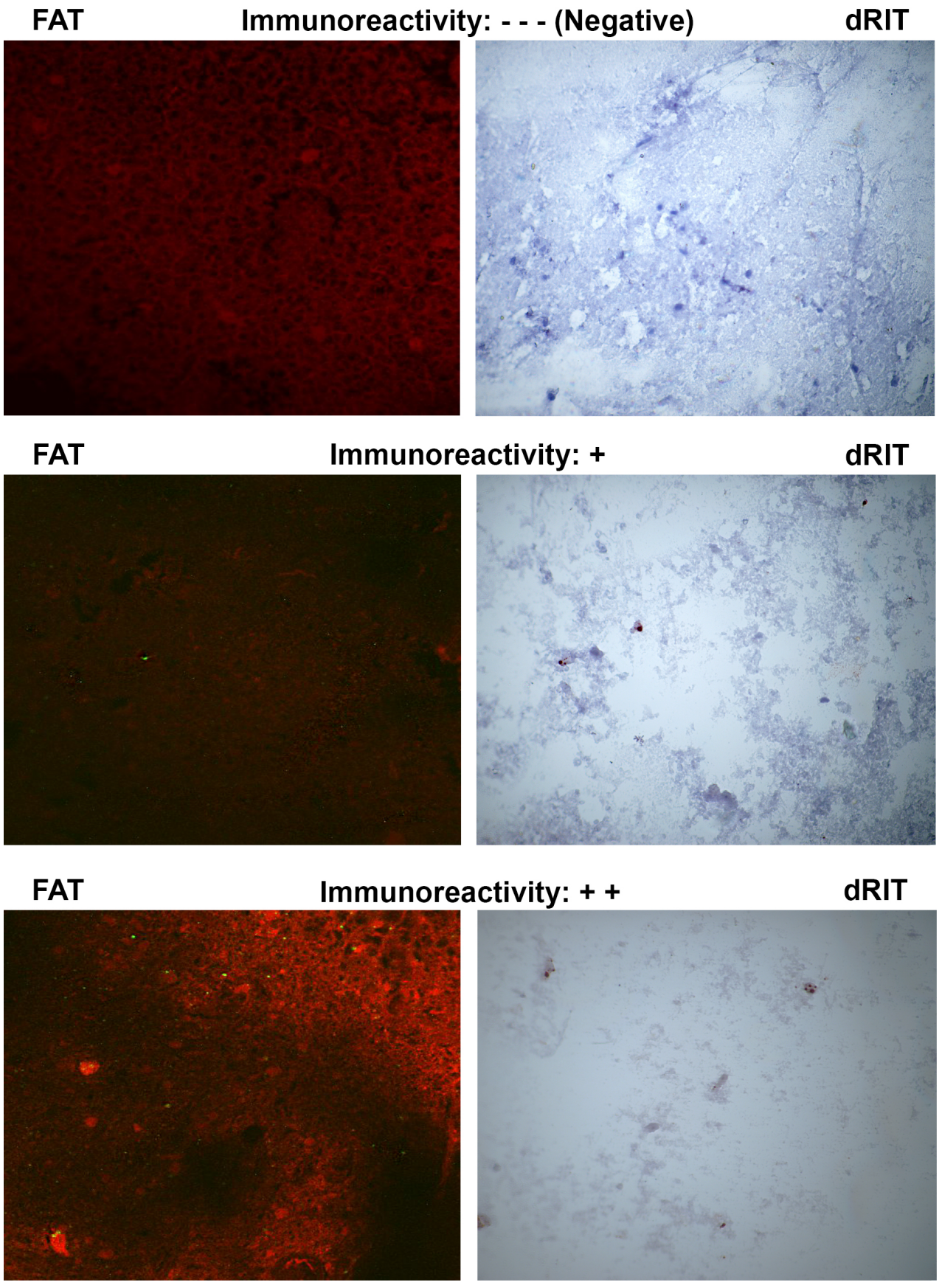
2.3.1. Biotinylation of the polyclonal antibody

Due to the nature of the “HABA/Avidin” complex, the level of biotinylation was determined based on the change in the absorbance values associated with both the “HABA/Avidin” and “HABA/Avidin/Biotin” complexes. The biotinylated polyclonal antibody displaced the HABA compound from the HABA/Avidin complex, resulting in a consistent drop in absorbance measured at 500 nm. The absorbance of the batches all dropped from approximately 0,590 absorbance units (AU) to 0,345 AU. The absorbance values associated with each of the three batches of biotinylated polyclonal antibodies (Batch 1 - 3) was inputted into the Pierce HABA calculator and the approximate molar ratio of biotin to protein was calculated accordingly. Based on the dropped absorbance observed in all three batches, and subsequent calculations derived from the “Pierce HABA calculator”, it was shown that the ratio of moles of biotin to moles of polyclonal antibody was 4.32:1, 3.31:1 and 3.09:1 respectively, indicating that successful levels of biotinylation had been achieved.

2.3.2. Evaluation of the dRIT using three different antibodies

The FAT was applied to all 250 CNS samples and the immunoreactivity score associated with each sample was determined based on both the staining intensity and antigen distribution observed with each sample (Figure 2.1). The dRIT diagnostic test relying on each of the three biotinylated antibodies (monoclonal antibody 1, monoclonal antibody 2 and the polyclonal antibody) was applied to the same 250 CNS samples and the respective levels of immunoreactivity was scored according to the staining intensity and antigen distribution observed with each sample. The immunoreactivity represented in Figure 2.1 was produced by the dRIT assay using the biotinylated polyclonal antibody preparation (ARC-OVI), but the immunoreactivity patterns were the same regardless of which biotinylated antibody was applied. As mentioned in section 2.2.6, the dRIT diagnostic assay resulted in two false positive results (664/12 and 711/12) when compared to the FAT. The first sample only produced a false positive result with the dRIT diagnostic assay relying on monoclonal antibody 2, while the second samples (711/12) resulted in a false positive result irrespective of which of the three-biotinylated antibodies was applied. Apart from the observed false positive results, the locally produced biotinylated polyclonal antibody (ARC-OVI) did not produce any false negative

results. On the other hand, monoclonal antibody 1 and monoclonal antibody 2 produced 34 and 19 false negative results respectively.



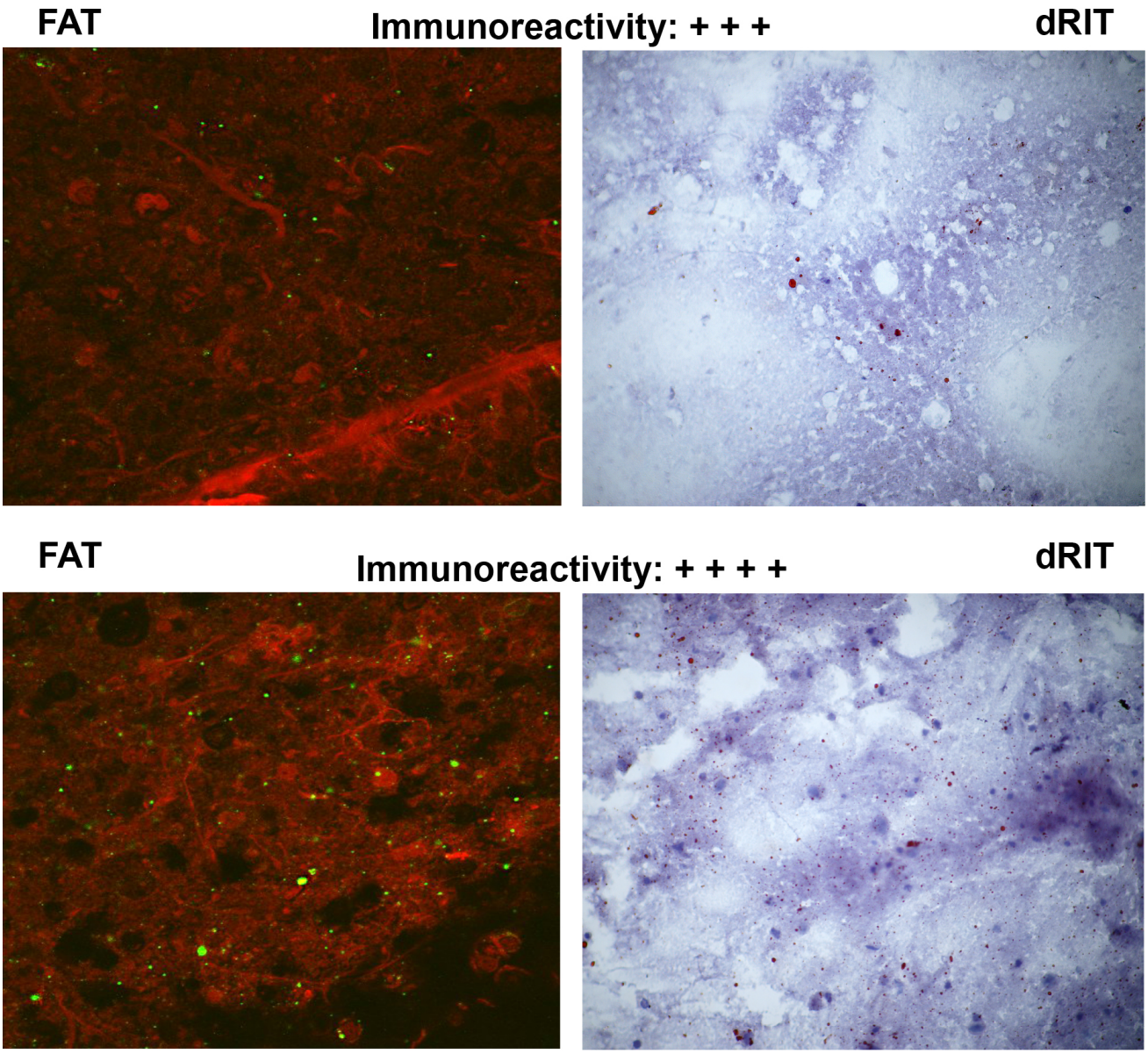


Figure 2.1. Visual representation of immunoreactivity scores associated with fluorescent antibody test and the direct, rapid immunohistochemical test assays

All immunoreactivity signs observed in the figure were based on the application of the locally produced polyclonal antibody (ARC-OVI). The fluorescent antibody test photos were taken at a magnification of 630x, and the direct, rapid immunohistochemical test photos were taken at a magnification of 200x. The immunoreactivity scores were based on the criteria stipulated in standard operating procedure for each of the assay, but a brief description was as follows:

- - -: No particles present in any of the fields. Sample is considered to be negative

+: The antigen is very scanty with one or more particles in less than 50% of the fields

++: The antigen is scanty with one or more particle present in less than 100%, but in more than 50%, of the fields

+++: The antigen is abundant with one or more particle present in every field, but the amount of antigen present can be counted properly

++++: The antigen is very abundant in every field and the amount of antigen present is “too numerous to count properly”

2.3.3. Investigation of false positive results

2.3.3.1. Real-time PCR amplification

The sample subjected to real-time PCR amplification (711/12) produced a statistical increase in the fluorescence over the background at a cycling threshold of 24,04 with an estimated copy number of $4{,}69 \times 10^4$ (Figure 2.2).

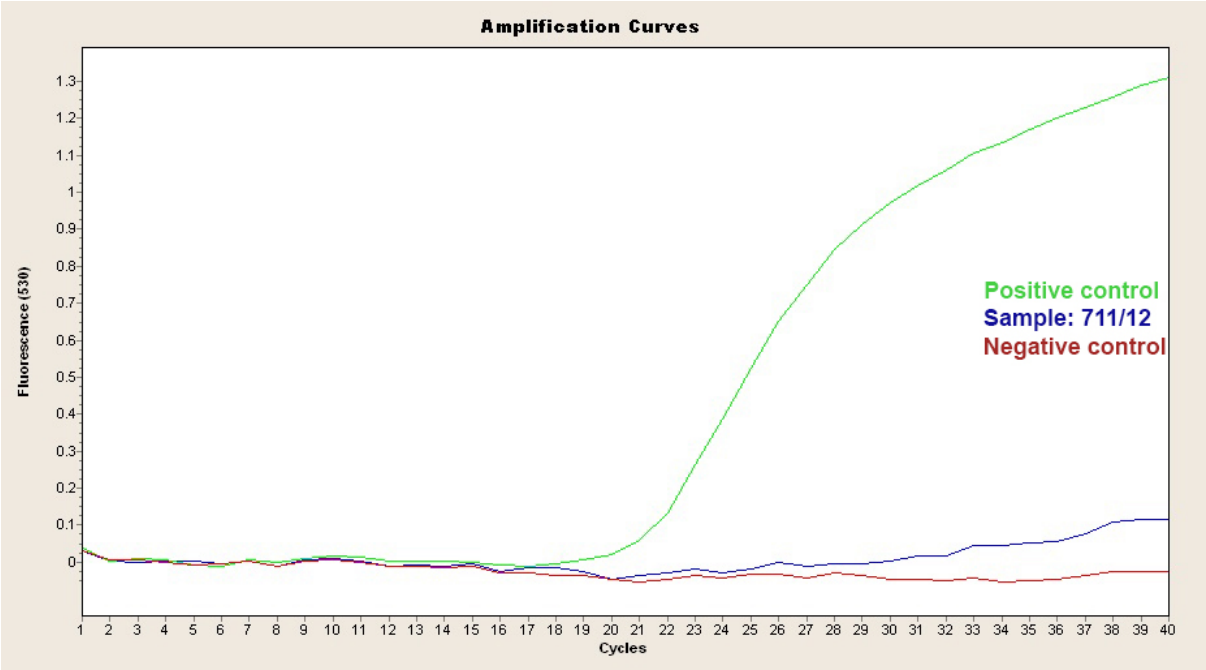


Figure 2.2. Real-time PCR amplification curve illustrating fluorescence increase for sample 711/12

The real time amplification curve, illustrated in the figure, showed the significant increase in the fluorescence associated with the sample that produced a dRIT positive result regardless of which of the three-biotinylated antibodies was applied. No amplification was observed for the negative control, while the positive control showed clear signs of an increase in fluorescence at a cycling threshold of 19,88.

2.3.3.2. hn-PCR amplification and sequence BLAST

After the first round of nucleic acid amplification, and subsequent 1% agarose gel electrophoresis analysis, only the positive control produced a distinct band. As such, a hn-PCR reaction was performed on the first round amplification of both samples (664/12 and 711/12). Although the undiluted PCR products primarily caused smears on the agarose gel, the diluted PCR product for sample 711/12 produced a clear band, while the size of the amplified product was confirmed with the inclusion of a λ ladder marker (Figure 2.3).

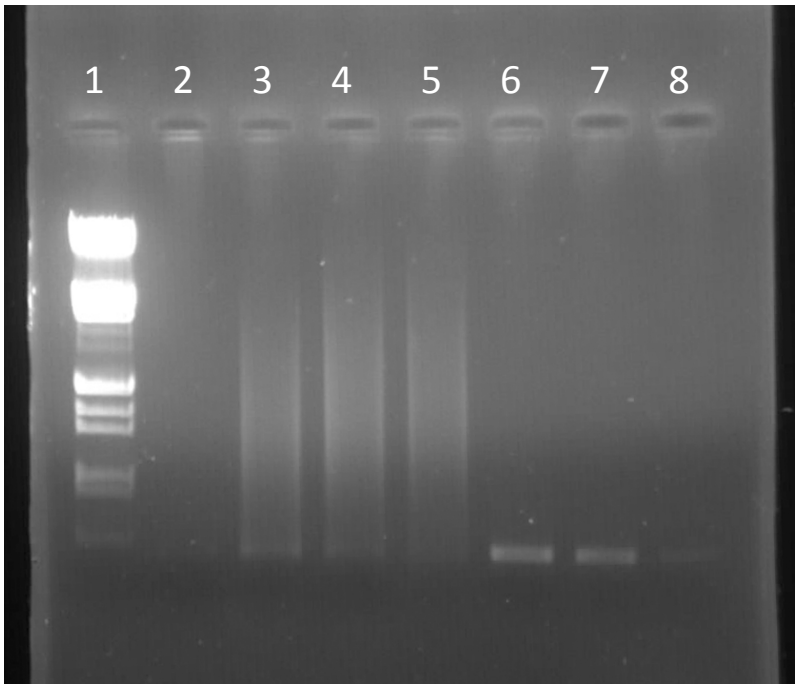


Figure 2.3. Agarose gel electrophoresis analysis of the hn-PCR reaction performed on sample 711/12

Lane1: λ marker. Lane 2: Negative control with no band. Lane 3: hn-PCR product of positive control (undiluted first round PCR product) Lane4: 10 µl of hn-PCR product of 711/12 (undiluted first round PCR product). Lane 6: Positive control of RABV (1:10 diluted first round PCR product). Lane 7: 10µl of Sample 711/12 (1:10 diluted first round PCR product). Lane 8: 5µl of Sample 711/12 (1:10 diluted first round PCR product). The size of the bands in Lane 6-8 corresponded with the correct band size (526 bp) as determined by the λ marker

The PCR amplicon (Lane 7-8, Figure 2.3) was purified and the forward and reverse sequence orientation was sequenced in order to create a trimmed consensus sequence of 466 nucleotides in length. The trimmed consensus sequence was subjected to a BLAST analysis on the GenBank website (www.ncbi.nlm.nih.gov). The BLAST result indicated that the nucleoprotein gene of the RABV had been amplified and that the sequence submitted to GenBank had a 97% maximum identity with the RABV 567/04 nucleoprotein sequence (GenBank Accession number: HM179505). The RABV 567/04 sequence belonged to the canid variant of RABV and was isolated from the Mpumalanga province of South Africa (Kgaladi, Nel, & Markotter, 2008). The obtained molecular evidence and consensus sequence indicated that the FAT had produced a false negative once applied to the sample and that the dRIT had in fact produced a true positive result.

Due to the fact that the hn-PCR amplification applied to samples 664/12 was negative, further analysis into the origin of the false positive was performed. In order to determine whether the observed false positive result in the case of sample 664/12 was due to non-specific binding of the second biotinylated monoclonal antibody, the dRIT diagnostic reaction was repeated but the biotinylated antibody was omitted from the

diagnostic reaction. The presence of immunoreactivity would have been indicative of the presence of EABA in the tissue sample, while the lack of immunoreactivity would have indicate that the false positive resulted due to the non-specific binding of the biotinylated antibody. Sample 664/12 showed no signs of immunoreactivity after the dRIT diagnostic assay was repeated without the presence of the second biotinylated monoclonal antibody. The results indicated that the immunoreactivity was due to the non-specific binding of the biotinylated monoclonal antibody and not due to EABA in the CNS sample.

2.3.3.3. Phylogenetic analysis of sequenced isolate derived from sample 711/12

The sequenced amplicon derived from sample 711/12 was aligned with selected canid and mongoose variants that had been chosen as representatives of the intrinsic nature of RABV in southern Africa. The phylogenetic analysis, based on a NJ tree relying on the Kimura-2 parameter (Figure 2.4), illustrated that the newly obtained sequence grouped with the canid variant of the RABV.

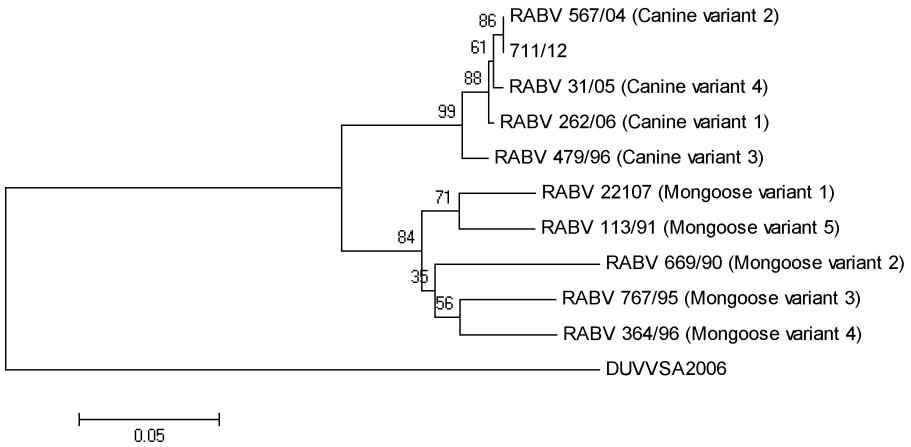


Figure 2.4. Phylogenetic tree of representatives of canine and mongoose variants of RABV circulating in Southern Africa

The phylogenetic analysis illustrated that sample 711/12 grouped with the canine variant of RABV, which was in agreement with the BLAST analysis performed in section 2.3.3.2. The relatively weak bootstrap support was most likely due to the relatively short sequence obtained from sequencing reaction. DUVV lyssavirus was used as a root for the neighbour joining tree using the Kimura-2 parameter.

2.3.4. Antigenic typing results

All false negative results were antigenically typed as mentioned in section 2.2.7, and it was found that all 36 samples contained the mongoose variant of RABV (Table 2.2). Monoclonal antibody 1 produced thirty-four false negative results, while

monoclonal antibody 2 produced nineteen false negative results. Out of the thirty-six false negative samples, seventeen samples produced false negative results in the case of both of the biotinylated monoclonal antibodies. This was indicative of the fact that the cocktail of biotinylated monoclonal antibodies would have miss-diagnosed seventeen true positive samples used in this study. The eight mongoose samples that produced true positive results using either monoclonal antibody 1 or monoclonal antibody 2 were also subjected to antigenic typing and were found to also contain the mongoose variant of RABV.

Table 2.2. Antigenic typing results of false negative samples		
Reference number	**Host species**	**Rabies virus variant**
CNS samples delivering false negative results with Monoclonal antibody 1		
756/99	Canid	Mongoose variant
601/99	Feline	Mongoose variant
620/99	Feline	Mongoose variant
114/11	Feline	Mongoose variant
376/11	Feline	Mongoose variant
660/11	Feline	Mongoose variant
261/12	Feline	Mongoose variant
382/12	Feline	Mongoose variant
540/99	Yellow mongoose	Mongoose variant
1087/99	Yellow mongoose	Mongoose variant
153/11	Yellow mongoose	Mongoose variant
177/11	Yellow mongoose	Mongoose variant
448/12	Yellow mongoose	Mongoose variant
502/12	Yellow mongoose	Mongoose variant
594/11	Black-backed jackal	Mongoose variant
1029/99	Bovine	Mongoose variant
1086/99	Bovine	Mongoose variant
CNS samples delivering false negative results with Monoclonal antibody 2		
306/12	Feline	Mongoose variant
529/99	Yellow mongoose	Mongoose variant
CNS samples delivering false negative results with both monoclonal antibodies		
1003/99	Canid	Mongoose variant
579/11	Canid	Mongoose variant
133/12	Canid	Mongoose variant
283/11	Feline	Mongoose variant
520/11	Feline	Mongoose variant
613/11	Feline	Mongoose variant
457/12	Feline	Mongoose variant
650/12	Feline	Mongoose variant
651/12	Feline	Mongoose variant
91/11	Yellow mongoose	Mongoose variant
99/11	Yellow mongoose	Mongoose variant
169/11	Yellow mongoose	Mongoose variant
010/12	Yellow mongoose	Mongoose variant
072/12	Yellow mongoose	Mongoose variant
100/12	Yellow mongoose	Mongoose variant
131/12	Yellow mongoose	Mongoose variant
107/12	Bovine	Mongoose variant

2.3.5. Sensitivity and specificity of the dRIT assay using different biotinylated antibodies

With the application of the dRIT test (section 2.2.5), and the subsequent confirmation with nucleic acid amplification techniques (2.2.6), it was shown that sample distribution in this chapter consisted of 201 positive samples and 49 negative samples used to evaluate the diagnostic efficacy of the dRIT diagnostic assay (Table 2.3).

Table 2.3. Diagnostic sensitivity, specificity and Cohen's Kappa measure of agreement of dRIT diagnostic assay using three biotinylated antibodies

FAT							
Biotinylated Antibodies	**True Positive**	**False Positive**	**True Negative**	**False Negative**	**Diagnostic Sensitivity***	**Diagnostic Specificity***	**Kappa Value***
Polyclonal Antibody	200	0	49	1	99,5% (97,25% -99,92%)	100% (92,68% -100%)	ND
dRIT							
Polyclonal Antibody	201	0	49	0	100% (98,16% -100%)	100% (92,68% -100%)	0.987 (0,963 - 1,000)
Monoclonal antibody #1	167	0	49	34	83,08% (77,17% - 87,99%)	100% (92,68% -100%)	0.649 (0,548 - 0,751)
Monoclonal antibody #2	182	1	48	19	90,55% (85,63% - 94,21%)	97,96% (89,10% - 99,66%)	0.767 (0,674 – 0,861)

* Value in brackets represented the 95% confidence interval (CI)
ND: "Not done" due to FAT being the reference test

The three individual dRIT reactions, each relying on one of the three-biotinylated antibodies, showed varying degrees of diagnostic efficacy based on their application to the 250 CNS tissue samples. The dRIT diagnostic assay relying on the polyclonal antibody preparation (ARC-OVI, Rabies division), that was biotinylated for the purpose of this study, resulted in no false negative or positive results (Figure 2.5 - 2.11; Table A2). The confirmation of the false negative sample (711/12) associated with the FAT diagnostic assay resulted in a diagnostic sensitivity of 99,5% (95% CI: 97,25% - 99,92%), while the diagnostic efficacy of the dRIT relying on the biotinylated polyclonal antibody was marginally higher with a diagnostic sensitivity and specificity of 100% (95% CI: Sensitivity: 98,16%-100% and specificity: 92,68% -100%).

The application of the dRIT test relying on the first biotinylated monoclonal antibody (Monoclonal antibody 1, CDC) produced thirty-four false negative results and no false positive results (Figure 2.5 - 2.11; Table A2). The high number of false negative samples (34/201) thus resulted in a reduced diagnostic sensitivity of 83,08% (95% CI: 77,17% - 87,99%) and a diagnostic specificity of 100% (95% CI: 92,68% -100%.) The application of the dRIT test relying on the second biotinylated monoclonal antibody (Monoclonal antibody 2, CDC) produced nineteen false negative results and a single

positive result (Figure 2.5 - 2.11; Table A2). Due to the presence of the false negative and false positive results the diagnostic sensitivity was 90,55% (95% CI: 85,63% - 94,21%) and the diagnostic specificity was 97,96% (95% CI: 89,10% - 99,66%).

Because of the relatively high number of false results obtained, the Cohen's kappa measure of agreement for each of the assays differed marginally. The single false negative result associated with the FAT resulted in a Cohens' kappa measure of agreement of 0.987 (95% CI: 0,963-1,000) for the dRIT diagnostic assay relying on the biotinylated polyclonal antibody, leading to an "almost perfect agreement" with the FAT test. The comparison of the FAT with the dRIT diagnostic assay relying on each of the two-biotinylated antibodies resulted in a Cohens' kappa measure of agreement of 0,649 (95% CI: 0,548-0,751) and 0,767 (95% CI: 0,674-0,861) respectively. As such, the dRIT relying on monoclonal antibody 1 and monoclonal antibody 2 each had a "substantial agreement" with the FAT diagnostic assay according to the pre-determined agreement criteria mentioned in section 2.2.8.3 (Landis & Koch, 2012).

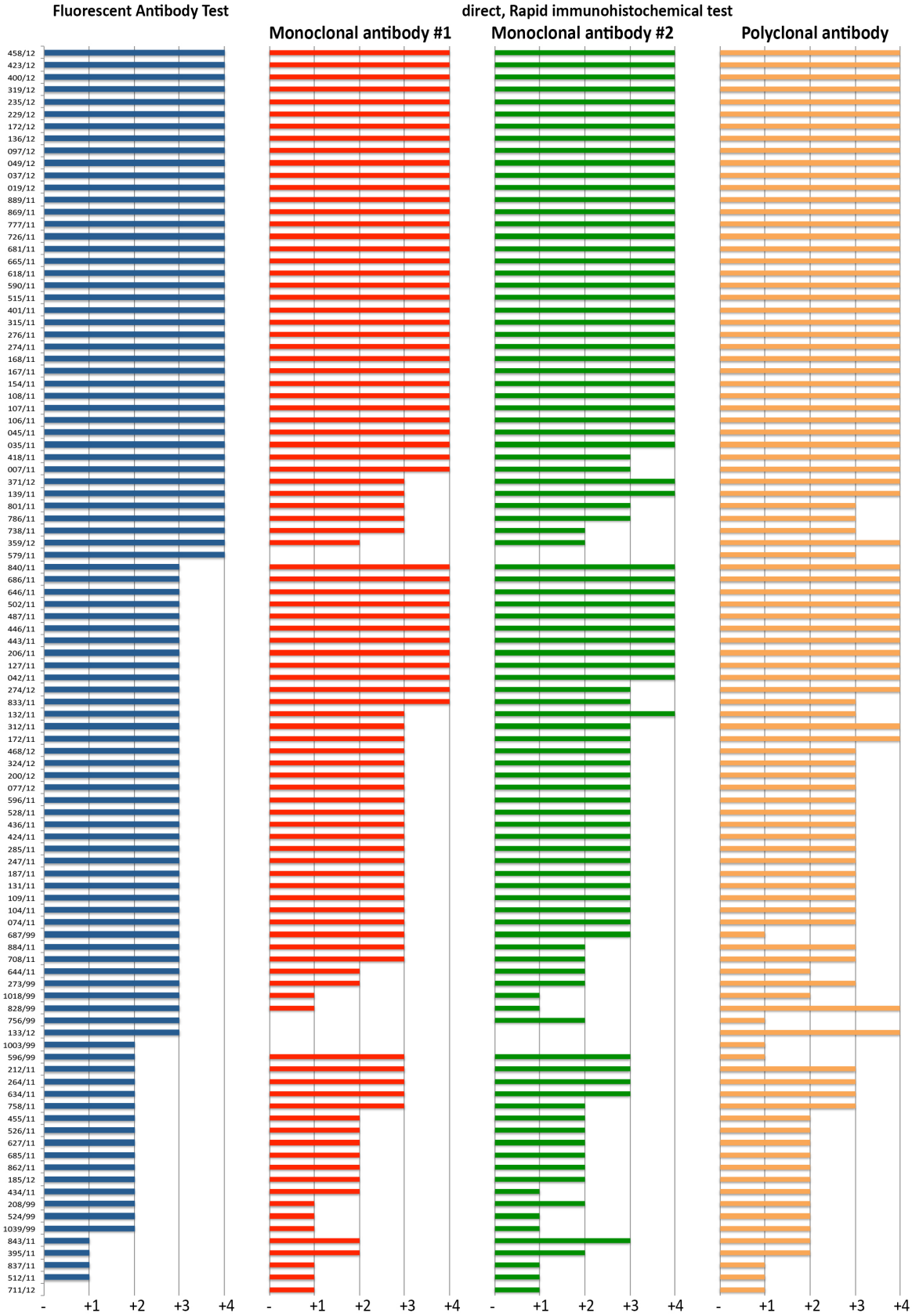


Figure 2.5. Immunoreactivity scores associated with known positive canine samples

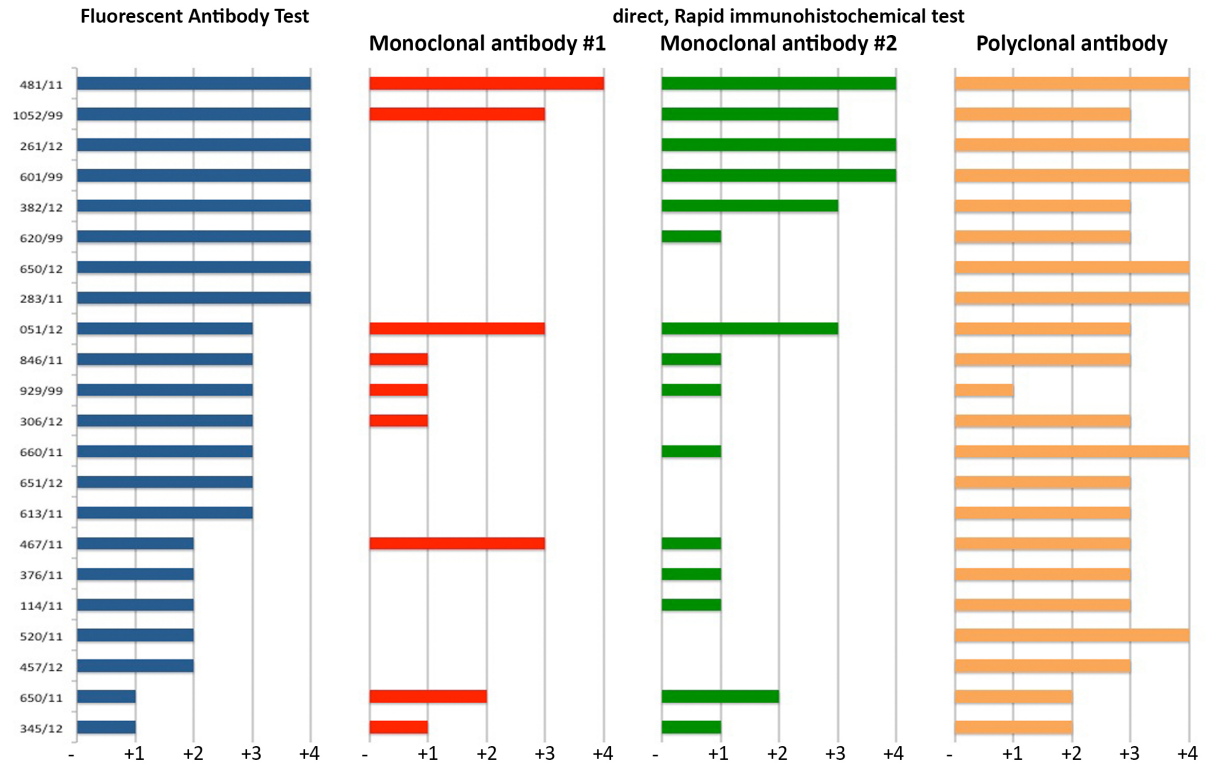


Figure 2.6. Immunoreactivity scores associated with known positive feline samples

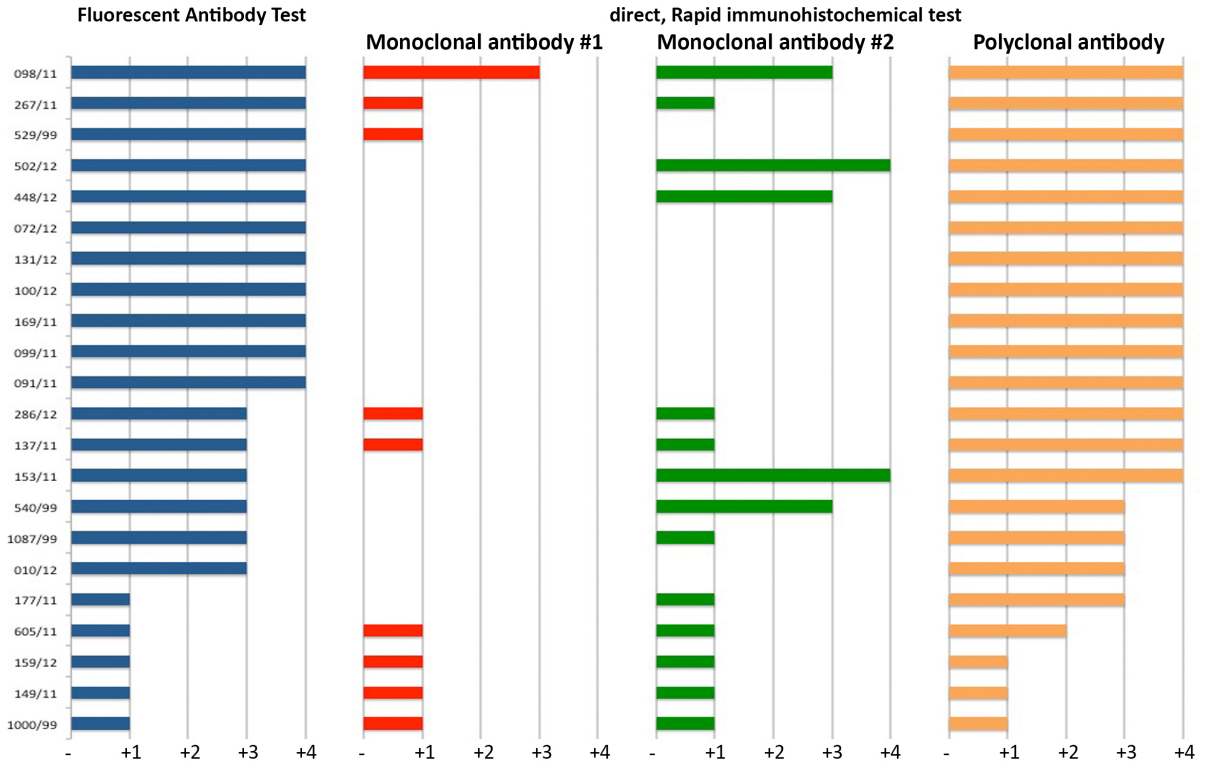


Figure 2.7. Immunoreactivity scores associated with known positive mongoose samples

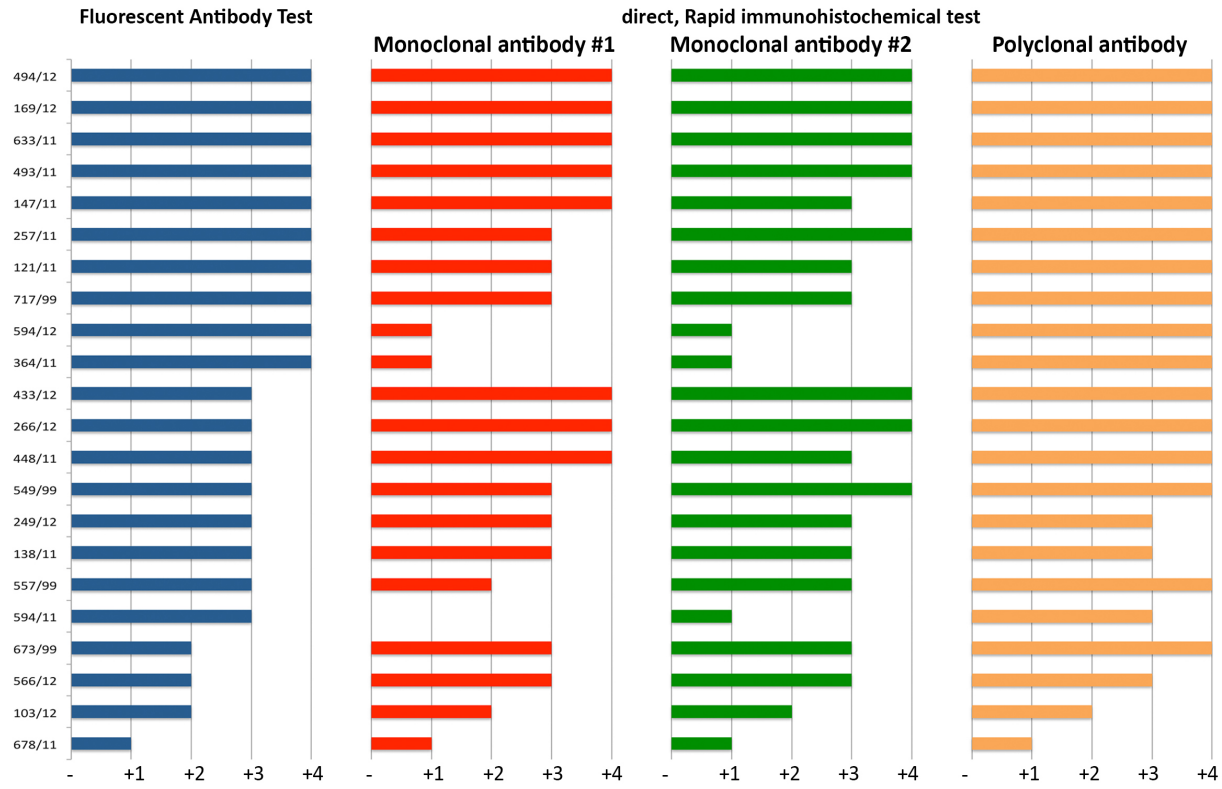


Figure 2.8. Immunoreactivity scores associated with known positive jackal samples

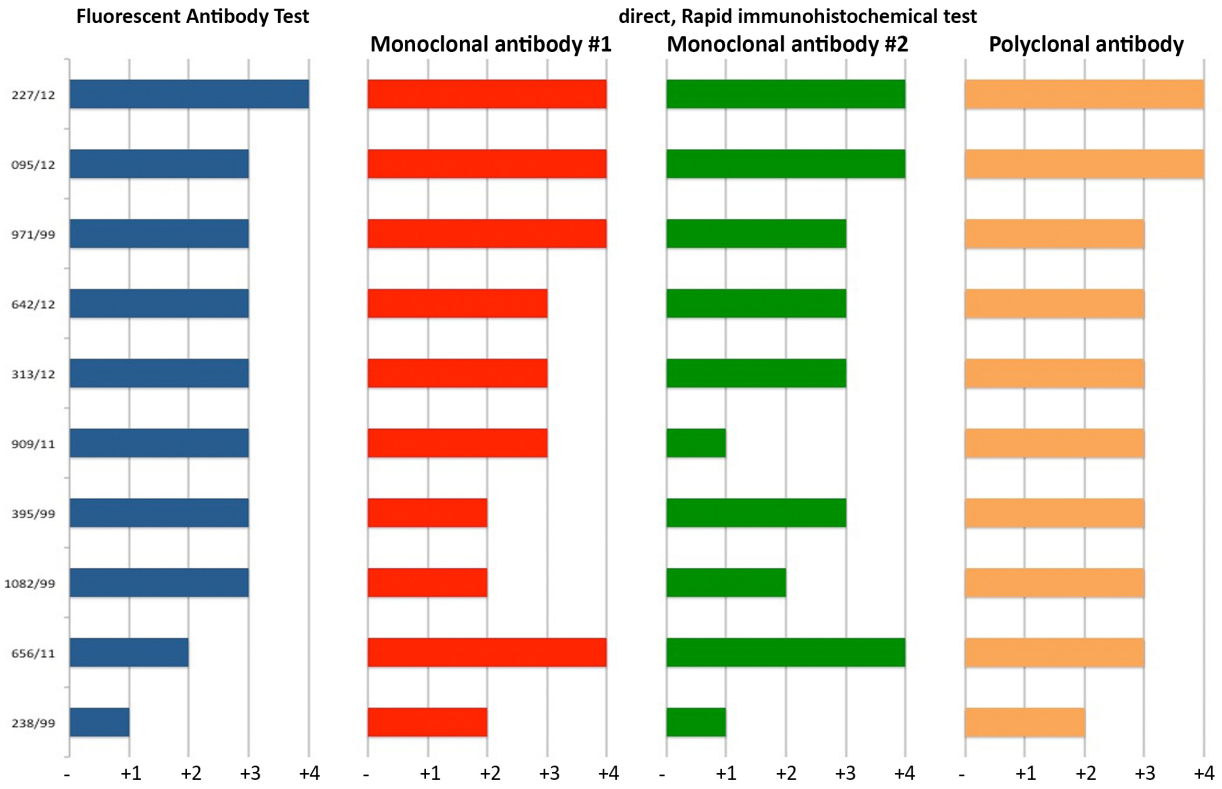


Figure 2.9. Immunoreactivity scores associated with known positive fox samples

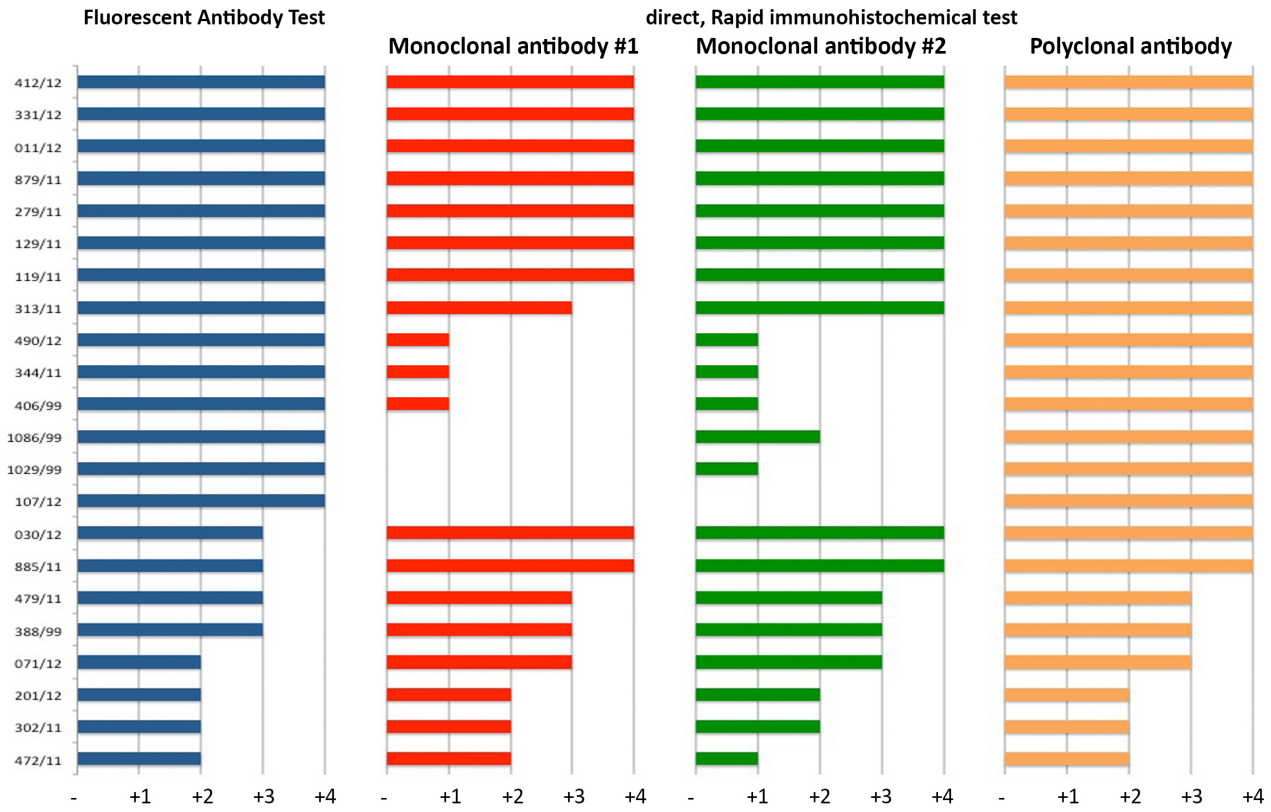


Figure 2.10. Immunoreactivity scores associated with known positive bovine samples

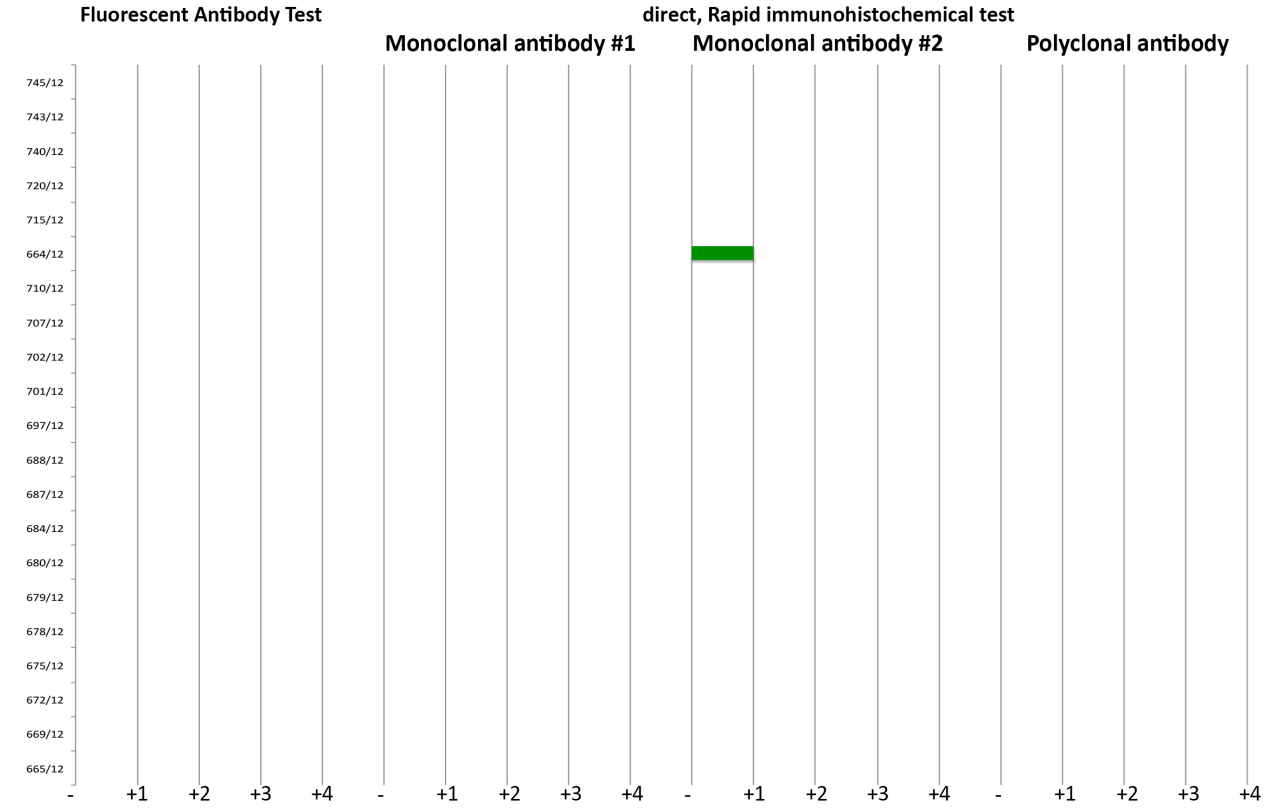


Figure 2.11. Immunoreactivity scores associated with known negative samples

2.4. Discussion

Considering the need for improved RABV surveillance across the African continent, the development of a diagnostic assay that is intended to be complimentary to the FAT needs to be applied and tested in a significant number of scenarios in order to obtain enough data to confirm the statistical efficacy and reproducibility of the assay. As was illustrated in this chapter, the widespread application of a novel diagnostic assay is also required to highlight any underlying limitations that could potentially influence the applicability of the diagnostic efficacy.

The main aim of this chapter was to determine whether a polyclonal antibody could be biotinylated and applied to the dRIT protocol as a replacement antibody preparation for the cocktail of biotinylated monoclonal antibodies supplied by the CDC. The evaluation of the dRIT assay utilizing the biotinylated polyclonal antibody thus involved the comparison of the diagnostic efficacy with that of the dRIT diagnostic assay relying on either of the two monoclonal antibodies that routinely make up the antibody cocktail applied in the dRIT diagnostic reaction as well as with the recommended FAT diagnostic assay. The work in this chapter clearly illustrated the fact that an alternative antibody preparation could be biotinylated and applied to the diagnostic assay instead of the cocktail of biotinylated monoclonal antibodies in order to achieve a working diagnostic assay.

As mentioned in the section 1.5.1.2, the FAT diagnostic assay is currently the only diagnostic assay that is recommended for the routine diagnosis of rabies by the WHO and OIE. The main reason for this recommendation is the high diagnostic efficacy associated with the diagnostic assay, resulting in test results that are considered to be conclusive when the test is performed by a trained technician. Despite this fact, a RABV positive sample included in this study was miss-diagnosed by the FAT assay on two separate occasions – once by the ARC-OVI, rabies division, at the time of the initial diagnosis of the sample and the second time when the FAT was performed in this study. Two possible reasons for the origin of the false results were singled out as the potential cause, and will be discussed briefly.

It is important to note that the standardised FAT protocol needs to be followed to precision using properly calibrated and maintained equipment in order to obtain accurate results. This fact was proven during a “proficiency test” held for SADC countries by the ARC-OVI, rabies division. During the course of the training held for 14 members from the SADC countries, 23 false negative and 9 false positive results were reported (C.T. Sabeta, 2011). The concluding findings of this training program was that, for a laboratory to be truly efficient at routine rabies diagnosis, the staff would need to undergo regular proficiency testing, while the facilities should maintain an ISO/CEI

17025 accreditation standard at all times. It is also accepted that the CNS tissue samples have to be as fresh as possible, because the deterioration of the sample tends to lead to an increased probability of obtaining a false negative result (Komalvarin et al., 1993; WHO, 2005). Without the presence of cold chains in most resource-limited developing countries, samples are routinely shipped in chemical preservatives at room temperature, which often leads to samples that have begun decomposing by the time the initial diagnosis is performed. Even though the specific sample that was miss-diagnosed by the FAT had been stored at 4 °C for nearly two months before the dRIT was applied, the diagnostic assay produced clear and distinct red inclusions that were easily observed. The fact that the dRIT diagnostic test, relying on the biotinylated polyclonal antibody, did not produce a false negative result was most thus most likely due to the fact that the dRIT results are easier to interpret regardless of the condition of the tissue sample.

The biotinylated polyclonal antibody was applied to the 250 CNS samples using the dRIT diagnostic assay, and the subsequent statistical analysis indicated that the dRIT had a diagnostic efficacy that was marginally better than that of the FAT diagnostic test. The Cohen's Kappa measure of agreement correlated this fact by describing the two tests as having an "almost perfect agreement" with each other. The results of the dRIT relying on the two-biotinylated monoclonal antibodies, applied to the same sample set, varied from those observed in with the FAT test. The dRIT diagnostic assay relying on monoclonal antibody 1 produced thirty-four false negative results, while the dRIT test relying on biotinylated monoclonal antibody 2 produced nineteen false negative results that were also associated with the mongoose variant. Further investigation of the false negative results showed that all the samples belonged to the mongoose variant of the RABV. Our study has been the first of its kind to include the mongoose variant of the RABV in the sample panel, and the results showed that the classical dRIT assay (using the two-biotinylated monoclonal antibodies) tested negative on some of these samples. The possible explanations for these results (which will be discussed in further detail in Chapter VI) could be that the working dilution of the two-biotinylated monoclonal antibodies could have been over-diluted, resulting in a monoclonal antibody concentration that was too low to detect the antigenic epitopes on the RABV nucleoprotein. The other possible reason could be that the antigenic epitopes, with which the monoclonal antibodies should interact, are less conserved in certain isolates. Any changes in the structure of the epitopes could render the antibody-antigen interaction ineffective and result in false negative results.

In conclusion, the work performed in this chapter was used to, not only, perform further validation of the established dRIT assay (relying on the biotinylated monoclonal antibodies supplied by the CDC), but to also validate the dRIT diagnostic test relying on

a locally produced biotinylated antibody preparation by applying the various dRIT diagnostic assays to a sample set derived from southern Africa. The results clearly highlighted that the modified dRIT assay (using the locally produced biotinylated polyclonal antibody preparation) had a considerably higher diagnostic efficacy once compared to that of the classical dRIT.

Chapter III

Application of the direct, rapid immunohistochemical test using acetone fixation

3.1. Introduction

The main determinant in the applicability of a diagnostic assay for the routine diagnosis of RABV is the diagnostic efficacy (diagnostic sensitivity and specificity discussed in section 2.2.7.1 and 2.2.7.2) associated with the given assay. As discussed in section 1.5.1.2, the FAT remains the only diagnostic test that is recommended for routine rabies diagnosis by the WHO and OIE (OIE, 2008b; WHO, 2005) due to the high diagnostic efficacy associated with the diagnostic assay. Although the diagnostic efficacy is a crucial determinant of the applicability of a diagnostic assay, it should not be considered the only deciding factor in resource-limited developing countries. Based on the initial investigations mentioned in section 2.3.6, the dRIT relying on the biotinylated polyclonal antibody (ARC-OVI) appears to be reliable in terms of complimenting the FAT diagnostic assay based on the fact that dRIT assay, applied in this study, had a diagnostic sensitivity and specificity marginally higher than that of FAT assay. Despite the high diagnostic sensitivity and specificity associated with the dRIT assay, the widespread application of the dRIT in resource-limited developing countries will also be influenced by the general ease of application associated with the test. The FAT test relies on a standardised acetone fixation protocol that is implemented by all laboratories that perform routine FAT rabies diagnosis (Dean et al., 1996). Considering the fact that the dRIT diagnostic test has been adapted from a basic immunohistochemical reaction, the standard operating procedure of the dRIT assay (Niezgoda & Rupprecht, 2006) relies on the fixation of tissue with the recommended fixation method for immunohistochemistry reactions, i.e. 10% neutral buffered formalin (Section 1.7.1) (Farmilo & Stead, 2009; Grizzle et al., 2008). To date, no studies included the standardised acetone fixation procedure in the dRIT diagnostic assay. The incorporation of acetone fixation could ease the implementation of the dRIT diagnostic assay as a complimentary diagnostic assay in developing African countries due to the available resources and knowledge associated with the fixation process.

The aim of this chapter was to determine whether the dRIT diagnostic assay could be adapted to accommodate the standardised acetone fixation step instead of the routine formalin fixation. Apart from obtaining a working dRIT reaction relying on acetone fixation, the secondary aim was to investigate whether the addition of an acetone fixation step would influence the diagnostic sensitivity and specificity associated with the biotinylated polyclonal antibody once applied to the diagnostic assay.

3.2. Materials and methods

3.2.1. Biotinylated polyclonal antibody

The dRIT diagnostic assay, applied in this chapter, relied on the biotinylated polyclonal anti-ribonucleoprotein antibody preparation (ARC-OVI, rabies division) described in section 2.2.1 and 2.2.2.

3.2.2. Sample selection and preparation

The sample set (Table A1) used in the previous chapter (section 2.2.3) was used to study both the diagnostic sensitivity and diagnostic specificity associated with the dRIT diagnostic assay relying on acetone fixation instead of the recommended formalin fixation step.

3.2.3. Fluorescent antibody test

The level of positivity associated with each of the 250 CNS tissue samples had been determined in the previous chapter (section 2.2.4, Table A3) and as such the FAT diagnostic test was not repeated.

3.2.4. dRIT using acetone fixation

The standard operating procedure associated with the dRIT assay (Niezgoda & Rupprecht, 2006) was adapted by replacing the recommended formalin fixation step (submerging slides in 10% neutral buffered formalin for ten minutes) with the standardised acetone fixation process used during the FAT test (submerging slides in cold filtered acetone (Associated Chemical Enterprises) for thirty minutes). The adapted dRIT diagnostic assay was first applied to both a positive and negative control derived from CNS tissue of mice (infected with the CVS strain of RABV) in triplicate to investigate the general efficacy of adapted diagnostic assay but no signs of immunoreactivity were observed.

3.2.4.1. Taguchi optimization of the adapted dRIT assay

To obtain a working dRIT reaction relying on acetone fixation, the Taguchi optimization process (Cobb & Clarkson, 1994; Jeney, Dobay, & Lengyel, 1999) was

applied to certain pre-selected variables associated with the diagnostic test (Table 3.1). The Taguchi protocol is a technique whereby the estimated effects of various components that form part of the diagnostic reaction, are tested in nine separate reactions. As shown in Table 3.1, each column represented the individual reaction components, and each row represented individual reaction levels (Cobb & Clarkson, 1994). Briefly, the following conditions were altered in nine separate dRIT reactions each relying on acetone fixation: antibody incubation time, streptavidin-peroxidase incubation time, chromogen incubation time and chromogen wash step time.

Table 3.1. Variables associated with the adapted dRIT test

		Levels		
	Variables	**A**	**B**	**C**
[1]	**Antibody Incubation time**	10 minutes	20 minutes	40 minutes
[2]	**Streptavidin Incubation time**	10 minutes	20 minutes	40 minutes
[3]	**Chromogen Incubation time**	5 minutes	10 minutes	25 minutes
[4]	**Chromogen wash step time**	2 minute	5 minutes	10 minutes

Variables (→)	**[1]**	**[2]**	**[3]**	**[4]**
Reaction number (↓)				
1	A	A	A	A
2	A	B	B	B
3	A	C	C	C
4	B	A	B	C
5	B	B	C	A
6	B	C	A	B
7	C	A	C	B
8	C	B	A	C
9	C	C	B	A

3.2.4.2. dRIT using acetone fixation

Based on the statistical analysis of the dRIT diagnostic assay discussed in Chapter II (section 2.3.6) it was shown that the locally produced biotinylated polyclonal antibody (ARC-OVI, rabies division) far out performed the biotinylated monoclonal antibodies in terms of diagnostic sensitivity and specificity once the dRIT diagnostic assay had been applied to the 250 CNS tissue samples derived from southern African mammalian species (Table A1). Therefore, the dRIT reaction relying on acetone fixation and the biotinylated polyclonal antibody was applied to all 250 CNS tissue samples used in this study. The dRIT diagnostic assay relying on acetone fixation and either of

the two-biotinylated monoclonal antibodies (CDC) was applied to a subset of 22 RABV positive CNS tissue samples to merely prove that the acetone fixation process did not influence the diagnostic process of the monoclonal antibodies in the adapted dRIT reaction (Table A4).

A single touch impression was made from the CNS tissue by placing a small amount of composite brain material on clean filter paper. After pressing a clear microscope slide (Marienfeld, Germany) down on top of the sample, the slide with the touch impressions was fixed in cold 100% acetone (Associated Chemical Enterprises) for thirty minutes. After the fixation period had passed, the slide was air dried for five minutes before the touch impression was re-hydrated by dip rinsing the slide in TPBS buffer (PBS containing 1% Tween80 (Merck chemicals)). The slide was submerged in 3% hydrogen peroxide (Merck chemicals) for ten minutes after which it was dip rinsed in fresh TPBS buffer. The excess buffer was shaken from the slide and the area surrounding the smear impression was blotted dry using fresh paper towel. The working concentration (1:220) of the biotinylated polyclonal antibody (ARC-OVI, Rabies division) was added to the touch impression until the whole impression was covered. The slide was placed in a humidity chamber and incubated at room temperature for twenty minutes, after which the slide was dip rinsed in fresh TPBS buffer. The excess buffer was shaken from the slide and the area surrounding the smear impression was blotted dry using fresh paper towel. The touch impression was covered in a ready-to-use solution of Streptavidin-peroxidase (Kirkegaard and Perry Laboratories), after which the slide was transferred to a humidity chamber. The humidity chamber was incubated at room temperature for forty minutes and after incubation the slide was dip rinsed in fresh TPBS buffer and the excess buffer was shaken from the slide before the area surrounding the smear impressions was blotted dry using fresh paper towel. A working solution of the 3-amino-9-ethylcarbazole (AEC) chromogen was made according to the instructions provided with the staining kit (AEC Chromogen Kit, Sigma-Aldrich). Two drops of supplied acetate buffer, one drop of supplied AEC chromogen and one drop of supplied 3% hydrogen peroxide was added to 4 ml of double distilled water. The solution was mixed sufficiently and stored at 4 °C until use. The impression on the slide was covered in the working solution of the 3-amino-9-ethylcarbazole (AEC) chromogen and after placing the slide in a humidity chamber the slide was incubated at room temperature for five minutes. After the staining time had lapsed, the slide was submerged in distilled water for five minutes. The touch impression was counterstained with a 1:2 dilution of Gill's formulation #2 (Sigma-Aldrich) for two minutes, after which the slide was dip rinsed in distilled water in order to wash away the residual counterstain. Finally, the slide was mounted with a water-soluble mounting medium (PBS/glycerol (Sigma-Aldrich) mixed 1:1), and examined by light microscopy (Nikon,

Alphashot YS) at both 200x and 400x magnification in order to score the respective immunoreactivity. A total of 40 fields were viewed by a single microscopist, and all samples originally producing “false” results (either negative or positive) were repeated a further two times to confirm the diagnosis. The immunoreactivity of the various samples was determined in a blind fashion by not relying on the FAT immunoreactivity scores to influence the interpretation of the dRIT results.

3.2.5. Statistical analysis of results

The statistical analysis of the dRIT diagnostic tests relying on acetone fixation was determined by comparing the dRIT results to the results obtained from the FAT diagnosis. The statistical analysis of the diagnostic efficacy was performed by assuming an exact binomial distribution (MedCalc® 12.2.1.0, Ostend Belgium).

3.3. Results

3.3.1. Taguchi optimization of the adapted dRIT assay

The main aim of the Taguchi optimization protocol was to determine whether the alteration of four variables associated with the dRIT diagnostic assay could be manipulated in nine separate optimization reactions in order to obtain a working diagnostic assay that could be applied to the CNS tissue samples obtained from southern Africa mammalian species. The final levels of each variable (Table 3.2), used to adapt the standard dRIT protocol, were chosen in order to have a dRIT diagnostic assay resulting in the best possible immunoreactivity, while avoiding high levels of non-specific background staining. Based on the Taguchi optimization protocol, a working dRIT reaction was obtained whereby the positive control had clear and abundant red inclusions on the blue neuronal background, while the negative control had no red inclusions whatsoever.

Table 3.2. Variables chosen after Taguchi optimization protocol of the acetone fixation dRIT protocol	
Variable(s)	**Chosen levels**
Antibody incubation time	20 minutes
Streptavidin incubation time	40 minutes
Chromogen incubation time	5 minutes
Chromogen wash step time	5 minutes

3.3.2. dRIT using acetone fixation

The dRIT test relying on the biotinylated polyclonal antibody, was applied to the 250 acetone fixed CNS tissue samples and the immunoreactivity scores associated with each of the samples was scored according to the staining intensity and antigen distribution observed with each sample. The same patterns and intensity of the red inclusions was observed as when using formalin fixation (Figure 2.1). The biotinylated polyclonal antibody resulted in no false negative or positive results subsequent to the application to the 250 CNS tissue samples (Figure 3.1 – 3.7, Table A3), while the adapted dRIT protocol (relying on the two individual biotinylated monoclonal antibodies), applied to the subset of known positive CNS samples, produced no false negative results (Figure 3.8 and Table A4). The samples included in the subset were chosen based on the fact that it had been showed in Chapter II that the two monoclonal antibodies, once applied in the dRIT assay to formalin fixed CNS tissue samples, produced true positive results.

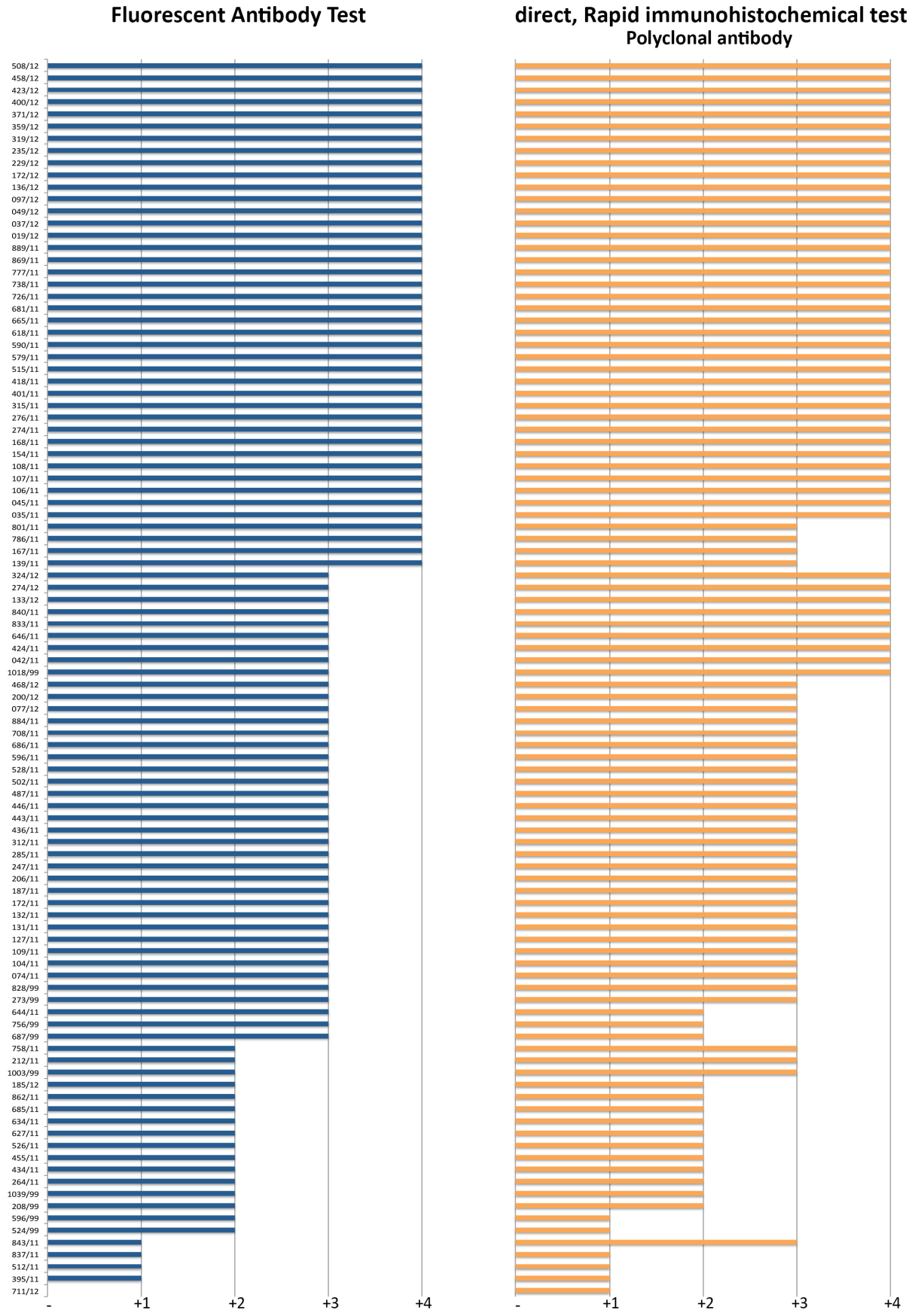


Figure 3.1. Immunoreactivity scores associated with known positive canine samples

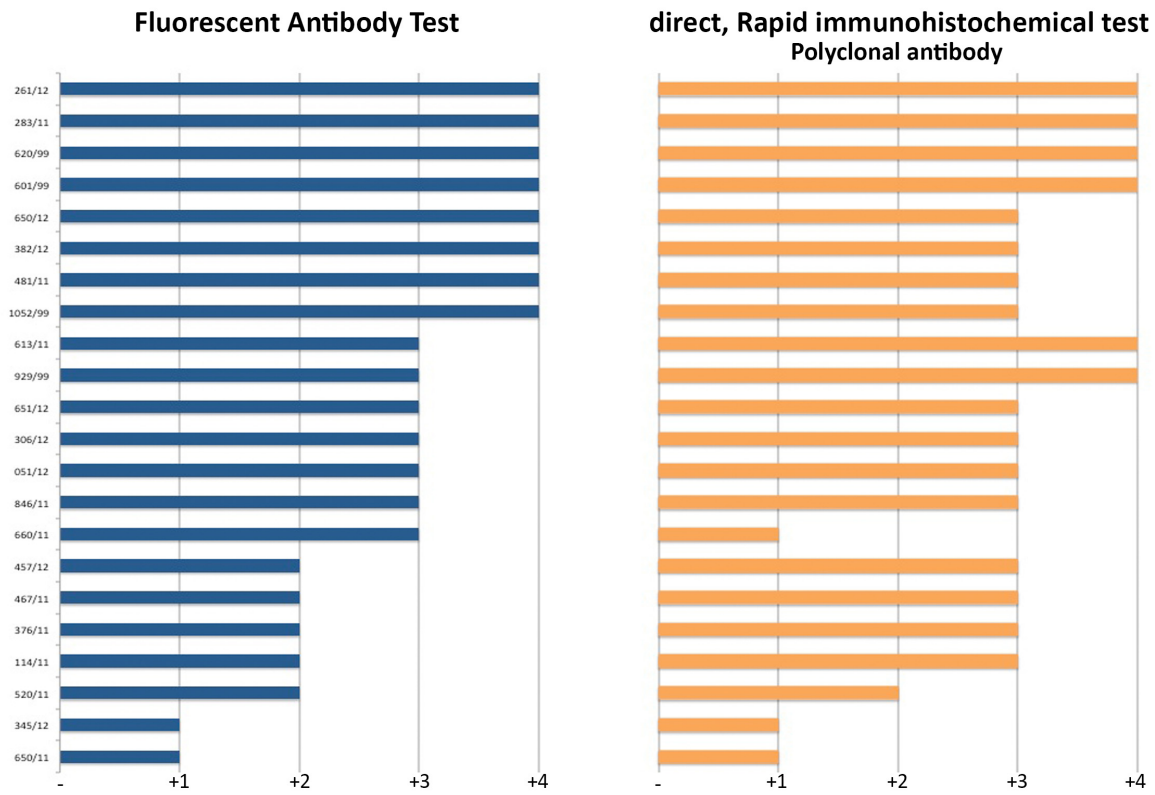


Figure 3.2. Immunoreactivity scores associated with known positive feline samples

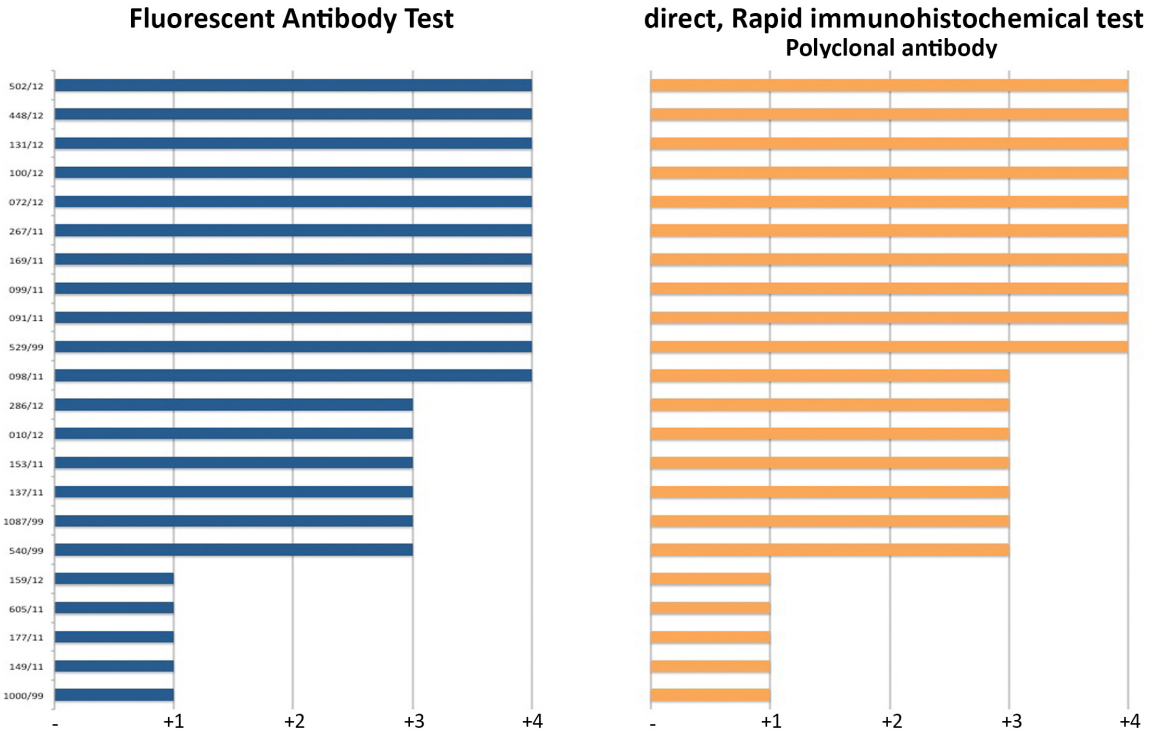


Figure 3.3. Immunoreactivity scores associated with known positive mongoose samples

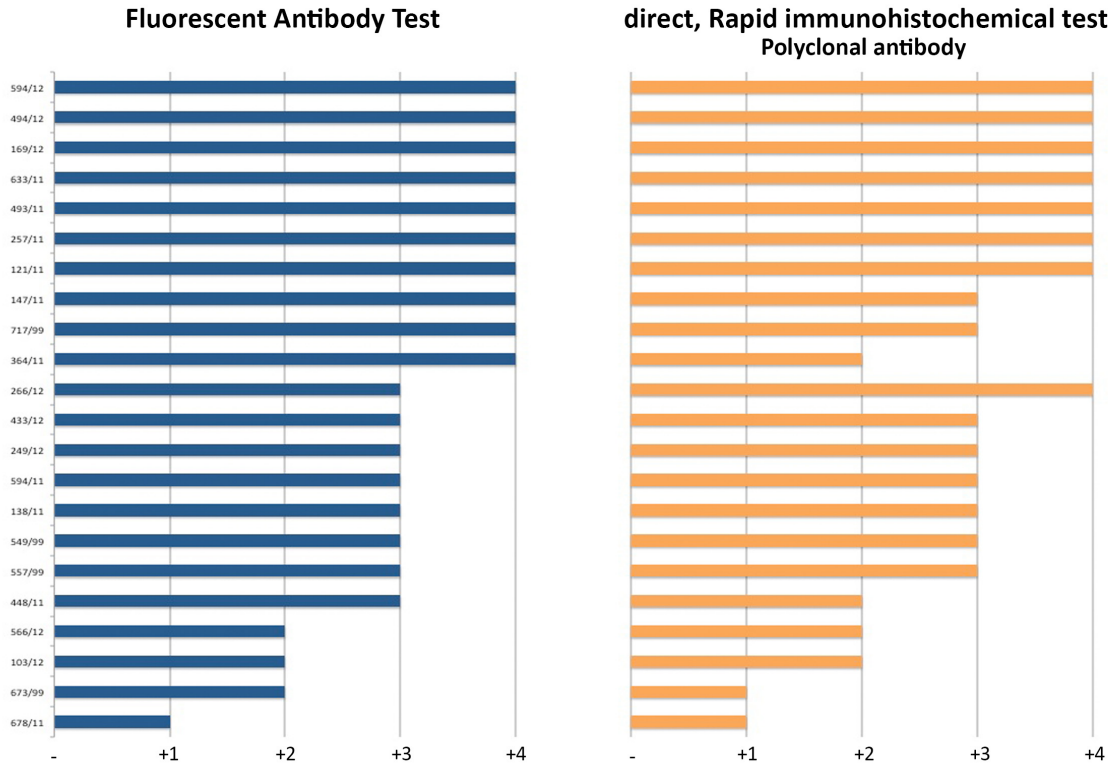


Figure 3.4. Immunoreactivity scores associated with known positive jackal samples

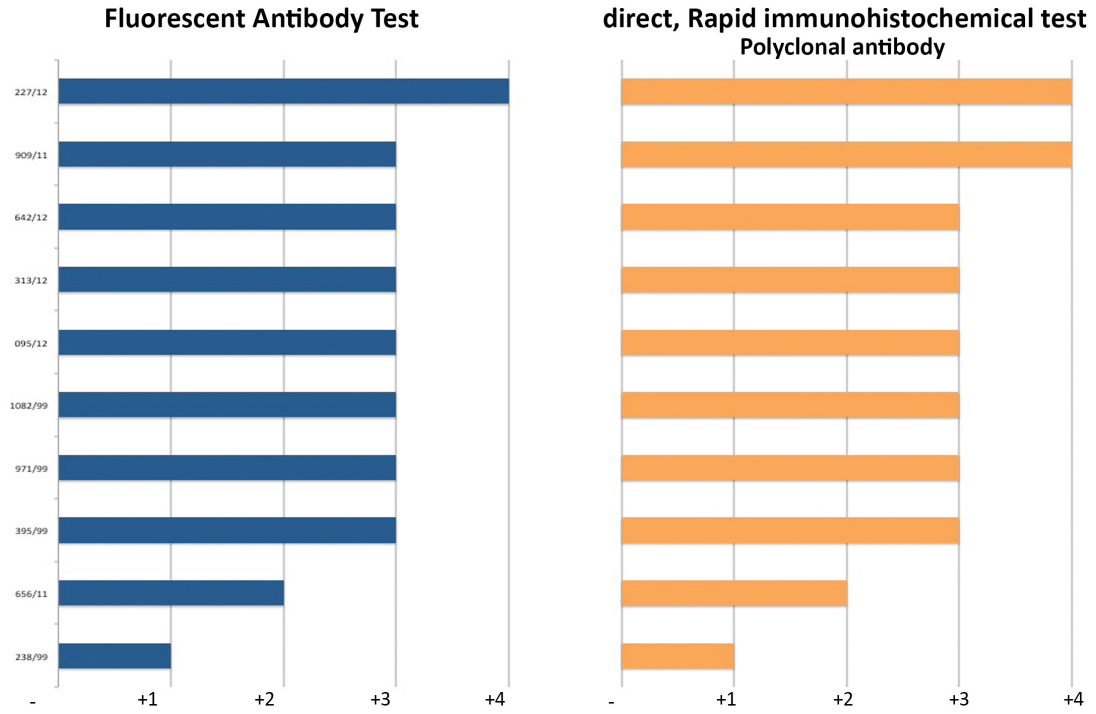


Figure 3.5. Immunoreactivity scores associated with known positive fox samples

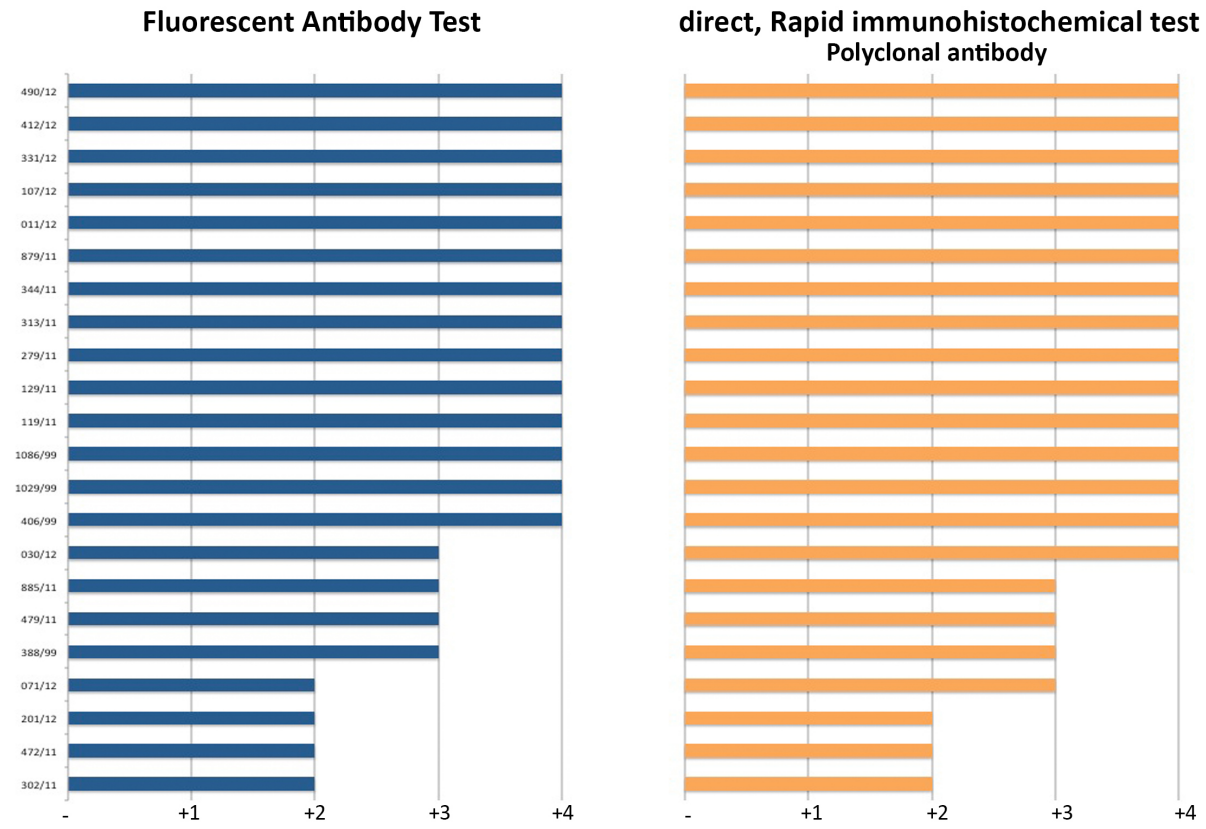


Figure 3.6. Immunoreactivity scores associated with known positive bovine samples

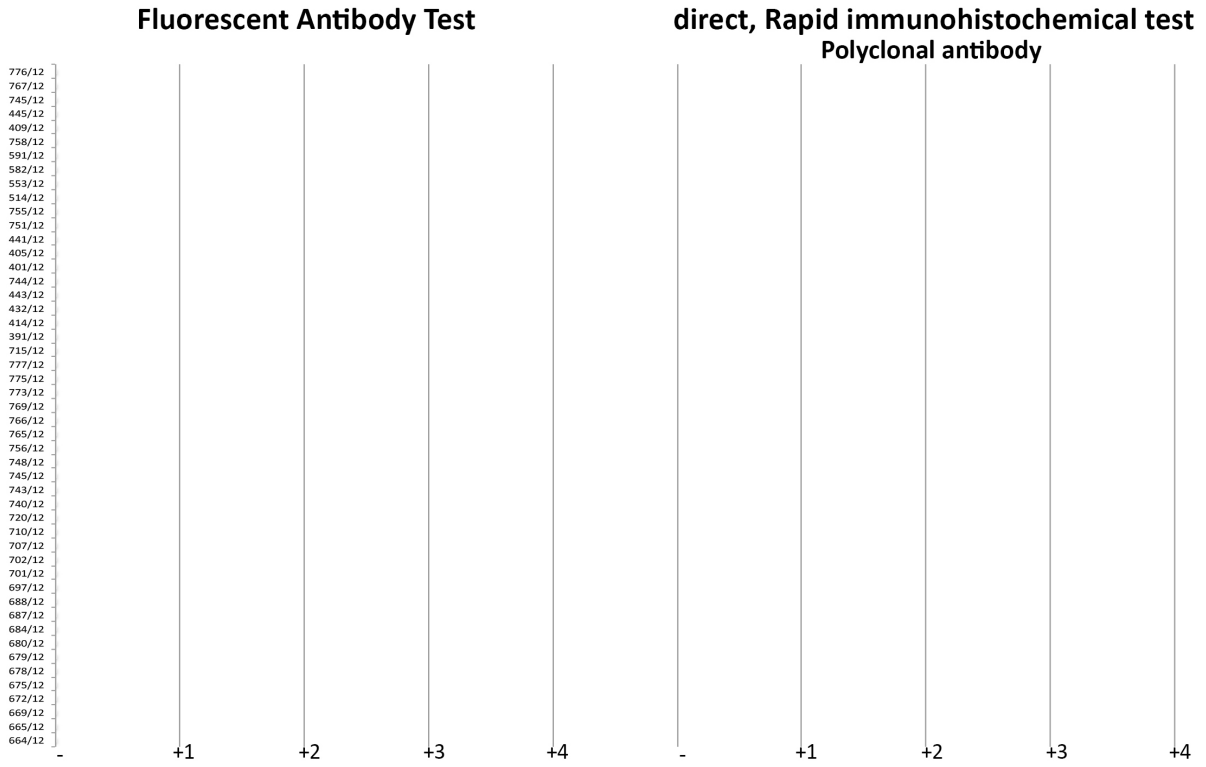


Figure 3.7. Immunoreactivity scores associated with known negative samples

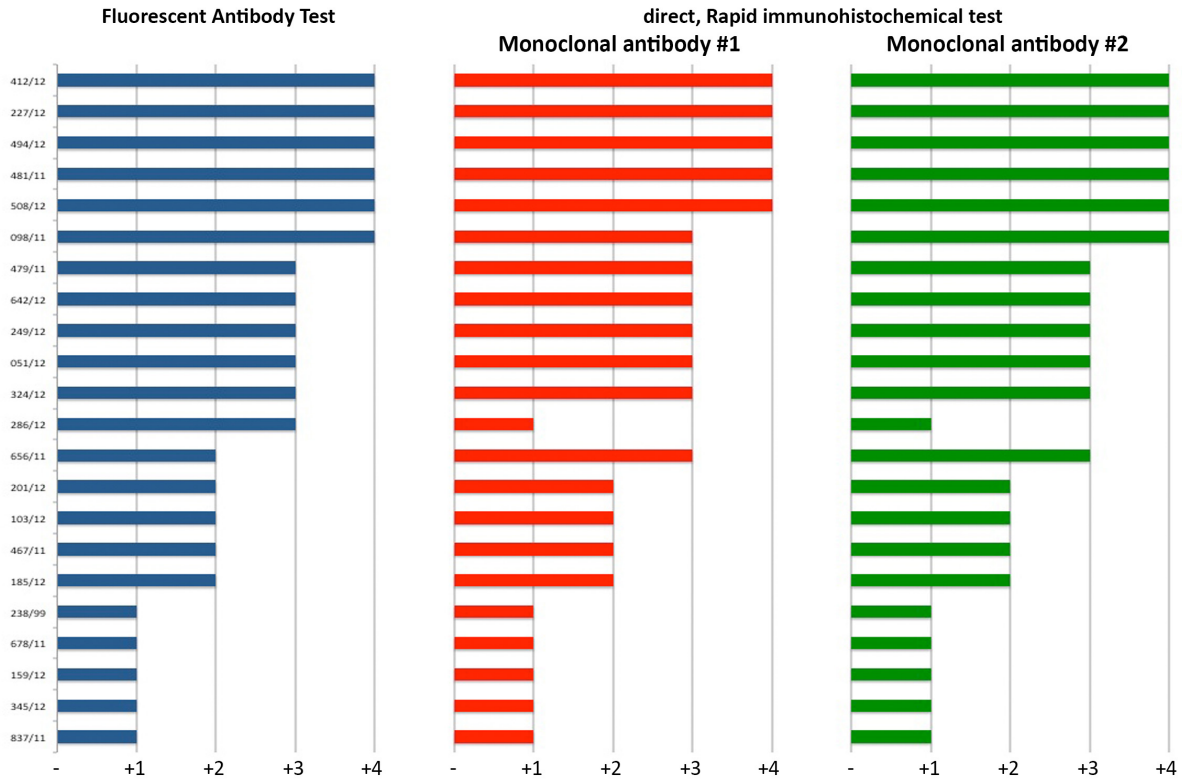


Figure 3.8.. Immunoreactivity scores associated with adapted dRIT protocol relying on biotinylated monoclonal antibodies

3.3.3. Statistical analysis of results

The comparison of the immunoreactivity scores between the dRIT relying on the biotinylated polyclonal antibody and the FAT diagnostic assay was used to determine the diagnostic efficacy of the two assays under investigation (Table 3.3).

Table 3.3. Diagnostic sensitivity, specificity and Cohen's Kappa measure of agreement of dRIT diagnostic assay using acetone fixation

FAT							
Biotinylated Antibodies	**True Positive**	**False Positive**	**True Negative**	**False Negative**	**Diagnostic Sensitivity***	**Diagnostic Specificity***	**Kappa Value***
Polyclonal Antibody	200	0	49	1	99,5% (97,25% -99,92%)	100% (92,68% -100%)	ND
dRIT							
Polyclonal Antibody	201	0	49	0	100% (98,16% -100%)	100% (92,68% -100%)	0.987 (0,963 - 1,000)

* Value in brackets represented the 95% confidence interval (CI)
ND: "Not done" due to FAT being the reference test

Based on the summarized results of this study (Table 3.3), it was illustrated that the polyclonal antibody, once applied to the dRIT diagnostic assay relying on acetone fixation, had a diagnostic sensitivity and specificity of 100% resulting in a diagnostic efficacy that was marginally higher than that of the FAT assay. Despite the small difference in diagnostic efficacy between the two assay, the Cohens' kappa measure of agreement (0,987; CI: 0,963-1,000) indicated that the adapted dRIT reaction had an "almost perfect agreement" with the FAT test according to the pre-determined categories stipulated in (Landis & Koch, 2012).

The small sample size used in the application of the adapted dRIT diagnostic reaction relying on the monoclonal antibody 1 and monoclonal antibody 2 was not substantial enough to validate any statistical analysis and as such the specific part of the research was merely used as a proof of concept to show that the adapted protocol could in fact be used with the biotinylated monoclonal antibodies supplied by the CDC.

3.4. Discussion

As mentioned in the introduction to this chapter (Section 2.1 and Section 3.1), the application of the dRIT as a widespread diagnostic assay in developing countries could potentially be limited by factors other than the aforementioned diagnostic efficacy or availability of the biotinylated monoclonal antibody supplied by the CDC. This is because resource-limited laboratories in developing country might experience another bottleneck in the application of the novel diagnostic assay due to the limited availability of chemicals and reagents required to perform the diagnostic test. Considering the fact that good quality 100% acetone is a not only an effective tissue fixative (section 1.7.1.2), but also a common chemical used in the medical, cosmetic and laboratory industry across the globe, it would be safe to assume that the chemical would be easily obtained regardless of geographic location.

Based on the afore mentioned information, the standard operating procedure of the dRIT assay was adapted in order to incorporate acetone fixation as an initial step in die diagnostic process. Due to the poorer tissue penetrative properties associated with acetone (section 1.7.1.2), replacing the formalin fixation with an acetone fixation step resulted in a diagnostic assay that was initially rendered ineffective. Based on the loss of efficacy of the diagnostic test, the adapted diagnostic assay was optimized using the Taguchi protocol whereby antibody, streptavidin-peroxidase and chromogen incubation times were altered in an ordered fashion. Even though the optimization process involved the adaption of the entire dRIT protocol, all the changes were based on altered

incubation periods with the chronological order of the dRIT reaction remaining constant. The adapted dRIT protocol was applied to the same sample set described in the previous chapter and the same immunological signs were observed in the tissue preparations regardless of whether formalin or acetone fixation had been applied.

Subsequent to the application of the adapted dRIT diagnostic assay to the aforementioned sample set, the statistical analysis of the results was evaluated and it was shown that the adapted dRIT assay relying on the biotinylated polyclonal antibody had a diagnostic efficacy marginally higher than that of the FAT diagnostic assay. More importantly, the results observed in this part of the study resembled that in Chapter II where the dRIT diagnostic assay relying on formalin fixation was performed on the sample set used in this part of the study. The fact that the same results were observed was thus indicative of the fact that the adaptation of the dRIT diagnostic protocol had not influenced the diagnostic capacity.

Even though the sample size associated with the application of the adapted dRIT protocol relying on either of the two-biotinylated monoclonal antibodies did not consist of a large enough sample set to enable the inferring of statistical results, the fact that clear and consistent immunoreactivity was observed highlighted the fact that the dRIT assay, using the cocktail of biotinylated monoclonal antibodies, could function as a routine diagnostic test once applied to acetone fixed tissue samples.

The research performed in this chapter did not only function in further validating the diagnostic potential of the dRIT diagnostic assay, but also highlighted the versatility associated with the given diagnostic assay. The standardised dRIT protocol was easily adapted in order to cater for the availability of dominant tissue fixating chemical used by many diagnostic facilities across the African continent, while still maintaining a high level of diagnostic efficacy. The versatility, and subsequent ease of application, associated with the dRIT diagnostic assay would be a beneficial characteristic in terms of improving widespread disease surveillance across the resource-limited developing countries.

Chapter IV

Diagnosis of representatives of African rabies-related lyssaviruses with the direct rapid immunohistochemical test

4.1. Introduction

The twelve members of the *Lyssavirus* genus are all characterised as zoonotic viruses that cause severe encephalomyelitis resulting in numerous neurological disorders in the infected host (Dietzgen et al., 2011; Jackson, 2002; Nel & Markotter, 2007). The most well-known lyssavirus, rabies virus, is enzootic throughout most of the world with the near global distribution of the rabies virus highlighting the important role that is played by the multiple mammalian reservoir species that facilitate the spread of the disease. Despite the wide range of reservoir species available to the RABV on the African continent it is important to note that bats (order: *Chiroptera*) play no part in rabies virus transmission, which is in contrast to what is observed on the North and South American continent (McColl, Tordo, & Aguilar Setien, 2000; J. S. Smith, Orciari, & Yager, 1995). Apart from the RABV, the African continent is known to host four of the recognized eleven rabies-related lyssaviruses (DUVV, LBV, MOKV and SHIBV) and one putative lyssavirus species (IKOV) (Dietzgen et al., 2011). These African rabies-related lyssaviruses, except for MOKV and the putative IKOV, relies solely on bats as the principle vector of transmission (Arai, Kuzmin, Kameoka, & Botvinkin, 2003; Badrane & Tordo, 2001; Dzikwi et al., 2010; I V Kuzmin et al., 2010; Nel & Markotter, 2007). Although most of the African rabies-related lyssavirus species rely on bats as the principle vector, occasional spill over events do occur during which the viral species spread to dead end mammalian hosts as discussed in section 1.2. The twelve lyssaviruses species can also be further grouped into phylogroups based on their antigenic and sequence diversity (Badrane, Bahloul, & Perrin, 2001; Dietzgen et al., 2011).

Phylogroup I includes all the known lyssavirus species apart from LBV, MOKV, SHIBV, WCBV and the putative IKOV species. Phylogroup II consists solely of African rabies-related lyssaviruses; LBV, MOKV and SHIBV (Badrane et al., 2001; Dietzgen et al., 2011). A series of recent studies have shown that the LBV species diversity was much more complex than initially thought, with the species consisting of four distinct lineages containing high levels of sequence divergence among them (Delmas et al., 2008; Markotter et al., 2008). Phylogroup III consists of a single viral isolate – WCBV, while the IKOV is proposed to not fit into any of the pre-existing phylogroups (Marston, Ellis, et al., 2012; Marston, Horton, et al., 2012). Although the dRIT has been applied to the CNS tissue of various RABV infected mammalian species, the diagnostic assay has not been applied to any of the antigenically distinct rabies-related lyssaviruses (Durr et al., 2008; Lembo et al., 2006; Madhusudana et al., 2012; Saturday et al., 2009).

The proposed aim of this chapter was to apply the dRIT diagnostic test to a panel

of selected representatives of the rabies-related lyssaviruses originating from the African continent. Due to the small amount of rabies-related isolates that have been discovered to date, a small sample size was used as a mere proof of concept to determine whether the dRIT test was capable of detecting the antigenically distinct rabies-related viruses that occur in southern Africa.

4.2. Materials and Methods

4.2.1. Biotinylated polyclonal antibody

The dRIT diagnostic assay relied on the biotinylated polyclonal anti-ribonucleoprotein antibody preparation (ARC-OVI, rabies division) described in section 2.2.1 and 2.2.2, and the two-biotinylated monoclonal antibodies (monoclonal antibody 1 and monoclonal antibody 2) (CDC).

4.2.2. Sample selection

A panel of seven rabies-related viral isolates (Table 4.1) were chosen as representative isolates of the inherent diversity of the known southern African rabies-related lyssaviruses. All the viral isolates were proliferated in suckling mouse brain according to an established protocol (Koprowski, 1996) (ARC-OVI Ethical approval, 15/4/P001) in order to obtain CNS tissue containing antigen associated with all the chosen viral isolates.

Table 4.1. Details of African rabies-related viruses used in this study

Virus	Host	Country of Origin	Lab ID nr.	Genbank accession nr.
LBV (Lineage A)	Bat (Rousettus aegyptiacus)	Unknown	LBVAFR 1999	EF547447
LBV (Lineage C)	Bat (Epomophorus whalbergii)	South Africa	LBVSA 2004	DQ 499945
MOKV (Group 1)	Feline	Zimbabwe	13270/82	AF319514
MOKV (Group 2)	Feline	South Africa	322/96	AF074813
MOKV (Group 3)	Feline	South Africa	252/97	AF074816
MOKV (Group 4)	Domestic cat	South Africa	112/96	AF074810
DUVV	Human	South Africa	DUVVSA 2006	DQ 676932

4.2.3. Fluorescent antibody test

The CNS tissue samples derived from the suckling mice that were inoculated with one of the seven representative rabies-related isolates (Table 4.1) were all subjected to FAT diagnosis in order to confirm the presence of viral antigen as well as

to score to level of immunoreactivity associated with each sample. The FAT protocol was followed according to the procedure discussed in section 2.2.4 (Dean et al., 1996).

4.2.4. Direct, rapid immunohistochemical test

All seven CNS samples subjected to FAT diagnosis were tested with the dRIT by creating three separate slides for each sample of CNS tissue in order to apply the two monoclonal antibodies (CDC) and the polyclonal antibody (ARC-OVI, Rabies division) separately. The dRIT protocol was followed according to the procedure discussed in section 2.2.5.

4.3. Results

4.3.1. Fluorescent antibody test

The FAT diagnostic assay was applied to all seven CNS tissue samples in order to determine whether the samples had a clear and strong immunoreactivity based on the presence of viral antigen (Table 4.2. and Figure 4.2). The immunoreactivity score associated with each sample was determined based on both the staining intensity and antigen distribution observed with each sample as described in the standard operating procedure for the FAT test. The observed immunoreactivity patterns mimicked those shown in Figure 2.1, and further photo documentation was thus not include in the chapter.

4.3.2. Direct, rapid immunohistochemical test

The dRIT diagnostic test relying on each of the three-biotinylated antibodies (monoclonal antibody 1, monoclonal antibody 2 and the polyclonal antibody) was applied to the same seven CNS tissue samples and the respective levels of immunoreactivity was scored according to the staining intensity and antigen distribution observed with each sample (Figure 4.2 and Table 4.2). The same levels of immunoreactivity were observed as displayed in Figure 2.1, with clear red inclusions being present on the blue neuronal background.

The three individual dRIT reactions (each relying on one of the three above mentioned biotinylated antibodies) showed equal levels of efficacy based on their application on the seven African rabies-related infected CNS samples. No false

negative results were obtained and all three biotinylated antibodies detected all seven of the African rabies-related isolates in the infected CNS tissue samples (Figure 4.2 and Table 4.2).

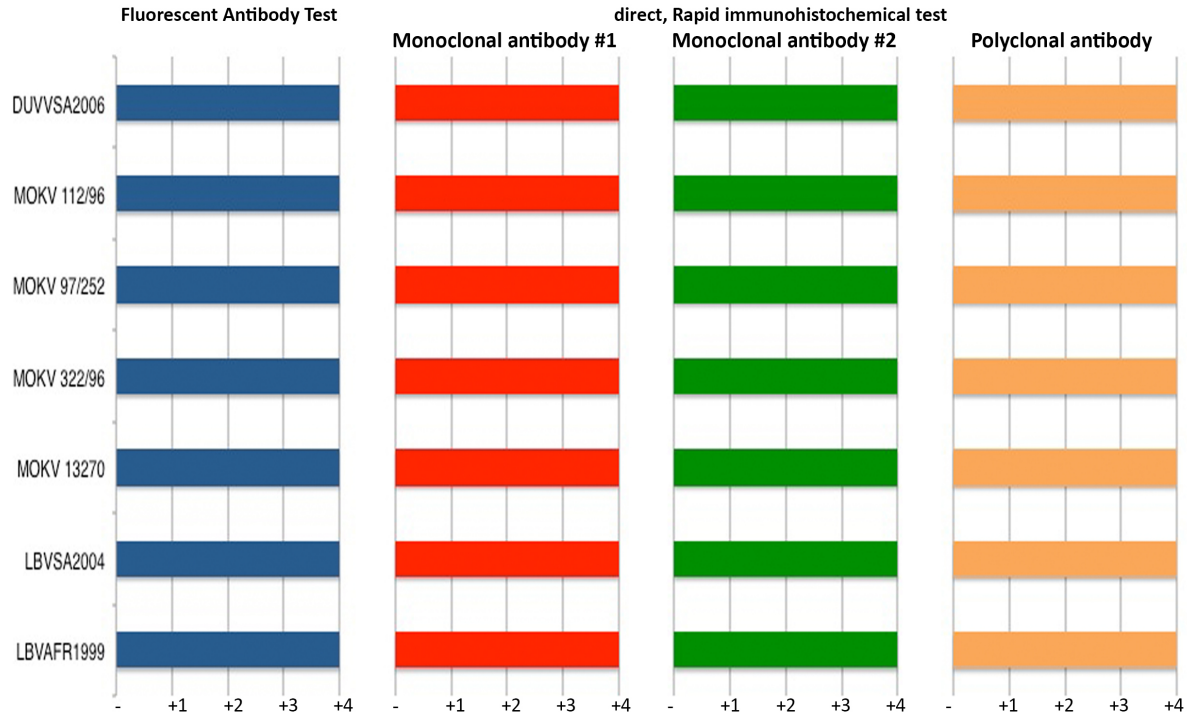


Figure 4.1. Immunoreactivity scores associated with African rabies related lyssaviruses

Table 4.2. Immunoreactivity scores associated with the dRIT diagnostic test of African rabies-related lyssaviruses

Sample information	FAT	direct, Rapid Immunohistochemical Test (dRIT)		
Sample Number		Monoclonal Antibody #1	Monoclonal Antibody #2	Polyclonal Antibody
LBVAFR 1999	+ + + +	+ + + +	+ + + +	+ + + +
LBVSA 2004	+ + + +	+ + + +	+ + + +	+ + + +
MOKV 13270/82	+ + + +	+ + + +	+ + + +	+ + + +
MOKV 322/96	+ + + +	+ + + +	+ + + +	+ + + +
MOKV 252/97	+ + + +	+ + + +	+ + + +	+ + + +
MOKV 112/96	+ + + +	+ + + +	+ + + +	+ + + +
DUVVSA2006	+ + + +	+ + + +	+ + + +	+ + + +

4.4. Discussion

As discussed in the introduction to this chapter (section 4.1), the diagnostic efficacy of novel diagnostic assay is an important factor in terms of the applicability of a diagnostic test. Irrespective of how well a diagnostic assay performs in terms of its diagnostic sensitivity, the diagnostic specificity must also be taken into consideration

since the diagnostic specificity of an assay acts as an indicator of how well a diagnostic test functions in detecting the antigenically distinct lyssaviruses. Although the occurrence of the antigenically distinct rabies-related viruses is extremely low in comparison to that of the RABV, the increase in surveillance by means of molecular amplification techniques has led to the increase of discovered rabies-related isolates over the past few years. To date, none of the published studies using the dRIT diagnostic assay mentioned in section 1.8.1 had included any of the antigenically distinct rabies-related lyssaviruses.

As such, the work performed in this chapter was used as proof of concept to determine whether the dRIT test (relying on either one of three distinct biotinylated antibodies) could be used to detect selected rabies-related lyssaviruses originating from the southern part of the African continent. After the dRIT diagnostic test had been applied to CNS tissue consisting of one of the seven available rabies-related isolates, the results indicated that the immunoreactivity observed in the dRIT test had a 100% agreement with the results observed for the FAT diagnostic assay, irrespective of the applied biotinylated antibody used in the reaction. The observed agreement between the FAT and dRIT assay based on the selected samples served to strengthen the applicability of the dRIT diagnostic assay in terms of diagnosing all the known viruses in the *Lyssavirus* genus.

Although the work in this chapter was not based on the application of the dRIT diagnostic assay on CNS tissues derived from *Chiropteran* (bat) species, the diagnostic premise would remain the same. The only possible drawback that could potentially be associated with the use of bat brain samples would be the presence of endogenous biotin associated specifically with the *Chiropteran* CNS tissue, which could result in false positive results. Should the effects of EABA, associated with the endogenous biotin, cause high levels of background staining, EABA-blocking chemicals could be applied to the diagnostic assay to prevent the presence of unnecessary false positive results (Miller, Kubier, Reynolds, Henry, & Turnbow, 1999; Wendelboe & Bisgaard, 2009).

Chapter V

Development of a simulation framework to determine the cost of performing lyssavirus diagnostic assays in developing countries

5.1. Introduction

The epizootic nature of Lyssaviruses across the globe has led to an emphasis with regards to developing novel diagnostic assays that can be used to ensure efficient diagnosis in developing countries where the prevalence of the disease remains largely under-reported (Knobel et al., 2005, 2007; Lembo et al., 2010). In developing countries the lack of diagnosis relying on the gold standard FAT occurs because of the low amount of diagnostic laboratories with adequate facilities and/or training to enable routine rabies diagnosis (Weyer & Blumberg, 2007). The resource-limited developing countries thus frequently rely on the older, inaccurate tests, such as the Seller's stain, to attempt some level of surveillance (Tierkel, 1973; Unpublished data from the 10th annual Southern and Eastern Africa Rabies Group meeting in Mozambique 2011). Despite the Seller's stain method being no longer an accepted diagnostic test for rabies diagnosis, it remains the only viable option in certain developing countries (Tepsumethanon et al., 2004; WHO, 2005).

Although the developmental diagnostic assays focus has shifted from the detection of antigen to the amplification of viral nucleic acid, the detection of viral antigen in CNS tissue remains the most effective and conclusive diagnostic mechanism. One such novel diagnostic assay for the detection of viral antigen, while seemingly ideal for distribution and application in developing countries, is the dRIT test. In this test the lyssavirus antigen is detected by means of an adapted immunohistochemical assay relying on a compound light microscope instead of the much more expensive fluorescent microscope required for FAT diagnosis (Durr et al., 2008; Niezgoda & Rupprecht, 2006). Although the dRIT diagnostic sensitivity and specificity has been shown to be equal to that of the FAT test in numerous pilot studies (Durr et al., 2008; Lembo et al., 2006; Madhusudana et al., 2012; Saturday et al., 2009), it does not display a true representation of the applicability of the dRIT diagnostic assay in developing countries such as Africa.

The criteria associated with the applicability of a diagnostic test is primarily driven by the statistical relevance (diagnostic sensitivity and diagnostic specificity) of the given test (OIE, 2008a). However certain key factors that influence the general implementation and application of the respective assay are not regularly taken into consideration. Although the diagnostic sensitivity and specificity of a diagnostic assay are still two of the most important indicators of the general efficacy of a diagnostic test designated to be used in the effective distribution of post-exposure prophylaxis after human exposure had occurred, they cannot be the only deciding factors in terms of determining whether one test would be more suited than another in terms of widespread

application of the diagnostic assay for routine surveillance. Apart from the statistical significance of a given test, the cost associated with performing routine diagnosis over a set time period cannot be overlooked. As such, the cost associated with the establishment of a diagnostic facility and subsequent diagnosis should be taken into consideration before a true representation of the applicability of a certain diagnostic assay can be made.

The aim of this chapter is to consider costs associated with the implementation and on-going maintenance of two hypothetical diagnostic assays in a pre-existing bio-safety level 2 (BSL 2) diagnostic facility situated in a resource-limited environment. For the purpose of the framework, it was assumed that the hypothetical facility contained all the basic laboratory equipment, such as: autoclaves, biosafety cabinets, pipettes, etc. The simulation framework thus included the financial comparisons of all the equipment and reagent associated costs associated with performing routine rabies diagnosis relying on either the FAT or the dRIT diagnostic assays. Cost estimations were used to predict the financial outcomes in low, medium and high throughput facilities over a set period of time to determine which of the diagnostic assays under investigation would be financially more viable or preferable than the other.

5.2. Description of the simulation framework

The research in this chapter was based on the development of a simulation framework used to underpin the cost of the routine diagnosis of rabies with two diagnostic assays, the FAT and dRIT, in a developing country setting. Although South Africa was used as a model country in the development of the simulation framework, the assumption was that the basic BSL 2 facilities needed to perform either of the two diagnostic assays would be available in some developing countries in Africa. The basic infrastructure would include a room of sufficient size with specific insulating requirements, running water and electricity and an environment facilitating telecommunications. Apart from the physical building it was assumed that the said BSL 2 facility contained a working biosafety cabinet and working autoclave, and was manned by staff that had undergone sufficient training in either diagnostic assay (WHO, 2005).

In order to obtain the best representation of the cost from the simulation framework, two sets of assumptions were developed. One on laboratory throughput and the other on cost data. These assumptions had a direct impact on the financial inferences associated with the two diagnostic assays under investigation.

5.2.1. Laboratory throughput

Three scenarios were evaluated for the laboratory throughput in order to determine the different financial effects of a low, medium or high number of samples delivered for routine diagnosis over multiple years. The throughput values for a low (n = 50 samples per annum) and a medium (n = 500 samples per annum) diagnostic facility were selected to represent a country with low sample submission, while a high throughput value (n = 2500 samples per annum) was selected to represent a country with high level of sample submissions. Along with the three theoretical throughput rates per annum, the average number of samples per day was calculated based on the average number of working days per annum in South Africa (Table 5.2).

5.2.2. Cost data

The cost data associated with both diagnostic assays was split into the direct and indirect costs to obtain a clearer representation of the various financial components associated with each test as well as the impact on the price per diagnostic reaction.

5.2.2.1. Direct cost

The direct cost is readily defined as: “a variable cost directly attributable to production (Bodie et al., 2009)”. For the sake of the research, the direct costs were all costs that were directly involved with performing the diagnostic assays. The direct cost for each of the two diagnostic assays was unique in certain respects, and similar in others. Certain direct costs were unique as far as specific reagents and equipment needed to perform the test were concerned, and others were similar such as labour and standard items of equipment required. Although the direct costs associated with routine diagnosis consisted of the price of the equipment and the reagents and the cost of the diagnostic technician, the three inputs were calculated separately and then combined. This was done to illustrate the make-up of the final total cost in its composite parts.

5.2.2.2. Indirect cost

The indirect cost is readily defined as: “a fixed cost that cannot be attributed directly to the production of a particular item and is incurred even when there is no output (Bodie et al., 2009).” For the sake of the research, the indirect costs were all costs that were not directly involved with the diagnostic process, but did influence the

efficacy of performing diagnosis. The costs of facilities, cleaning staff and communications were not factored into the simulation framework due to the fact that the simulation framework was based on the use of pre-existing BSL 2 facilities that would be utilized for diagnosis.

5.2.2.3. Determination of direct cost elements and pricing

In the simulation framework, the equipment costs consisted of two categories:

- equipment required by both diagnostic assays such as a fridge/freezer combination, forceps, scissors, glass submersion chambers and humidity boxes.
- unique equipment such as the fluorescent microscope and incubator for the FAT test and a compound light microscope for the dRIT test.

Two prices were obtained for the diagnostic equipment that was unique to each of the two diagnostic tests, a low cost and a high cost option to cater for facilities with either a low or high start-up capital availability.

The reagent costs component of the simulation framework was also split into two categories:

- Specialised reagents: For the FAT test the FITC-antibody labelling kit and for dRIT the antibody biotinylation kit were selected based on the fact that the reagents were standardised with the diagnostic assays, and as such the prices were obtained from the suppliers used during the study. The FITC-antibody labelling kit was chosen because the kit relied on the same FITC compound (Isomer 1) used by the Onderstepoort Veterinary Institute (OVI) Rabies unit for the routine FITC labelling of anti-ribonucleoprotein polyclonal antibody in order to facilitate FAT diagnosis. The EZ-Link® Sulfo-NHS-Biotinylation Kit was chosen as the biotinylation kit is recommended by the CDC for the routine biotinylation of antibodies for the purpose of performing the dRIT diagnostic assay.
- General reagents for FAT would be 100% acetone, Phosphate buffered saline (PBS), microscope slides and cover slips. For dRIT the reagents would be 10% Neutral buffered formalin, 3% hydrogen peroxide, Streptavidin-peroxidase, AEC chromogen kit, Phosphate buffered saline (PBS), Gills #2 Haematoxylin, Distilled water, Tween80, microscope slides and cover slips. These reagents do not need standardization during the process of diagnosis and therefore the best available commercial prices were chosen.

The simulation framework was designed to incorporate the direct cost associated with the diagnostic technician in order to establish the direct costs involved in performing routine diagnosis.

5.3. Determination of direct cost

5.3.1. Direct cost

As stated previously, the direct cost was defined as all cost directly involved in performing either of the two diagnostic tests. The direct cost was allocated into three sections, equipment cost, reagent cost and cost of the diagnostic technician.

5.3.1.1. Direct cost of equipment

The direct cost associated with the equipment required to perform either of the diagnostic test was determined for the initial year of investigation. The availability of low and high start-up capital was taken into consideration in the pricing of the equipment (Table 5.1).

Table 5.1. Direct cost of equipment required to perform the diagnostic assays		
- Low start-up capital -		
	FAT	*dRIT*
Common equipment	**Rand**	**Rand**
Refrigerator/Freezer unit	2 304	2 304
Forceps and Scissors	350	350
Glass slide submersion chamber	40	40
Humidity chamber	520	520
Filter paper	77	77
Clock timer	350	350
F10 concentrated disinfectant	177	177
Nitrile gloves (100 per pack)	60	60
1,5ml Eppendorf tubes (500 tubes)	147	147
Pipette tips	520	1 075
Mounting medium	245	245
Sub total:	**4 790**	**4 790**
Unique equipment		
37 °C Incubator	8 265	---
Required microscope	26 811	3 576
Total equipment cost:	**R39 866**	**R8 366**
- High start-up capital -		
	FAT	*dRIT*
Common equipment	**Rand**	**Rand**
Refrigerator/Freezer unit	2 304	2 304
Forceps and Scissors	350	350
Glass slide submersion chamber	40	40
Humidity chamber	520	520
Filter paper	77	77
Clock timer	350	350
F10 concentrated disinfectant	177	177
Nitrile gloves (100 per pack)	60	60
1,5ml Eppendorf tubes (500)	147	147
Pipette tips	1 075	1 075
Mounting medium	245	245
Sub total	**4 790**	**4 790**
Unique equipment		
Incubator	14 310	---
Required microscope	101 218	47 302
Total equipment cost:	**R120 318**	**R52 092**

5.3.1.2. Direct cost of reagents

Unlike the equipment cost component of the direct costs, the cost of the reagents was directly influenced by the number of samples subjected to a single diagnostic run with either of the two diagnostic assays. The diagnostic run was defined as a group of samples that were simultaneously taken through all the steps of the diagnostic process. During the process of performing diagnosis, the samples were subjected to one of two possible processes:

- *Touch impression of samples on slide was covered with a specific reagent*:

$$\frac{Total\ volume\ of\ reagent\ purchased}{Volume\ of\ reagent\ used\ to\ cover\ sample} = Number\ of\ slides\ diagnosed\ per\ volume\ reagent$$

$$\frac{Reagent\ price\ for\ the\ volume\ purchased}{Number\ of\ slides\ diagnosed\ per\ volume} = Price\ per\ diagnostic\ slide$$

- *Slides were submerged in a glass submersion chamber* with a volume of 140 ml. The price of the final volume reagent used was divided by the number of slides diagnosed per day in order to determine the final price per diagnostic slide:

$$\frac{Total\ volume\ of\ reagent\ purchased}{Volume\ of\ reagent\ used\ per\ diagnostic\ run} = Number\ of\ runs\ per\ volume\ reagent$$

$$\frac{Reagent\ price\ for\ the\ volume\ purchased}{Number\ of\ available\ diagnostic\ runs} = Price\ per\ diagnostic\ run$$

$$\frac{Price\ per\ diagnostic\ run}{Average\ number\ of\ slides\ per\ run} = Price\ per\ diagnostic\ slide$$

The average laboratory throughput assumed in section 5.2.1, determined the average number of samples diagnosed per run. It was assumed that all samples delivered to a laboratory were included in a single diagnostic run at the end of the workday in order to prevent reagent wastage. Since the average number of samples was used to determine the reagent cost per diagnostic reaction, the average number of slides per run was determined according average laboratory throughput mentioned previously (Table 5.2).

Table 5.2. Average diagnostic laboratory throughput incorporated in the simulation framework			
Throughput classification	**Average number of samples per annum**	**Average number of samples per five day work week**	**Average number of samples per day ◆**
Low	50	1	0,2§
Medium	500	10	2
High	2500	50	10
◆ Assume each year in South Africa consists of 249 working days (Days excluded from the 365 days per annum: weekends, public holidays and public holidays that fall on a Sunday were moved to Monday as regularly happens in South Africa) § One slides on one day of the five day work week			

5.3.1.2.1. Average price of reagents per diagnostic run of the FAT diagnostic assay

1) FluoroTag™ FITC conjugation kit @ R4048 + Unlabelled anti-ribonucleoprotein polyclonal antibody @ R600 (ARC-OVI) = R4648

For the sake of the simulation framework, it was assumed that ARC-OVI, Rabies division, had supplied the unlabelled anti-ribonucleoprotein at a pre-determined concentration for a fee of R600 (included in the antibody labelling price).

Each kit contained a single spin column that produced 3 ml of FITC labelled antibody. The spin column was re-used five times to supply 15 ml FITC labelled antibody per kit. The antibody working dilution (1:1000) produced 15 000 ml of FITC-labelled antibody at the working concentration. Each slide (containing one touch impression) required approximately 0.05 ml of FITC-labelled antibody per slide.

$$\frac{15\,000\ ml\ FITC-labelled\ antibody\ per\ kit}{0.05\ ml\ FITC-labelled\ antibody\ per\ slide} = 300\,000\ slides\ per\ kit$$

$$\frac{R\ 4\ 648\ per\ kit}{300\,000\ slides\ per\ kit} = R0{,}015\ per\ slide$$

2) 2.5 L 100% Acetone @ R598

$$\frac{2500\ ml\ per\ bottle\ of\ 100\%\ acetone}{140\ ml\ acetone\ per\ diagnostic\ run} = 17\ runs\ per\ bottle$$

$$\frac{R\ 598\ per\ 2{,}5\ L\ of\ 100\%\ acetone}{17\ diagnostic\ runs\ per\ bottle} = R35{,}18\ per\ 140\ ml\ of\ 100\%\ acetone$$

1 Sample per run – Low throughput facility	2 Samples per run – Medium throughput facility	10 Samples per run – High throughput facility
$\frac{R35{,}18\ per\ 140\ ml}{1\ slide\ per\ 140\ ml}$ R35,18	$\frac{R35{,}18\ per\ 140\ ml}{2\ slides\ per\ 140\ ml}$ R17,59	$\frac{R35{,}18\ per\ 140\ ml}{10\ slides\ per\ 140\ ml}$ R3,52

3) 1L 10xPBS @ R300

The standard operating procedure for the dRIT diagnostic assay required the use of 1xPBS.

$$\frac{10\ 000\ ml\ 1xPBS\ per\ bottle}{140\ ml\ 1xPBS\ per\ diagnostic\ run} = 71\ runs\ per\ bottle$$

$$\frac{R300\ per\ 10L\ of\ 1xPBS}{71\ runs\ per\ bottle} = R4{,}23$$

1 Sample per run – Low throughput facility	2 Samples per run – Medium throughput facility	10 Samples per run – High throughput facility
$\frac{R4{,}23\ per\ 140\ ml}{1\ slide\ per\ 140\ ml}$ R 4,23	$\frac{R4{,}23\ per\ 140\ ml}{2\ slides\ per\ 140\ ml}$ R 2,12	$\frac{R4{,}23\ per\ 140\ ml}{10\ slides\ per\ 140\ ml}$ R 0,42

4) Microscope slides (pack of 50) @ R32,8

$$\frac{R32{,}8\ per\ pack}{50\ slides\ per\ pack} = R0{,}66\ per\ slide$$

5) Cover slips (pack of 100) @ R46

$$\frac{R46\ per\ pack}{100\ cover\ slips\ per\ pack} = R0{,}46\ per\ cover\ slip$$

5.3.1.2.2. Average price of reagents per diagnostic run of the dRIT diagnostic assay

6) EZ-Link® Sulfo-NHS-Biotinylation Kit @ R4136 + Unlabelled anti-ribonucleoprotein polyclonal antibody @ R600 (ARC-OVI) = R4736

For the sake of the simulation framework, it was assumed that ARC-OVI, Rabies division, had supplied the unlabelled anti-ribonucleoprotein at a pre-determined concentration for a fee of R600 (included in the antibody labelling price).

Each kit contained five spin columns that produced 2 ml of biotinylated antibody each. Thus, each kit produced 10ml of stock concentration biotinylated antibody. The antibody working dilution (1:220) produced 2200 ml of biotinylated antibody. Each slide (containing one touch impression) required approximately 0.05 ml of biotinylated antibody.

$$\frac{2200\ ml\ biotinylated\ antibody\ per\ kit}{0.05\ ml\ biotinylated\ antibody\ per\ slide} = 44\ 000\ slides\ per\ kit$$

$$\frac{R4\ 736}{44\ 000\ slides\ per\ kit} = R0{,}10\ per\ slide$$

7) 10% Neutral buffered formalin (20L) @ R1305

$$\frac{20\ 000ml\ of\ 10\%\ Neutral\ buffered\ formalin}{140\ ml\ 10\%\ Neutral\ buffered\ formalin} = 142\ runs\ per\ bottle$$

$$\frac{R1305\ for\ 20L\ of\ 10\%\ neutral\ buffered\ formalin}{142\ runs\ per\ bottle\ of\ 10\%\ neutral\ buffered\ formalin} = R9{,}19\ per\ run$$

1 Sample per run – Low throughput facility	2 Samples per run – Medium throughput facility	10 Samples per run – High throughput facility
$\frac{R9{,}19 per\ 140\ ml}{1\ slide\ per\ 140\ ml}$ R 9,19	$\frac{R9{,}19 per\ 140\ ml}{2\ slides\ per\ 140\ ml}$ R 4,60	$\frac{R9{,}19 per\ 140\ ml}{10\ slides\ per\ 140\ ml}$ R 0,92

8) 30% Hydrogen peroxide (2,5L) @ R117

The standard operating procedure for the dRIT diagnostic assay required the use of 3% Hydrogen peroxide.

$$\frac{25\ 000\ ml\ per\ bottle\ of\ 3\%\ hydrogen\ peroxide}{140\ ml\ per\ run} = 178\ runs\ per\ bottle$$

$$\frac{R117\ per\ bottle\ of\ 3\%\ hydrogen\ peroxide}{178\ runs\ per\ bottle} = R0{,}66\ per\ run$$

1 Sample per run – Low throughput facility	2 Samples per run – Medium throughput facility	10 Samples per run – High throughput facility

$\frac{R0{,}66\ per\ 140\ ml}{1\ slide\ per\ 140\ ml}$	$\frac{R0{,}66\ per\ 140\ ml}{2\ slides\ per\ 140\ ml}$	$\frac{R0{,}66\ per\ 140\ ml}{10\ slides\ per\ 140\ ml}$
R 0,66	R 0,33	R 0,07

9) Streptavidin-peroxidase (100ml, Ready-to-use) @ R2986

The ready-to-use reagent required approximately 0,05 ml to cover the single touch impression made on each slide.

$$\frac{100\ ml\ per\ bottle\ of\ Streptavidin-peroxidase}{0.05\ ml\ per\ slide\ of\ Streptavidin-peroxidase} = 2000\ slides\ per\ bottle$$

$$\frac{R2986\ per\ bottle}{2000\ slides\ per\ bottle} = R1{,}49\ per\ slide$$

10) AEC Chromogen kit @ R1336

The AEC staining kit produces 150 ml of the AEC chromogen according to the information provided by the supplier. Each slide required approximately 0.05 ml of chromogen to cover the single touch impression made on each slide.

$$\frac{150ml\ per\ AEC\ chromogen\ staining\ kit}{0{,}05\ ml\ chromogen\ per\ slide} = 3000\ slides\ per\ bottle$$

$$\frac{R1336\ per\ AEC\ chromogen\ staining\ kit}{3000\ slides\ per\ AEC\ chromogen\ staining\ kit} = R0{,}45\ per\ slide$$

11) Gills #2 Haematoxylin (1L) @ R630

According to the standard operating procedure of the dRIT diagnostic assay, the Gills #2 Haematoxylin solution is diluted 1:2 and made once a week (each week consisted of five working days). The volume of the full submersion chamber was 140 ml and 70 ml Gills #2 solution was required to ensure sufficient dilution.

$$\frac{2000ml\ per\ bottle\ Gills\ \#2\ solution}{70\ ml\ Gills\ \#2\ solution\ per\ week} = 28\ weeks\ per\ bottle$$

$$\frac{R630\ per\ bottle\ of\ Gills\ \#2\ Haematoxylin}{28\ weeks\ per\ bottle\ of\ Gills\ \#2\ Haematoxylin} = R22{,}5\ per\ week$$

1 Sample per run – Low throughput facility	**2 Samples per run – Medium throughput facility**	**10 Samples per run – High throughput facility**

$\frac{R22{,}5\ per\ 140\ ml}{1\ slide\ per\ week}$	$\frac{R22{,}5\ per\ 140\ ml}{10\ slide\ per\ week}$	$\frac{R22{,}5\ per\ 140\ ml}{50\ slide\ per\ week}$
R 22,50	R 2,25	R 0,45

12) 1L (x10) PBS @ R300

The standard operating procedure for the dRIT diagnostic assay required the use of 1xPBS. The three individual PBS wash steps with each wash step relying on 140 ml. The final volume of 1xPBS per diagnostic run was thus 420 ml.

$$\frac{10\ 000ml\ per\ bottle\ of\ 1xPBS}{420\ ml\ per\ run} = 23\ runs\ per\ bottle$$

$$\frac{R300\ per\ 20\ L\ of\ 1xPBS}{23\ runs\ per\ bottle} = R13{,}04\ per\ run$$

1 Sample per run – Low throughput facility	**2 Samples per run – Medium throughput facility**	**10 Samples per run – High throughput facility**
$\frac{R13{,}04\ per\ 140\ ml}{1\ slide\ per\ 140\ ml}$	$\frac{R13{,}04\ per\ 140\ ml}{2\ slides\ per\ 140\ ml}$	$\frac{R13{,}04\ per\ 140\ ml}{10\ slides\ per\ 140\ ml}$
R 13,04	R6,52	R 1,30

13) Tween80 (500ml) @ R155

According to the standard operating procedure of the dRIT diagnostic assay, for every 990 ml of PBS, 10 ml of Tween80 had to be added. Thus, for every 460 ml of PBS, 4,6 ml of Tween 80 was required.

$$\frac{500ml\ per\ bottle\ of\ Tween80}{4{,}6\ ml\ Tween80\ per\ run} = 108\ runs\ per\ bottle\ of\ Tween80$$

$$\frac{R155\ per\ bottle\ of\ Tween80}{108\ runs\ per\ bottle\ of\ Tween80} = R1{,}44\ per\ run$$

1 Sample per run – Low throughput facility	**2 Samples per run – Medium throughput facility**	**10 Samples per run – High throughput facility**

$\frac{R1{,}44\ per\ 140\ ml}{1\ slide\ per\ 140\ ml}$	$\frac{R1{,}44\ per\ 140\ ml}{2\ slides\ per\ 140\ ml}$	$\frac{R1{,}44\ per\ 140\ ml}{10\ slides\ per\ 140\ ml}$
R 1,44	R 0,72	R 0,14

14) Distilled Water (5L) @ R21

The standard operating procedure for the dRIT diagnostic assay required 130 ml distilled water per run (126 ml to dilute hydrogen peroxide to 3% and 4 ml for the AEC chromogen kit) as well as 14 ml per day (70 ml per week for the dilution of the Gills #2 solution).

$$\frac{5000ml\ per\ bottle\ of\ 5L\ distilled\ water}{144\ ml\ distilled\ water\ per\ day} = 34\ runs\ per\ bottle$$

$$\frac{R21\ per\ 5\ L\ distilled\ water}{34\ runs\ per\ 5\ L\ of\ distilled\ water} = R0{,}62\ per\ run$$

1 Sample per run – Low throughput facility	**2 Samples per run – Medium throughput facility**	**10 Samples per run – High throughput facility**
$\frac{R0{,}62\ per\ 140\ ml}{1\ slide\ per\ 140\ ml}$	$\frac{R0{,}62\ per\ 140\ ml}{2\ slides\ per\ 140\ ml}$	$\frac{R0{,}62\ per\ 140\ ml}{10\ slides\ per\ 140\ ml}$
R 0,62	R 0,31	R 0,06

15) Microscope slides (pack of 50) @ R32,8

$$\frac{R32{,}8\ per\ pack}{50\ slides\ per\ pack} = R0{,}66\ per\ slide$$

16) Cover slips (pack of 100) @ R46

$$\frac{R46\ per\ pack}{100\ cover\ slips\ per\ pack} = R0{,}46\ per\ cover\ slip$$

The total reagents costs were summarized in Table 5.3, along with the "total reagent cost per annum" which was calculated by multiplying the total reagent cost per run with the amount of samples diagnosed per year.

50 samples per annum – 1 slide per diagnostic run

$Reagent\ cost\ calculated\ for\ one\ slide\ per\ diagnostic\ run\ x\ 50\ samples\ per\ year$

$= total\ reagent\ cost\ per\ annum$

500 samples per annum – 2 slides per diagnostic run

$$Reagent\ cost\ calculated\ for\ two\ slide\ per\ diagnostic\ run\ x\ 500\ samples\ per\ year$$
$$= total\ reagent\ cost\ per\ annum$$

2500 samples per annum – 10 slides per diagnostic run

$$Reagent\ cost\ calculated\ for\ ten\ slide\ per\ diagnostic\ run\ x\ 2500\ samples\ per\ year$$
$$= total\ reagent\ cost\ per\ annum$$

Table 5.3. Summary of direct cost of reagents required to perform the FAT and dRIT diagnostic assays			
Fluorescent antibody test			
	1 slide per run	**2 slides per run**	**10 slides per run**
1) FITC labelled antibody	R0,02	R0,02	R0,02
2) 100% acetone	R35,18	R17,59	R3,52
3) 1xPBS	R4,23	R2,12	R0,42
4) Microscope slide	R0,66	R0,66	R0,66
5) Cover slip	R0,46	R0,46	R0,46
Total reagent cost per diagnostic test:	**R40,55**	**R20,88**	**R5,08**
Total reagent cost per annum			
50 samples per annum	R2 028		
500 samples per annum		R10 440	
2500 samples per annum			R12 700
direct, Rapid immunohistochemical test			
	1 slide per run	**2 slides per run**	**10 slides per run**
6)Biotinylated antibody	R0,10	R0,10	R0,10
7)Neutral buffered formalin	R9,19	R4,60	R0,92
8) 3% Hydrogen peroxide	R0,66	R0,33	R0,07
9) Streptavidin-peroxidase	R1,49	R1,49	R1,49
10) AEC chromogen	R0,45	R0,45	R0,45
11) Gills #2 formula	R22,50	R2,25	R0,45
12) 1xPBS	R13,04	R6,52	R1.30
13) Tween80	R1,44	R0,72	R0,14
14) Distilled Water	R0,62	R0,31	R0,06
15) Microscope slide	R0,66	R0,66	R0,66
16) Cover slip	R0,46	R0,46	R0,46
Total reagent cost per diagnostic test:	**R50,61**	**R17,89**	**R6,1**
Total reagent cost per annum			
50 samples per annum	R2 531		
500 samples per annum		R 8 945	
2500 samples per annum			R15 250

5.3.2.3. Direct cost of staff performing diagnosis

Although the diagnostic technicians cost would be a fixed expenditure per annum, the diagnostic procedure could not be performed without this input and therefore the cost was included as a direct cost in the simulation framework. The cost for a person with the necessary qualification and experience was estimated to be R210 000 per annum.

5.4. Total direct cost per annum for the first year of analysis

Based on the details set out in section 5.3, the total direct cost for the first year was calculated (Table 5.4). Equipment cost was depreciated in one year for the purpose of this summary but it should be noted that this skewed the outcome somewhat.
The next level of analysis was used to explain the statement in more detail.

Table 5.4. Total direct cost per annum for the first year of analysis						
-Fluorescent antibody test -						
	Low start-up capital			*High start-up capital*		
	50 samples per annum	**500 samples per annum**	**2500 samples per annum**	**50 samples per annum**	**500 samples per annum**	**2500 samples per annum**
Equipment cost	R39 866	R39 866	R39 866	R120 318	R120 318	R120 318
Reagent cost	R2 028	R10 440	R12 700	R2 028	R10 440	R12 700
Diagnostic technician's cost	R210 000	R210 000	R210 000	R210 000	R210 000	R210 000
Total direct cost:	**R251 894**	**R260 306**	**R262 566**	**R332 346**	**R340 758**	**R343 018**
-direct, Rapid immunohistochemical test -						
	Low start-up capital			*High start-up capital*		
	50 samples per annum	**500 samples per annum**	**2500 samples per annum**	**50 samples per annum**	**500 samples per annum**	**2500 samples per annum**
Equipment cost	R8 366	R8 366	R8 366	R52 092	R52 092	R52 092
Reagent cost	R2 531	R8 945	R15 250	R2 531	R8 945	R15 250
Diagnostic technician's cost	R210 000	R210 000	R210 000	R210 000	R210 000	R210 000
Total direct cost:	**R220 897**	**R227 311**	**R233 616**	**R264 623**	**R271 037**	**R277 342**

5.5. Multi-year analysis of total direct cost

For the purpose of this analysis the cost of equipment was depreciated over a one year, five year and ten year period. Were the direct costs of the equipment weren't incorporated in this manner, the costs associated with the equipment would not be efficiently factored into the "cost per diagnostic reaction" and this would have led to a higher initial diagnostic reaction cost and a much lower diagnostic reaction cost thereafter (Table 5.5). The cost of reagents and the annual cost of the diagnostic technician were calculated for the same periods as the equipment cost, taking the following into account:

- Reagent costs: the annual cost for the reagents were adjusted by an annual inflationary increase of 5%.
- Diagnostic technician: Although an annual salary increase is not compulsory under South African law, a 5% annual increase in cost was allowed for to cater for inflationary increases.

Table 5.5. Multi-year analysis of total direct cost						
-Fluorescent antibody test -						
50 samples per annum	***Low start-up capital***			***High start-up capital***		
	1 year	**5 years**	**10 years**	**1 year**	**5 years**	**10 years**
Equipment cost	R39 866	R7 973	R3 987	R120 318	R24 064	R12 032
Reagent cost	R2 028	R2 465	R3 146	R2 028	R2 465	R3 146
Diagnostic technician's cost	R210 000	R255 256	R325 779	R210 000	R255 256	R325 779
Total direct cost:	**R251 894**	**R265 694**	**R332 912**	**R332 346**	**R281 785**	**R340 957**
500 samples per annum	***Low start-up capital***			***High start-up capital***		
	1 year	**5 years**	**10 years**	**1 year**	**5 years**	**10 years**
Equipment cost	R39 866	R7 973	R3 987	R120 318	R24 064	R12 032
Reagent cost	R10 440	R12 690	R16 196	R10 440	R12 690	R16 196
Diagnostic technician's cost	R210 000	R255 256	R325 779	R210 000	R255 256	R325 779
Total direct cost:	**R260 306**	**R275 919**	**R345 962**	**R340 758**	**R292 010**	**R354 007**
2500 samples per annum	***Low start-up capital***			***High start-up capital***		
	1 year	**5 years**	**10 years**	**1 year**	**5 years**	**10 years**
Equipment cost	R39 866	R7 973	R3 987	R120 318	R24 064	R12 032
Reagent cost	R12 700	R15 437	R19 702	R12 700	R15 437	R19 702
Diagnostic technician's cost	R210 000	R255 256	R325 779	R210 000	R255 256	R325 779
Total direct cost:	**R262 566**	**R278 666**	**R349 648**	**R343 018**	**R294 757**	**R357 513**

Table 5.5. Multi-year analysis of total direct cost (continued)						
-direct, Rapid immunohistochemical test -						
50 samples per annum	***Low start-up capital***			***High start-up capital***		
	1 year	**5 years**	**10 years**	**1 year**	**5 years**	**10 years**
Equipment cost	R8 366	R1 673	R837	R52 092	R10 418	R5 209
Reagent cost	R2 531	R3 076	R3 926	R2 531	R3 076	R3 926
Diagnostic technician's salary	R210 000	R255 256	R325 779	R210 000	R255 256	R325 779
Total direct cost:	**R220 897**	**R260 005**	**R330 542**	**R264 623**	**R268 750**	**R334 914**
500 samples per annum	***Low start-up capital***			***High start-up capital***		
	1 year	**5 years**	**10 years**	**1 year**	**5 years**	**10 years**
Equipment cost	R8 366	R1 673	R837	R52 092	R10 418	R5 209
Reagent cost	R8 945	R10 873	R13 877	R8 945	R10 873	R13 877
Diagnostic technician's salary	R210 000	R255 256	R325 779	R210 000	R255 256	R325 779
Total direct cost:	**R227 311**	**R267 802**	**R340 493**	**R271 037**	**R276 547**	**R344 865**
2500 samples per annum	***Low start-up capital***			***High start-up capital***		
	1 year	**5 years**	**10 years**	**1 year**	**5 years**	**10 years**
Equipment cost	R8 366	R1 673	R837	R52 092	R10 418	R5 209
Reagent cost	R15 250	R18 536	R23 658	R15 250	R18 536	R23 658
Diagnostic technician's salary	R210 000	R255 256	R325 779	R210 000	R255 256	R325 779
Total direct cost:	**R233 616**	**R275 465**	**R350 274**	**R277 342**	**R284 210**	**R354 646**

5.6. Determination of the total indirect cost

The total indirect cost, associated with performing either of the two diagnostic assays under investigation, for the first year of investigation is summarised in Table 5.6. The only indirect cost that would be attributed to each of the two diagnostic assays, while being excluded from the indirect costs associated with the use of a pre-existing BSL 2 facility, would be the cost of the annual microscope service as well as the vaccination of the diagnostic technician. Smaller sundry indirect costs, such as insurance considered but in the final analysis the impact of indirect cost was not a determining factor overall.

Table 5.6. Total indirect cost per annum for the first year of analysis

		Per annum
		Rand
Vaccination of diagnostic technician■		315
Sub-total:		**R315**
Servicing of fluorescent microscope (FAT)		4 000,00
Servicing of light microscope (dRIT)		1 345,20
Total indirect cost per annum	**FAT**	**R4315,00**
	dRIT	**R1660,20**

■ Total vaccination cost associated with completing the full course of three vaccinations on day: 0, 7 and 21 at an average of $35 ($30-40; (Shwiff, Anderson, & Hampson, 2013)) for the full course.

5.7. Multi-year analysis of indirect cost

As with the direct costs, the indirect costs were also calculated over the same one, five and ten year period, taking the following assumptions into consideration:

- Vaccination cost: In order to ensure the safety of the laboratory technician, an annual booster consisting of one dose of vaccine per year (after the initial 3 course

vaccination process) is recommended. The price per vaccine was calculated to be approximately $12 per vial with an annual price increase of 5%.

- Microscope servicing costs: Annual servicing of all microscopes is recommended in order to ensure effective diagnosis in accredited diagnostic facilities. As such, the annual servicing fee was adjusted by taking a 5% annual inflation increase into consideration.

The total indirect costs calculated for the specific annual time periods were summarized in Table 5.7.

Table 5.7. Multi-year analysis of total indirect cost			
- Fluorescent antibody test -			
	1 Year	**5 years**	**10 years**
Vaccination of diagnostic technician	R315	R131	R168
Fluorescent microscope annual service	R4 000	R4 862	R6 205
Total indirect cost per annum:	**R4 314**	**R4 993**	**R6 373**
- direct, Rapid immunohistochemical test -			
	1 Year	**5 years**	**10 years**
Vaccination of diagnostic technician	R315	R131	R168
Compound light microscope annual service	R1 345	R1 635	R2 087
Total indirect cost per annum:	**R1 660**	**R1 766**	**R2 255**

5.8. Total cost of diagnosis

The total cost (direct and indirect cost) for each diagnostic assay is summarised in Table 5.8, and the total price per diagnostic assay was determined by dividing the final cost value by the average number of samples per year. The final cost per diagnostic assay, as summarized in Table 5.8, for the low and high start-up capital facilities were plotted and visually represented in Figure 5.1.

Table 5.5. Total cost of diagnosis						
Low start-up capital						
50 samples per annum	**FAT 1 year**	**dRIT 1 year**	**FAT 5 years**	**dRIT 5 years**	**FAT 10 years**	**dRIT 10 years**
Direct cost:	R251 894,00	R220 897,00	R265 694,00	R260 005,00	R332 912,00	R330 542,00
Indirect cost:	R4 314,00	R1 660,00	R4 993,00	R1 766,00	R6 373,00	R2 255,00
Total cost:	**R256 208,00**	**R222 557,00**	**R270 687,00**	**R261 711,00**	**R339 285,00**	**R332 797,00**
Total cost per diagnostic test	**R5 124,16**	**R4 451,14**	**R5 413,74**	**R5 235,42**	**R6 785,70**	**R6 655,94**
500 samples per annum	**FAT 1 year**	**dRIT 1 year**	**FAT 5 years**	**dRIT 5 years**	**FAT 10 years**	**dRIT 10 years**
Direct cost:	R260 306,00	R227 311,00	R275 919,00	R267 802,00	R345 962,00	R340 493,00
Indirect cost:	R4 314,00	R1 660,00	R4 993,00	R1 766,00	R6 373,00	R2 255,00
Total cost:	**R264 620,00**	**R228 971,00**	**R280 912,00**	**R269 568,00**	**R352 335,00**	**R342 748,00**
Total cost per diagnostic test	**R529,24**	**R457,94**	**R561,82**	**R539,14**	**R704,67**	**R685,50**
2500 samples per annum	**FAT 1 year**	**dRIT 1 year**	**FAT 5 years**	**dRIT 5 years**	**FAT 10 years**	**dRIT 10 years**
Direct cost:	R262 566,00	R233 616,00	R278 666,00	R275 465,00	R349 648,00	R350 274,00
Indirect cost:	R4 314,00	R1 660,00	R4 993,00	R1 766,00	R6 373,00	R2 255,00
Total cost:	**R266 880,00**	**R235 276,00**	**R283 659,00**	**R277 231,00**	**R356 012,00**	**R352 529,00**
Total cost per diagnostic test	**R106,75**	**R94,11**	**R113,46**	**R110,89**	**R142,40**	**R141,01**

Table 5.5. Total cost of diagnosis (continued)						
High start-up capital						
50 samples per annum	**FAT 1 year**	**dRIT 1 year**	**FAT 5 years**	**dRIT 5 years**	**FAT 10 years**	**dRIT 10 years**
Direct cost:	R332 346,00	R264 623,00	R281 785,00	R268 750,00	R340 957,00	R334 914,00
Indirect cost:	R4 314,00	R1 660,00	R4 993,00	R1 766,00	R6 373,00	R2 255,00
Total direct cost:	**R336 660,00**	**R266 283,00**	**R286 778,00**	**R270 516,00**	**R347 330,00**	**R337 169,00**
Total cost per diagnostic test	**R6 733,20**	**R5 325,66**	**R5 735,56**	**R5 410,32**	**R6 946,60**	**R6 743,38**
500 samples per annum	**FAT 1 year**	**dRIT 1 year**	**FAT 5 years**	**dRIT 5 years**	**FAT 10 years**	**dRIT 10 years**
Direct cost:	R340 758,00	R271 037,00	R292 010,00	R276 547,00	R354 007,00	R344 865,00
Indirect cost:	R4 314,00	R1 660,00	R4 993,00	R1 766,00	R6 373,00	R2 255,00
Total direct cost:	**R345 072,00**	**R272 697,00**	**R297 003,00**	**R278 315,00**	**R360 380,00**	**R347 120,00**
Total cost per diagnostic test	**R690,14**	**R545,39**	**R594,01**	**R556,63**	**R720,76**	**R694,24**
2500 samples per annum	**FAT 1 year**	**dRIT 1 year**	**FAT 5 years**	**dRIT 5 years**	**FAT 10 years**	**dRIT 10 years**
Direct cost:	R343 018,00	R277 342,00	R294 757,00	R284 210,00	R357 513,00	R354 646,00
Indirect cost:	R4 314,00	R1 660,00	R4 993,00	R1 766,00	R6 373,00	R2 255,00
Total direct cost:	**R347 332,00**	**R279 002,00**	**R299 750,00**	**R285 976,00**	**R363 886,00**	**R356 901,00**
Total cost per diagnostic test	**R138,93**	**R111,60**	**R119,90**	**R114,39**	**R145,55**	**R142,76**

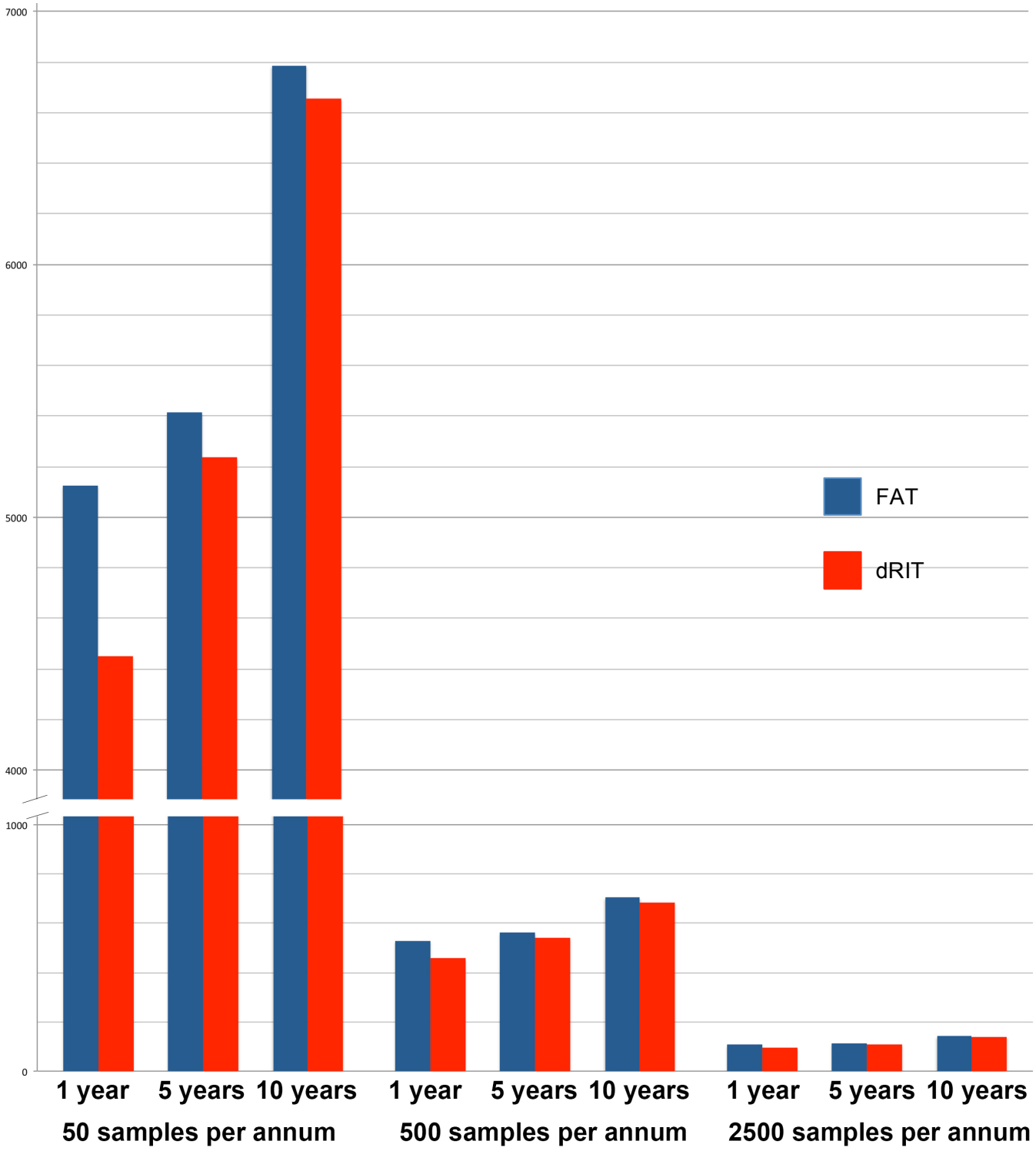


Figure 5.1. Total cost of diagnosis for a low start-up capital facility

Graphical representation of the total costs involved in performing either the FAT or dRIT diagnostic assays using a low start-up capital. Despite the different theoretical annual throughputs, the cost associated with the dRIT assay was lower than that of the FAT diagnostic assay.

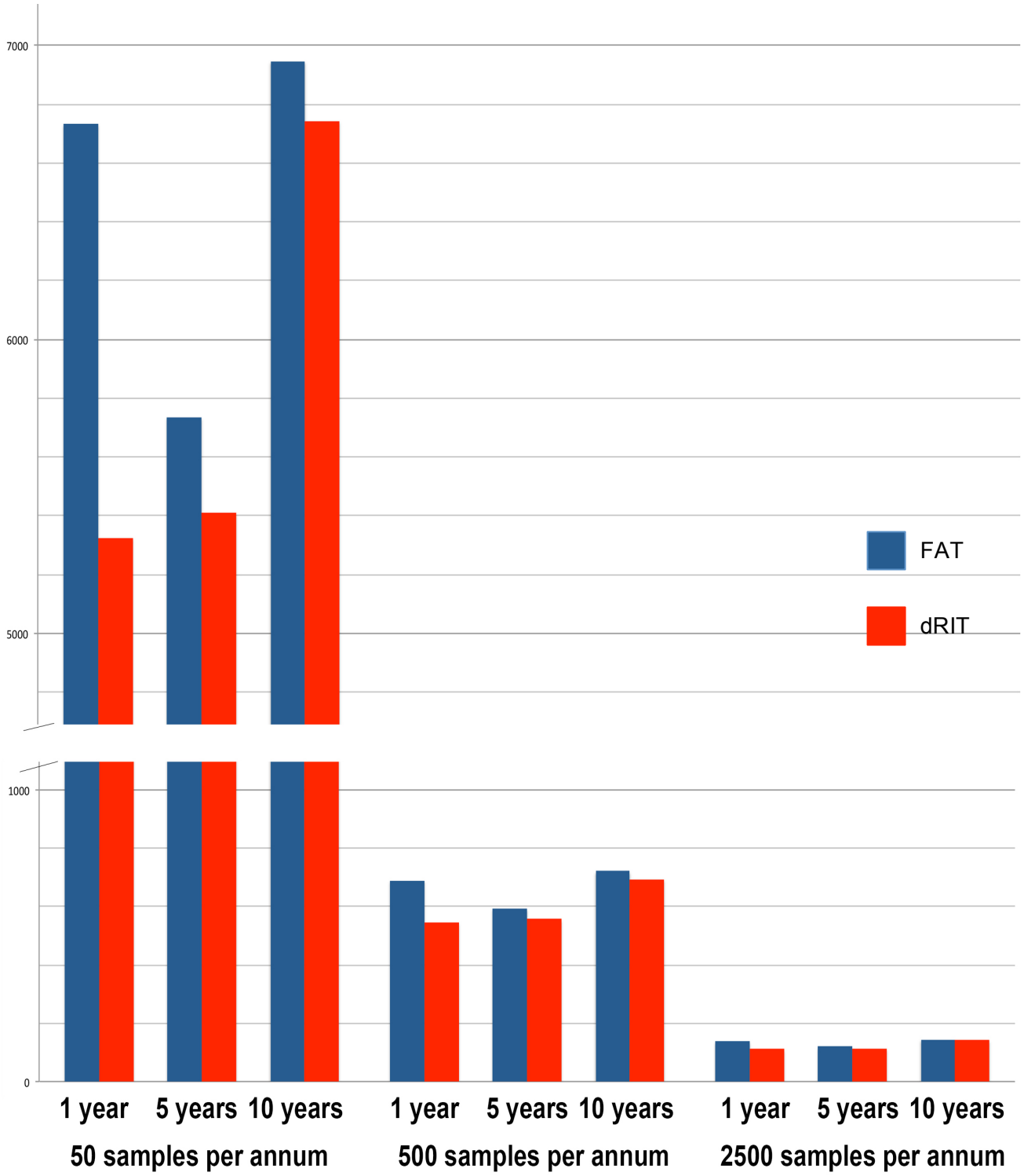


Figure 5.2. Total cost of diagnosis for a high start-up capital facility

Graphical representation of the total costs involved in performing either the FAT or dRIT diagnostic assays using a high start-up capital. Despite the different theoretical annual throughputs, the cost associated with the dRIT assay was lower than that of the FAT diagnostic assay.

5.9. Discussion

Although the dRIT test has only been applied in a limited number of studies in developing countries, the results have indicated a high level of consistency between the FAT and dRIT tests in terms of their diagnostic sensitivity and specificity. Extensive testing carried out in South Africa in relation to this study has further confirmed the aforementioned findings. Based on the results obtained from these previous studies, most researchers advocated the widespread application of the dRIT test in developing countries where little or no routine rabies diagnosis takes place. Two major reasons are cited to support this recommendation:

1. the dRIT test requires a basic compound light microscope compared to the significantly more expensive fluorescent microscope
2. the immunoreactivity scores are easier to interpret in the dRIT diagnostic assay.

The compound light microscope is indeed a less expensive item of equipment than the fluorescent one, however it is only a single cost that forms part of a much bigger system of inter-linked costs pertaining to equipment, reagents and labour cost required to perform a diagnostic assay. The limited data available in respect of the cost per diagnostic test resulted in a simulation framework being developed to establish the various costs involved in a newly build diagnostic facility situated in a developing country. Because the diagnostic facilities would receive a varying number of samples per annum, three theoretical throughput rates (50, 500 and 2500 samples per annum) were used in the simulation framework to predict the effect that the varying number of samples would have on the price per diagnosis. The application of the simulation framework was used to forecast the theoretical cost per diagnostic test for the two assays. From the results obtained from the simulation framework certain key fact became apparent.

The initial investment in terms of the capital expenditure required to set up a diagnostic facility for each of the methodologies differed significantly. This difference was due to the high cost of the specific equipment required to perform the FAT diagnostic test, consisting of mainly a fluorescent microscope and a 37 °C incubator, while the dRIT diagnostic test required only a compound light microscope. This difference is evident in both the low (FAT: R39 866 vs. dRIT: R8 366) and high (FAT: R120 318 vs. dRIT: R52 092) start-up capital assumptions. Although the total costs were calculated for a one, five and ten year period, the cost values for the first year were used only to illustrate the high initial capital investment. The cost values associated with the first year are thus irrelevant since it is unlikely that any country would invest in the establishment of a specific diagnostic facility for a single year. Even

allowing for a margin of error, the cost per diagnostic test in each of the theoretical low, medium and high throughput facilities and over the various depreciation time periods did not vary significantly. The total cost per diagnostic test did, however, marginally favour the dRIT diagnostic assay in all scenarios. The only cost factor that was not included in the simulation framework was the costs associated with transporting a sample to the specific diagnostic facility. Even though this cost would have had a big influence on the price per diagnostic assay, the cost would be the same for either the FAT or dRIT test resulting in a value that would not contribute to the difference observed in the “cost per diagnostic test” for each of the diagnostic assay.

The high level of “cost per diagnostic test” in the low throughput scenario (approximately 50 samples per annum) highlighted the potential reason for limited routine rabies diagnosis in resource-limited developing countries. The number of samples tested in a facility has a hugely significant effect on the cost per test due to the relatively high fixed costs involved. This supports the view that regional diagnostic facilities would be more economical. Further, consideration should be given to the testing of samples originating from multiple countries in a single regional diagnostic facility, as this would be the most cost effective approach to routine rabies diagnosis in developing countries.

Chapter VI

Concluding remarks

Throughout this study, the importance of competent and routine rabies diagnosis has been highlighted. Indeed, effective surveillance and diagnostic capabilities play a key role in fathoming the true severity of the disease across both developing and developed countries (Knobel et al., 2005). As discussed in this study, the dRIT diagnostic assay has been identified as a diagnostic assay that has the potential to complement the gold standard FAT diagnostic test. Since the initial publication of the dRIT standard operating procedure (Niezgoda & Rupprecht, 2006) five peer-reviewed publications based on the dRIT diagnostic assay has been published (Durr et al., 2008; Lembo et al., 2006; Madhusudana et al., 2012; Saturday et al., 2009; Tao et al., 2008). The dRIT diagnostic assay currently uses a "ready-to-use" cocktail of biotinylated monoclonal antibodies. The antibody cocktail, supplied by the CDC, consists of two individual biotinylated monoclonal antibodies that are each directed towards a unique epitope on the lyssavirus nucleoprotein. The fact that the CDC is the sole supplier of a biotinylated antibody preparation for application in the dRIT diagnostic assay can be singled out as a potential limitation preventing the widespread application of the diagnostic test.

In my study, I have aimed to investigate the possibility of biotinylating a polyclonal antibody preparation that had been produced at the ARC-OVI in South Africa. In this specific study, the locally produced biotinylated polyclonal antibody was used in the dRIT diagnostic assay that was applied to a panel of southern African samples. In order to compare the diagnostic efficacy of the newly biotinylated polyclonal antibody, the two individual biotinylated monoclonal antibodies, routinely applied as the antibody cocktail by the CDC, were included in the study. The two-biotinylated monoclonal antibodies were supplied as ready-to-use reagents with a pre-determined working concentration and were applied in two separate dRIT diagnostic assays (each using one of the two monoclonal antibodies) to the same sample set of southern African samples.

The efficacy of the modified dRIT diagnostic assay, once compared to the classical dRIT assay (using the two biotinylated monoclonal antibodies), was found to be superior when applied to samples from southern Africa. The dRIT assay, in which the locally produced biotinylated polyclonal antibody preparation had been used, produced 100% accuracy in RABV detection. In contrast, the dRIT diagnostic assay using either of the two-biotinylated monoclonal antibodies (CDC) produced a number of false negative results. Upon further investigation, it was shown that all the false negative samples belonged to the mongoose variant of the RABV. To my knowledge, this was the first time that the dRIT diagnostic assay had a reduced diagnostic efficacy since its first application to actual CNS tissue samples in 2006 (Lembo et al., 2006). The inclusion of the mongoose variant in this study thus highlighted a potential

shortcoming associated with the application of the dRIT diagnostic assay using the cocktail of biotinylated monoclonal antibodies supplied by the CDC. Two plausible scenarios that could explain the origin of the false negative results will be discussed further.

(I) The first possible explanation for the observed false negative results would be that the biotinylated monoclonal antibodies had been over-diluted. Thus, the working dilution (as prepared by CDC prior to shipment) was not optimal for the cohort of southern African samples that were tested in this study. Prior to performing the FAT diagnostic assay using a new batch of FITC-conjugated antibodies, the working dilution of the conjugate is determined. The determination of the working dilution involves a titration series of the conjugate on representative antigenic types based on the RABV variants that are prevalent in the geographical area (Dean et al., 1996; OIE, 2008b; WHO, 2005). Without determining the optimal working dilution, the diagnostic assay cannot be optimised and the diagnostic assay could potentially produce false results. The same would of course apply to the dRIT diagnostic assay should. In this study, the CDC-determined working dilution of the monoclonal antibody cocktail had been used, which was the same approach published for the five previous studies in which the dRIT was evaluated at various locations around the world. The results presented here suggested that it should be considered best practise for any laboratory performing the dRIT diagnostic test to receive stock concentrations of the biotinylated antibody preparations, which will allow the assay to be optimized locally prior to its application in routine rabies diagnosis.

Although the immunoreactivity scores obtained in this study did not provide a quantitative value for the viral titre present in each sample, it did provide a general insight into the relative concentration of antigens in the individual samples. In my hands, most of the false negative results produced by the classical dRIT (using the two-biotinylated monoclonal antibodies) were found to produce high levels of immunoreactivity in the FAT and modified dRIT test (using the biotinylated polyclonal antibody preparation). Although the exact viral titre could not be determined, these false negative results did not appear to obviously correlate with low viral titres (based on the high (+3/+4) FAT positivity once applied to the samples). A quantitative real-time PCR assay should be applied in future research to determine whether a specific virus copy number can be determined for a true representation of a cut-off point in the viral titre.

(II) The second possible explanation for the observed negative results could be that the monoclonal antibodies failed to associate with their respective epitopes on the nucleoprotein of the mongoose variant. Monoclonal antibodies are designed to interact with a single antigenic epitope, and should a single amino acid change occur in the same region as the epitope, the interaction between the antigenic site and the antibody

might be prevented. In contrast to the use of monoclonal antibodies, the use of polyclonal antibodies that interact with multiple epitopes may be advantageous (M. Boenisch, 2009). Because polyclonal antibodies interact with multiple antigenic epitopes, small changes in single epitopes will not prevent the antibody from binding to the given antigen. This clear advantage should thus be brought into consideration when investigating and selecting future antibody preparations for diagnostic assays relying on the direct detection of viral antigen.

Irrespective of the origin of the false negative results observed in this study, it is clear that antibody preparations need to be supplied as stock concentrations that can be used to perform diagnostic assay validation and optimization prior to performing routine diagnosis. As shown in this study, the determination of the working dilution is especially important if the assay is applied to geographical niches where unique virus variants are known to occur endemically.

Without the widespread application of a developing assay, potential limitations associated with the novel diagnostic assay will not be identified, which could result in false confidence in the diagnostic results. As such, inter-laboratory ring trials need to be established whereby multiple biotinylated antibody preparations can be produced and applied in the dRIT diagnostic assay. The inclusion of diverse sample sets in these inter-laboratory trials will either single out one biotinylated antibody preparation as a universal reagent, or it will enable the establishment of an antibody cocktail consisting of globally produced biotinylated antibodies.

Apart from the small differences in the diagnostic efficacy of the FAT and dRIT assays investigated in this study, no real discriminatory factor could be discerned. Most of the pilot studies that did consider financial implications only considered the cost of the fluorescent and compound light microscopes, while all other costs were disregarded. Further evaluation of published data revealed that no in-depth analysis into the true financial implications associated with performing either the FAT or dRIT diagnostic assays had been carried out to date. As such, we undertook to perform an in-depth cost analysis to investigate the true cost of performing either the FAT or dRIT diagnostic assay. The development of the simulation framework provided accurate data regarding the cost associated with performing routine rabies diagnosis in a resource-limited developing country.

In this study I have investigated multiple criteria associated with the “ideal” diagnostic assay, with the deciding criteria being a high diagnostic efficacy, high versatility and a low financial costs associated with performing routine diagnosis. Although the previous criteria are important to consider, a constant drive to develop new diagnostic assays that are cheaper, faster and safer for the diagnostic technician is prominent. This is certainly not a modern occurrence as rabies diagnosis has been

constantly developing and improving since the initial application of the Seller's stain. As modern technology develops, one can observe a similar trend in the development of novel diagnostic assays that are becoming more advanced.

Although the application of molecular amplification has increased in the developing world, the costs associated with thermocycling devices and reagents still act as a barrier that prevents the widespread application of the assays. Another drawback associated with molecular amplification is that such methods are all particularly predisposed to contamination, which could result in false positive results. As such, the establishment and maintenance of the infrastructure needed to avoid the contamination is often underestimated. Despite the drawbacks that are currently observed, cheaper thermocycling machines and reagents will almost certainly become available as the field of molecular amplification develops. Until such time, however, it is my opinion that the widespread application of molecular amplification will not become a viable option for the resource-limited developing countries.

Lateral flow immunochromatography kits are routinely used as diagnostic assays for numerous disease such as human immunodeficiency virus, Hepatitis C virus, tuberculosis (Corstjens et al., 2007), rotavirus (Buser, Risch, Rutz, Mannang, & Munzinger, 2001), dengue haemorrhagic fever (Chakravarti, Gur, Berry, & Mathur, 2000), scrub typhus (Ching et al., 2001), *Streptococcus pneumonia* (Dominguez et al., 2001), malaria (Mens, van Amerongen, Sawa, Kager, & Schallig, 2008) and Hepatitis B virus (Shin, Kim, Shin, Chung, & Heo, 2001). Despite the widespread application of the diagnostic assay to such a broad range of infectious diseases, it is not a recommended diagnostic assay for rabies. The use of lateral flow immunochromatography is not recommended for rabies diagnosis because of the reduced diagnostic sensitivity associated with the assay, but recent studies have shown that the signal amplification techniques can be used to increase the diagnostic sensitivity of the assay (Linares, Kubota, Michaelis, & Thalhammer, 2012).

Although these novel diagnostic assays have shown promise in terms becoming more viable options for supplementing the FAT diagnostic assay, they are still being developed to their fullest potential. It is my opinion that, although those diagnostic tools have potential future applications, the widespread implementation of the highly reliable diagnostic assays that are currently available should be encouraged. In this study, and the five published dRIT pilot studies, the dRIT diagnostic assay has been shown to be one such option. In this study alone we have shown that the dRIT test, using the biotinylated polyclonal antibody, has a diagnostic sensitivity and specificity that is equal to the FAT, and could be definitely be more cost effective in the long run. These factors alone justify the further evaluation of the dRIT diagnostic assay on a global scale.

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Appendix

Table A1. Brain samples from various species used for the validation of the biotinylated antibodies

Number	Species	Sample number	Year of isolation	Province of origin	Number	Species	Sample number	Year of isolation	Province of origin
1	CAN	208/99	1999	KwaZulu-Natal	88	CAN	172/12	2012	Freestate
2	CAN	273/99	1999	Eastern Cape	89	CAN	185/12	2012	Limpopo
3	CAN	524/99	1999	Eastern Cape	90	CAN	200/12	2012	Mpumalanga
4	CAN	596/99	1999	Mpumalanga	91	CAN	229/12	2012	Limpopo
5	CAN	687/99	1999	Eastern Cape	92	CAN	235/12	2012	Limpopo
6	CAN	756/99	1999	Freestate	93	CAN	274/12	2012	Mpumalanga
7	CAN	828/99	1999	Freestate	94	CAN	319/12	2012	Mpumalanga
8	CAN	1003/99	1999	Freestate	95	CAN	324/12	2012	Limpopo
9	CAN	1018/99	1999	Mpumalanga	96	CAN	359/12	2012	Freestate
10	CAN	1039/99	1999	Freestate	97	CAN	371/12	2012	Mpumalanga
11	CAN	007/11	2011	Freestate	98	CAN	400/12	2012	Limpopo
12	CAN	035/11	2011	Limpopo	99	CAN	423/12	2012	Limpopo
13	CAN	042/11	2011	Mpumalanga	100	CAN	458/12	2012	Freestate
14	CAN	045/11	2011	Mpumalanga	101	CAN	468/12	2012	Freestate
15	CAN	074/11	2011	Limpopo	102	CAN	508/12	2012	Limpopo
16	CAN	104/11	2011	Limpopo	103	CAN	664/12	2012	Limpopo
17	CAN	106/11	2011	Mpumalanga	104	CAN	665/12	2012	North West
18	CAN	107/11	2011	Mpumalanga	105	CAN	669/12	2012	Gauteng
19	CAN	108/11	2011	Mpumalanga	106	CAN	672/12	2012	Mpumalanga
20	CAN	109/11	2011	Mpumalanga	107	CAN	675/12	2012	Limpopo
21	CAN	127/11	2011	Mpumalanga	108	CAN	678/12	2012	Gauteng
22	CAN	131/11	2011	Mpumalanga	109	CAN	679/12	2012	Gauteng
23	CAN	132/11	2011	Mpumalanga	110	CAN	680/12	2012	Gauteng
24	CAN	139/11	2011	Limpopo	111	CAN	684/12	2012	Freestate
25	CAN	154/11	2011	Mpumalanga	112	CAN	687/12	2012	Gauteng
26	CAN	167/11	2011	Limpopo	113	CAN	688/12	2012	North West
27	CAN	168/11	2011	Mpumalanga	114	CAN	697/12	2012	Freestate
28	CAN	172/11	2011	Mpumalanga	115	CAN	701/12	2012	North West
29	CAN	187/11	2011	Limpopo	116	CAN	702/12	2012	Mpumalanga
30	CAN	206/11	2011	Gauteng	117	CAN	707/12	2012	Mpumalanga
31	CAN	212/11	2011	Mpumalanga	118	CAN	710/12	2012	Mpumalanga
32	CAN	247/11	2011	Limpopo	119	CAN	711/12	2012	Mpumalanga
33	CAN	264/11	2011	Limpopo	120	CAN	715/12	2012	Freestate
34	CAN	274/11	2011	Mpumalanga	121	CAN	720/12	2012	Western Cape
35	CAN	276/11	2011	Mpumalanga	122	CAN	740/12	2012	Mpumalanga
36	CAN	285/11	2011	Mpumalanga	123	CAN	743/12	2012	Gauteng
37	CAN	312/11	2011	Limpopo	124	CAN	745/12	2012	Gauteng
38	CAN	315/11	2011	Limpopo	125	CAN	748/12	2012	Gauteng
39	CAN	395/11	2011	Mpumalanga	126	CAN	756/12	2012	Limpopo
40	CAN	401/11	2011	Limpopo	127	CAN	765/12	2012	Limpopo
41	CAN	418/11	2011	Mpumalanga	128	CAN	766/12	2012	Limpopo
42	CAN	424/11	2011	Limpopo	129	CAN	769/12	2012	Freestate
43	CAN	434/11	2011	Limpopo	130	CAN	773/12	2012	Mpumalanga
44	CAN	436/11	2011	Limpopo	131	CAN	775/12	2012	Freestate
45	CAN	443/11	2011	Eastern Cape	132	CAN	777/12	2012	Mpumalanga
46	CAN	446/11	2011	Limpopo	133	FEL	601/99	1999	Freestate
47	CAN	455/11	2011	Freestate	134	FEL	620/99	1999	Freestate
48	CAN	487/11	2011	Limpopo	135	FEL	929/99	1999	Freestate
49	CAN	502/11	2011	Mpumalanga	136	FEL	1052/99	1999	North West
50	CAN	512/11	2011	Limpopo	137	FEL	114/11	2011	Freestate
51	CAN	515/11	2011	Limpopo	138	FEL	283/11	2011	Freestate
52	CAN	526/11	2011	Mpumalanga	139	FEL	376/11	2011	Freestate
53	CAN	528/11	2011	Limpopo	140	FEL	467/11	2011	Mpumalanga
54	CAN	579/11	2011	Freestate	141	FEL	481/11	2011	Gauteng
55	CAN	590/11	2011	Limpopo	142	FEL	520/11	2011	North West
56	CAN	596/11	2011	Freestate	143	FEL	613/11	2011	Freestate
57	CAN	618/11	2011	Limpopo	144	FEL	650/11	2011	Zimbabwe (country)
58	CAN	627/11	2011	Limpopo	145	FEL	660/11	2011	Freestate
59	CAN	634/11	2011	Freestate	146	FEL	846/11	2011	Freestate
60	CAN	644/11	2011	Limpopo	147	FEL	051/12	2012	Mpumalanga
61	CAN	646/11	2011	Limpopo	148	FEL	261/12	2012	Northern Cape
62	CAN	665/11	2011	Limpopo	149	FEL	306/12	2012	Freestate
63	CAN	681/11	2011	Limpopo	150	FEL	345/12	2012	Eastern Cape
64	CAN	685/11	2011	Gauteng	151	FEL	382/12	2012	Freestate
65	CAN	686/11	2011	Mpumalanga	152	FEL	457/12	2012	North West
66	CAN	708/11	2011	Mpumalanga	153	FEL	650/12	2012	Freestate
67	CAN	726/11	2011	Mpumalanga	154	FEL	651/12	2012	Freestate
68	CAN	738/11	2011	Limpopo	155	FEL	391/12	2012	Gauteng
69	CAN	758/11	2011	Limpopo	156	FEL	414/12	2012	Gauteng
70	CAN	777/11	2011	Freestate	157	FEL	432/12	2012	Freestate
71	CAN	786/11	2011	Limpopo	158	FEL	443/12	2012	Gauteng
72	CAN	801/11	2011	Mpumalanga	159	FEL	744/12	2012	Gauteng
73	CAN	833/11	2011	Mpumalanga	160	CPEN	529/99	1999	Freestate
74	CAN	837/11	2011	Limpopo	161	CPEN	540/99	1999	Eastern Cape
75	CAN	840/11	2011	Limpopo	162	CPEN	1000/99	1999	Freestate
76	CAN	843/11	2011	Eastern Cape	163	CPEN	1087/99	1999	Eastern Cape
77	CAN	862/11	2011	Limpopo	164	CPEN	091/11	2011	Freestate
78	CAN	869/11	2011	Mpumalanga	165	CPEN	098/11	2011	Freestate
79	CAN	884/11	2011	Northern Cape	166	CPEN	099/11	2011	North West
80	CAN	889/11	2011	Limpopo	167	CPEN	137/11	2011	Freestate
81	CAN	019/12	2012	Limpopo	168	CPEN	149/11	2011	North West
82	CAN	037/12	2012	Mpumalanga	169	CPEN	153/11	2011	Eastern Cape
83	CAN	049/12	2012	Limpopo	170	CPEN	169/11	2011	Freestate
84	CAN	077/12	2012	Eastern Cape	171	CPEN	177/11	2011	Freestate
85	CAN	097/12	2012	Limpopo	172	CPEN	267/11	2011	Freestate
86	CAN	133/12	2012	Freestate	173	CPEN	605/11	2011	Freestate
87	CAN	136/12	2012	Limpopo	174	CPEN	010/12	2012	Freestate

CAN – Canid; FEL – Feline; CPEN – Yellow mongoose; CMES – Black-backed jackal; OMEG – Bat-eared fox; BOV - Bovine

Table A1. Brain samples from various species used for the validation of the biotinylated antibodies

Number	Species	Sample number	Year of isolation	Province of origin
175	CPEN	072/12	2012	Freestate
176	CPEN	100/12	2012	Freestate
177	CPEN	131/12	2012	Freestate
178	CPEN	159/12	2012	Freestate
179	CPEN	286/12	2012	Freestate
180	CPEN	448/12	2012	Freestate
181	CPEN	502/12	2012	Freestate
182	CPEN	401/12	2012	Mpumalanga
183	CPEN	405/12	2012	North West
184	CPEN	441/12	2012	Gauteng
185	CPEN	751/12	2012	Freestate
186	CPEN	755/12	2012	North West
187	CMES	557/99	1999	KwaZulu-Natal
188	CMES	549/99	1999	KwaZulu-Natal
189	CMES	673/99	1999	KwaZulu-Natal
190	CMES	717/99	1999	KwaZulu-Natal
191	CMES	121/11	2011	Limpopo
192	CMES	138/11	2011	Limpopo
193	CMES	147/11	2011	Limpopo
194	CMES	257/11	2011	Northern Cape
195	CMES	364/11	2011	Freestate
196	CMES	448/11	2011	Limpopo
197	CMES	493/11	2011	Northern Cape
198	CMES	594/11	2011	Freestate
199	CMES	633/11	2011	Limpopo
200	CMES	678/11	2011	Freestate
201	CMES	103/12	2012	Limpopo
202	CMES	169/12	2012	Northern Cape
203	CMES	249/12	2012	Limpopo
204	CMES	266/12	2012	Northern Cape
205	CMES	433/12	2012	Limpopo
206	CMES	494/12	2012	Gauteng
207	CMES	566/12	2012	North West
208	CMES	594/12	2012	Freestate
209	CMES	514/12	2012	Mpumalanga
210	CMES	553/12	2012	Freestate
211	CMES	582/12	2012	North West
212	CMES	591/12	2012	Mpumalanga
213	OMEG	238/99	1999	Northern Cape
214	OMEG	395/99	1999	Northern Cape
215	OMEG	971/99	1999	Northern Cape
216	OMEG	1082/99	1999	Northern Cape
217	OMEG	656/11	2011	Freestate
218	OMEG	909/11	2011	Freestate
219	OMEG	095/12	2012	Freestate
220	OMEG	227/12	2012	Limpopo
221	OMEG	313/12	2012	Northern Cape
222	OMEG	642/12	2012	Freestate
223	OMEG	758/12	2012	Northern Cape
224	BOV	388/99	1999	Mpumalanga
225	BOV	406/99	1999	Eastern Cape
226	BOV	1029/99	1999	North West
227	BOV	1086/99	1999	Mpumalanga
228	BOV	119/11	2011	Limpopo
229	BOV	129/11	2011	Mpumalanga
230	BOV	279/11	2011	Limpopo
231	BOV	302/11	2011	Mpumalanga
232	BOV	313/11	2011	Limpopo
233	BOV	344/11	2011	Freestate
234	BOV	472/11	2011	Mpumalanga
235	BOV	479/11	2011	Freestate
236	BOV	879/11	2011	Freestate
237	BOV	885/11	2011	Eastern Cape
238	BOV	011/12	2012	Limpopo
239	BOV	030/12	2012	Mpumalanga
240	BOV	071/12	2012	Limpopo
241	BOV	107/12	2012	Freestate
242	BOV	201/12	2012	Mpumalanga
243	BOV	331/12	2012	Mpumalanga
244	BOV	412/12	2012	Gauteng
245	BOV	490/12	2012	Freestate
246	BOV	409/12	2012	Freestate
247	BOV	445/12	2012	Freestate
248	BOV	745/12	2012	Gauteng
249	BOV	767/12	2012	Freestate
250	BOV	776/12	2012	Limpopo

CAN – Canid; FEL – Feline; CPEN – Yellow mongoose; CMES – Black backed jackal; OMEG – Bat eared fox; BOV - Bovine

Table A2. Immunoreactivity scores associated with the dRIT diagnostic test relying on three biotinylated antibodies						
Sample information			FAT	dRIT		
Sample Number	Year	Species		Monoclonal antibody 1	Monoclonal antibody 2	Polyclonal antibody
208/99	1999	CAN	++	+	++	++
273/99	1999	CAN	+++	++	++	+++
524/99	1999	CAN	++	+	+	++
596/99	1999	CAN	++	+++	+++	+
687/99	1999	CAN	+++	+++	+++	+
756/99	1999	CAN	+++	- - -	++	+
828/99	1999	CAN	+++	+	+	++++
1003/99	1999	CAN	++	- - -	- - -	+
1018/99	1999	CAN	+++	+	+	++
1039/99	1999	CAN	++	+	+	++
007/11	2011	CAN	++++	++++	+++	++++
035/11	2011	CAN	++++	++++	++++	++++
042/11	2011	CAN	+++	++++	++++	++++
045/11	2011	CAN	++++	++++	++++	++++
074/11	2011	CAN	+++	+++	+++	+++
104/11	2011	CAN	+++	+++	+++	+++
106/11	2011	CAN	++++	++++	++++	++++
107/11	2011	CAN	++++	++++	++++	++++
108/11	2011	CAN	++++	++++	++++	++++
109/11	2011	CAN	+++	+++	+++	+++
127/11	2011	CAN	+++	++++	++++	++++
131/11	2011	CAN	+++	+++	+++	+++
132/11	2011	CAN	+++	+++	++++	+++
139/11	2011	CAN	++++	+++	++++	++++
154/11	2011	CAN	++++	++++	++++	++++
167/11	2011	CAN	++++	++++	++++	++++
168/11	2011	CAN	++++	++++	++++	++++

172/11	2011	CAN	+++	+++	+++	++++
187/11	2011	CAN	+++	+++	+++	+++
206/11	2011	CAN	+++	++++	++++	++++
212/11	2011	CAN	++	+++	+++	+++
247/11	2011	CAN	+++	+++	+++	+++
264/11	2011	CAN	++	+++	+++	+++
274/11	2011	CAN	++++	++++	++++	++++
276/11	2011	CAN	++++	++++	++++	++++
285/11	2011	CAN	+++	+++	+++	+++
312/11	2011	CAN	+++	+++	+++	++++
315/11	2011	CAN	++++	++++	++++	++++
395/11	2011	CAN	+	++	++	++
401/11	2011	CAN	++++	++++	++++	++++
418/11	2011	CAN	++++	++++	+++	++++
424/11	2011	CAN	+++	+++	+++	+++
434/11	2011	CAN	++	++	+	++
436/11	2011	CAN	+++	+++	+++	+++
443/11	2011	CAN	+++	++++	++++	++++
446/11	2011	CAN	+++	++++	++++	++++
455/11	2011	CAN	++	++	++	++
487/11	2011	CAN	+++	++++	++++	++++
502/11	2011	CAN	+++	++++	++++	++++
512/11	2011	CAN	+	+	+	+
515/11	2011	CAN	++++	++++	++++	++++
526/11	2011	CAN	++	++	++	++
528/11	2011	CAN	+++	+++	+++	+++
579/11	2011	CAN	++++	---	---	+++
590/11	2011	CAN	++++	++++	++++	++++
596/11	2011	CAN	+++	+++	+++	+++
618/11	2011	CAN	++++	++++	++++	++++
627/11	2011	CAN	++	++	++	++
634/11	2011	CAN	++	+++	+++	+++

644/11	2011	CAN	+++	++	++	++
646/11	2011	CAN	+++	++++	++++	++++
665/11	2011	CAN	++++	++++	++++	++++
681/11	2011	CAN	++++	++++	++++	++++
685/11	2011	CAN	++	++	++	++
686/11	2011	CAN	+++	++++	++++	++++
708/11	2011	CAN	+++	+++	++	+++
726/11	2011	CAN	++++	++++	++++	++++
738/11	2011	CAN	++++	+++	++	+++
758/11	2011	CAN	++	+++	++	+++
777/11	2011	CAN	++++	++++	++++	++++
786/11	2011	CAN	++++	+++	+++	+++
801/11	2011	CAN	++++	+++	+++	+++
833/11	2011	CAN	+++	++++	+++	+++
837/11	2011	CAN	+	+	+	+
840/11	2011	CAN	+++	++++	++++	++++
843/11	2011	CAN	+	++	+++	++
862/11	2011	CAN	++	++	++	++
869/11	2011	CAN	++++	++++	++++	++++
884/11	2011	CAN	+++	+++	++	+++
889/11	2011	CAN	++++	++++	++++	++++
019/12	2012	CAN	++++	++++	++++	++++
037/12	2012	CAN	++++	++++	++++	++++
049/12	2012	CAN	++++	++++	++++	++++
077/12	2012	CAN	+++	+++	+++	+++
097/12	2012	CAN	++++	++++	++++	++++
133/12	2012	CAN	+++	---	---	++++
136/12	2012	CAN	++++	++++	++++	++++
172/12	2012	CAN	++++	++++	++++	++++
185/12	2012	CAN	++	++	++	++
200/12	2012	CAN	+++	+++	+++	+++
229/12	2012	CAN	++++	++++	++++	++++

235/12	2012	CAN	++++	++++	++++	++++
274/12	2012	CAN	+++	++++	+++	++++
319/12	2012	CAN	++++	++++	++++	++++
324/12	2012	CAN	+++	+++	+++	+++
359/12	2012	CAN	++++	++	++	++++
371/12	2012	CAN	++++	+++	++++	++++
400/12	2012	CAN	++++	++++	++++	++++
423/12	2012	CAN	++++	++++	++++	++++
458/12	2012	CAN	++++	++++	++++	++++
468/12	2012	CAN	+++	+++	+++	+++
508/12	2012	CAN	++++	++++	++++	++++
664/12	2012	CAN	---	---	+	---
665/12	2012	CAN	---	---	---	---
669/12	2012	CAN	---	---	---	---
672/12	2012	CAN	---	---	---	---
675/12	2012	CAN	---	---	---	---
678/12	2012	CAN	---	---	---	---
679/12	2012	CAN	---	---	---	---
680/12	2012	CAN	---	---	---	---
684/12	2012	CAN	---	---	---	---
687/12	2012	CAN	---	---	---	---
688/12	2012	CAN	---	---	---	---
697/12	2012	CAN	---	---	---	---
701/12	2012	CAN	---	---	---	---
702/12	2012	CAN	---	---	---	---
707/12	2012	CAN	---	---	---	---
710/12	2012	CAN	---	---	---	---
711/12	2012	CAN	---	+	+	+
715/12	2012	CAN	---	---	---	---
720/12	2012	CAN	---	---	---	---
740/12	2012	CAN	---	---	---	---
743/12	2012	CAN	---	---	---	---

745/12	2012	CAN	---	---	---	---
748/12	2012	CAN	---	---	---	---
756/12	2012	CAN	---	---	---	---
765/12	2012	CAN	---	---	---	---
766/12	2012	CAN	---	---	---	---
769/12	2012	CAN	---	---	---	---
773/12	2012	CAN	---	---	---	---
775/12	2012	CAN	---	---	---	---
777/12	2012	CAN	---	---	---	---
601/99	1999	FEL	++++	---	++++	++++
620/99	1999	FEL	++++	---	+	+++
929/99	1999	FEL	+++	+	+	+
1052/99	1999	FEL	++++	+++	+++	+++
114/11	2011	FEL	++	---	+	+++
283/11	2011	FEL	++++	---	---	++++
376/11	2011	FEL	++	---	+	+++
467/11	2011	FEL	++	+++	+	+++
481/11	2011	FEL	++++	++++	++++	++++
520/11	2011	FEL	++	---	---	++++
613/11	2011	FEL	+++	---	---	+++
650/11	2011	FEL	+	++	++	++
660/11	2011	FEL	+++	---	+	++++
846/11	2011	FEL	+++	+	+	+++
051/12	2012	FEL	+++	+++	+++	+++
261/12	2012	FEL	++++	---	++++	++++
306/12	2012	FEL	+++	+	---	+++
345/12	2012	FEL	+	+	+	++
382/12	2012	FEL	++++	---	+++	+++
457/12	2012	FEL	++	---	---	+++
650/12	2012	FEL	++++	---	---	++++

651/12	2012	FEL	+++	---	---	+++
391/12	2012	FEL	---	---	---	---
414/12	2012	FEL	---	---	---	---
432/12	2012	FEL	---	---	---	---
443/12	2012	FEL	---	---	---	---
744/12	2012	FEL	---	---	---	---
529/99	1999	CPEN	++++	+	---	++++
540/99	1999	CPEN	+++	---	+++	+++
1000/99	1999	CPEN	+	+	+	+
1087/99	1999	CPEN	+++	---	+	+++
091/11	2011	CPEN	++++	---	---	++++
098/11	2011	CPEN	++++	+++	+++	++++
099/11	2011	CPEN	++++	---	---	++++
137/11	2011	CPEN	+++	+	+	++++
149/11	2011	CPEN	+	+	+	+
153/11	2011	CPEN	+++	---	++++	++++
169/11	2011	CPEN	++++	---	---	++++
177/11	2011	CPEN	+	---	+	+++
267/11	2011	CPEN	++++	+	+	++++
605/11	2011	CPEN	+	+	+	++
010/12	2012	CPEN	+++	---	---	+++
072/12	2012	CPEN	++++	---	---	++++
100/12	2012	CPEN	++++	---	---	++++
131/12	2012	CPEN	++++	---	---	++++
159/12	2012	CPEN	+	+	+	+
286/12	2012	CPEN	+++	+	+	++++
448/12	2012	CPEN	++++	---	+++	++++
502/12	2012	CPEN	++++	---	++++	++++
401/12	2012	CPEN	---	---	---	---
405/12	2012	CPEN	---	---	---	---

441/12	2012	CPEN	---	---	---	---
751/12	2012	CPEN	---	---	---	---
755/12	2012	CPEN	---	---	---	---
557/99	1999	CMES	+++	++	+++	++++
549/99	1999	CMES	+++	+++	++++	++++
673/99	1999	CMES	++	+++	+++	++++
717/99	1999	CMES	++++	+++	+++	++++
121/11	2011	CMES	++++	+++	+++	++++
138/11	2011	CMES	+++	+++	+++	+++
147/11	2011	CMES	++++	++++	+++	++++
257/11	2011	CMES	++++	+++	++++	++++
364/11	2011	CMES	++++	+	+	++++
448/11	2011	CMES	+++	++++	+++	++++
493/11	2011	CMES	++++	++++	++++	++++
594/11	2011	CMES	+++	---	+	+++
633/11	2011	CMES	++++	++++	++++	++++
678/11	2011	CMES	+	+	+	+
103/12	2012	CMES	++	++	++	++
169/12	2012	CMES	++++	++++	++++	++++
249/12	2012	CMES	+++	+++	+++	+++
266/12	2012	CMES	+++	++++	++++	++++
433/12	2012	CMES	+++	++++	++++	++++
494/12	2012	CMES	++++	++++	++++	++++
566/12	2012	CMES	++	+++	+++	+++
594/12	2012	CMES	++++	+	+	++++
514/12	2012	CMES	---	---	---	---
553/12	2012	CMES	---	---	---	---
582/12	2012	CMES	---	---	---	---
591/12	2012	CMES	---	---	---	---

238/99	1999	OMEG	+	++	+	++
395/99	1999	OMEG	+++	++	+++	+++
971/99	1999	OMEG	+++	++++	+++	+++
1082/99	1999	OMEG	+++	++	++	+++
656/11	2011	OMEG	++	++++	++++	+++
909/11	2011	OMEG	+++	+++	+	+++
095/12	2012	OMEG	+++	++++	++++	++++
227/12	2012	OMEG	++++	++++	++++	++++
313/12	2012	OMEG	+++	+++	+++	+++
642/12	2012	OMEG	+++	+++	+++	+++
758/12	2012	OMEG	- - -	- - -	- - -	- - -
388/99	1999	BOV	+++	+++	+++	+++
406/99	1999	BOV	++++	+	+	++++
1029/99	1999	BOV	++++	- - -	+	++++
1086/99	1999	BOV	++++	- - -	++	++++
119/11	2011	BOV	++++	++++	++++	++++
129/11	2011	BOV	++++	++++	++++	++++
279/11	2011	BOV	++++	++++	++++	++++
302/11	2011	BOV	++	++	++	++
313/11	2011	BOV	++++	+++	++++	++++
344/11	2011	BOV	++++	+	+	++++
472/11	2011	BOV	++	++	+	++
479/11	2011	BOV	+++	+++	+++	+++
879/11	2011	BOV	++++	++++	++++	++++
885/11	2011	BOV	+++	++++	++++	++++
011/12	2012	BOV	++++	++++	++++	++++
030/12	2012	BOV	+++	++++	++++	++++
071/12	2012	BOV	++	+++	+++	+++
107/12	2012	BOV	++++	- - -	- - -	++++
201/12	2012	BOV	++	++	++	++

331/12	2012	BOV	++++	++++	++++	++++
412/12	2012	BOV	++++	++++	++++	++++
490/12	2012	BOV	++++	+	+	++++
409/12	2012	BOV	---	---	---	---
445/12	2012	BOV	---	---	---	---
745/12	2012	BOV	---	---	---	---
767/12	2012	BOV	---	---	---	---
776/12	2012	BOV	---	---	---	---

CAN – Canid; FEL – Feline; CPEN – Yellow mongoose; CMES – Black-backed jackal; OMEG – Bat-eared fox; BOV - Bovine

Table A3. Immunoreactivity scores associated with the adapted dRIT diagnostic test relying on the biotinylated polyclonal antibody				
Sample information			FAT	dRIT
Sample Number	Year	Species		Polyclonal antibody
208/99	1999	CAN	++	++
273/99	1999	CAN	+++	+++
524/99	1999	CAN	++	+
596/99	1999	CAN	++	+
687/99	1999	CAN	+++	++
756/99	1999	CAN	+++	++
828/99	1999	CAN	+++	+++
1003/99	1999	CAN	++	+++
1018/99	1999	CAN	+++	++++
1039/99	1999	CAN	++	++
007/11	2011	CAN	++++	++++
035/11	2011	CAN	++++	++++
042/11	2011	CAN	+++	++++
045/11	2011	CAN	++++	++++
074/11	2011	CAN	+++	+++
104/11	2011	CAN	+++	+++
106/11	2011	CAN	++++	++++
107/11	2011	CAN	++++	++++
108/11	2011	CAN	++++	++++
109/11	2011	CAN	+++	+++
127/11	2011	CAN	+++	+++
131/11	2011	CAN	+++	+++
132/11	2011	CAN	+++	+++
139/11	2011	CAN	++++	+++
154/11	2011	CAN	++++	++++
167/11	2011	CAN	++++	+++
168/11	2011	CAN	++++	++++
172/11	2011	CAN	+++	+++
187/11	2011	CAN	+++	+++
206/11	2011	CAN	+++	+++
212/11	2011	CAN	++	+++
247/11	2011	CAN	+++	+++
264/11	2011	CAN	++	++
274/11	2011	CAN	++++	++++
276/11	2011	CAN	++++	++++
285/11	2011	CAN	+++	+++
312/11	2011	CAN	+++	+++
315/11	2011	CAN	++++	++++
395/11	2011	CAN	+	+
401/11	2011	CAN	++++	++++
418/11	2011	CAN	++++	++++
424/11	2011	CAN	+++	++++
434/11	2011	CAN	++	++
436/11	2011	CAN	+++	+++
443/11	2011	CAN	+++	+++
446/11	2011	CAN	+++	+++
455/11	2011	CAN	++	++

487/11	2011	CAN	+++	+++
502/11	2011	CAN	+++	+++
512/11	2011	CAN	+	+
515/11	2011	CAN	++++	++++
526/11	2011	CAN	++	++
528/11	2011	CAN	+++	+++
579/11	2011	CAN	++++	++++
590/11	2011	CAN	++++	++++
596/11	2011	CAN	+++	+++
618/11	2011	CAN	++++	++++
627/11	2011	CAN	++	++
634/11	2011	CAN	++	++
644/11	2011	CAN	+++	++
646/11	2011	CAN	+++	++++
665/11	2011	CAN	++++	++++
681/11	2011	CAN	++++	++++
685/11	2011	CAN	++	++
686/11	2011	CAN	+++	+++
708/11	2011	CAN	+++	+++
726/11	2011	CAN	++++	++++
738/11	2011	CAN	++++	++++
758/11	2011	CAN	++	+++
777/11	2011	CAN	++++	++++
786/11	2011	CAN	++++	+++
801/11	2011	CAN	++++	+++
833/11	2011	CAN	+++	++++
837/11	2011	CAN	+	+
840/11	2011	CAN	+++	++++
843/11	2011	CAN	+	+++
862/11	2011	CAN	++	++
869/11	2011	CAN	++++	++++
884/11	2011	CAN	+++	+++
889/11	2011	CAN	++++	++++
019/12	2012	CAN	++++	++++
037/12	2012	CAN	++++	++++
049/12	2012	CAN	++++	++++
077/12	2012	CAN	+++	+++
097/12	2012	CAN	++++	++++
133/12	2012	CAN	+++	++++
136/12	2012	CAN	++++	++++
172/12	2012	CAN	++++	++++
185/12	2012	CAN	++	++
200/12	2012	CAN	+++	+++
229/12	2012	CAN	++++	++++
235/12	2012	CAN	++++	++++
274/12	2012	CAN	+++	++++
319/12	2012	CAN	++++	++++
324/12	2012	CAN	+++	++++
359/12	2012	CAN	++++	++++
371/12	2012	CAN	++++	++++
400/12	2012	CAN	++++	++++

423/12	2012	CAN	++++	++++
458/12	2012	CAN	++++	++++
468/12	2012	CAN	+++	+++
508/12	2012	CAN	++++	++++
664/12	2012	CAN	---	---
665/12	2012	CAN	---	---
669/12	2012	CAN	---	---
672/12	2012	CAN	---	---
675/12	2012	CAN	---	---
678/12	2012	CAN	---	---
679/12	2012	CAN	---	---
680/12	2012	CAN	---	---
684/12	2012	CAN	---	---
687/12	2012	CAN	---	---
688/12	2012	CAN	---	---
697/12	2012	CAN	---	---
701/12	2012	CAN	---	---
702/12	2012	CAN	---	---
707/12	2012	CAN	---	---
710/12	2012	CAN	---	---
711/12	2012	CAN	---	+
715/12	2012	CAN	---	---
720/12	2012	CAN	---	---
740/12	2012	CAN	---	---
743/12	2012	CAN	---	---
745/12	2012	CAN	---	---
748/12	2012	CAN	---	---
756/12	2012	CAN	---	---
765/12	2012	CAN	---	---
766/12	2012	CAN	---	---
769/12	2012	CAN	---	---
773/12	2012	CAN	---	---
775/12	2012	CAN	---	---
777/12	2012	CAN	---	---
601/99	1999	FEL	++++	++++
620/99	1999	FEL	++++	++++
929/99	1999	FEL	+++	++++
1052/99	1999	FEL	++++	+++
114/11	2011	FEL	++	+++
283/11	2011	FEL	++++	++++
376/11	2011	FEL	++	+++
467/11	2011	FEL	++	+++
481/11	2011	FEL	++++	+++
520/11	2011	FEL	++	++
613/11	2011	FEL	+++	++++
650/11	2011	FEL	+	+
660/11	2011	FEL	+++	+
846/11	2011	FEL	+++	+++
051/12	2012	FEL	+++	+++
261/12	2012	FEL	++++	++++

306/12	2012	FEL	+++	+++
345/12	2012	FEL	+	+
382/12	2012	FEL	++++	+++
457/12	2012	FEL	++	+++
650/12	2012	FEL	++++	+++
651/12	2012	FEL	+++	+++
391/12	2012	FEL	- - -	- - -
414/12	2012	FEL	- - -	- - -
432/12	2012	FEL	- - -	- - -
443/12	2012	FEL	- - -	- - -
744/12	2012	FEL	- - -	- - -
529/99	1999	CPEN	++++	++++
540/99	1999	CPEN	+++	+++
1000/99	1999	CPEN	+	+
1087/99	1999	CPEN	+++	+++
091/11	2011	CPEN	++++	++++
098/11	2011	CPEN	++++	+++
099/11	2011	CPEN	++++	++++
137/11	2011	CPEN	+++	+++
149/11	2011	CPEN	+	+
153/11	2011	CPEN	+++	+++
169/11	2011	CPEN	++++	++++
177/11	2011	CPEN	+	+
267/11	2011	CPEN	++++	++++
605/11	2011	CPEN	+	+
010/12	2012	CPEN	+++	+++
072/12	2012	CPEN	++++	++++
100/12	2012	CPEN	++++	++++
131/12	2012	CPEN	++++	++++
159/12	2012	CPEN	+	+
286/12	2012	CPEN	+++	+++
448/12	2012	CPEN	++++	++++
502/12	2012	CPEN	++++	++++
401/12	2012	CPEN	- - -	- - -
405/12	2012	CPEN	- - -	- - -
441/12	2012	CPEN	- - -	- - -
751/12	2012	CPEN	- - -	- - -
755/12	2012	CPEN	- - -	- - -
557/99	1999	CMES	+++	+++
549/99	1999	CMES	+++	+++
673/99	1999	CMES	++	+
717/99	1999	CMES	++++	+++
121/11	2011	CMES	++++	++++
138/11	2011	CMES	+++	+++
147/11	2011	CMES	++++	+++
257/11	2011	CMES	++++	++++
364/11	2011	CMES	++++	++
448/11	2011	CMES	+++	++
493/11	2011	CMES	++++	++++

594/11	2011	CMES	+++	+++
633/11	2011	CMES	++++	++++
678/11	2011	CMES	+	+
103/12	2012	CMES	++	++
169/12	2012	CMES	++++	++++
249/12	2012	CMES	+++	+++
266/12	2012	CMES	+++	++++
433/12	2012	CMES	+++	+++
494/12	2012	CMES	++++	++++
566/12	2012	CMES	++	++
594/12	2012	CMES	++++	++++
514/12	2012	CMES	- - -	- - -
553/12	2012	CMES	- - -	- - -
582/12	2012	CMES	- - -	- - -
591/12	2012	CMES	- - -	- - -
238/99	1999	OMEG	+	+
395/99	1999	OMEG	+++	+++
971/99	1999	OMEG	+++	+++
1082/99	1999	OMEG	+++	+++
656/11	2011	OMEG	++	++
909/11	2011	OMEG	+++	++++
095/12	2012	OMEG	+++	+++
227/12	2012	OMEG	++++	++++
313/12	2012	OMEG	+++	+++
642/12	2012	OMEG	+++	+++
758/12	2012	OMEG	- - -	- - -
388/99	1999	BOV	+++	+++
406/99	1999	BOV	++++	++++
1029/99	1999	BOV	++++	++++
1086/99	1999	BOV	++++	++++
119/11	2011	BOV	++++	++++
129/11	2011	BOV	++++	++++
279/11	2011	BOV	++++	++++
302/11	2011	BOV	++	++
313/11	2011	BOV	++++	++++
344/11	2011	BOV	++++	++++
472/11	2011	BOV	++	++
479/11	2011	BOV	+++	+++
879/11	2011	BOV	++++	++++
885/11	2011	BOV	+++	+++
011/12	2012	BOV	++++	++++
030/12	2012	BOV	+++	++++
071/12	2012	BOV	++	+++
107/12	2012	BOV	++++	++++
201/12	2012	BOV	++	++
331/12	2012	BOV	++++	++++
412/12	2012	BOV	++++	++++
490/12	2012	BOV	++++	++++
409/12	2012	BOV	- - -	- - -

445/12	2012	BOV	- - -	- - -
745/12	2012	BOV	- - -	- - -
767/12	2012	BOV	- - -	- - -
776/12	2012	BOV	- - -	- - -
CAN – Canid; FEL – Feline; CPEN – Yellow mongoose; CMES – Black-backed jackal; OMEG – Bat-eared fox; BOV - Bovine				

TABLE A4. Immunoreactivity scores associated with the adapted dRIT diagnostic test relying on biotinylated monoclonal antibodies					
Sample information			**FAT**	**direct, Rapid Immunohistochemical Test (dRIT)**	
Sample Number	**Year**	**Species**		**Monoclonal antibody 1**	**Monoclonal antibody 2**
508/12	2012	CAN	++++	++++	++++
324/12	2012	CAN	+++	+++	+++
185/12	2012	CAN	++	++	++
837/11	2011	CAN	+	+	+
481/11	2011	FEL	++++	++++	++++
051/12	2012	FEL	+++	+++	+++
467/11	2011	FEL	++	++	++
345/12	2012	FEL	+	+	+
098/11	2011	CPEN	++++	+++	++++
286/12	2012	CPEN	+++	+	+
159/12	2012	CPEN	+	+	+
494/12	2012	CMES	++++	++++	++++
249/12	2012	CMES	+++	+++	+++
103/12	2012	CMES	++	++	++
678/11	2011	CMES	+	+	+
227/12	2012	OMEG	++++	++++	++++
642/12	2012	OMEG	+++	+++	+++
656/11	2011	OMEG	++	+++	+++
238/99	1999	OMEG	+	+	+
412/12	2012	BOV	++++	++++	++++
479/11	2011	BOV	+++	+++	+++
201/12	2012	BOV	++	++	++
CAN – Canid; FEL – Feline; CPEN – Yellow mongoose; CMES – Black-backed jackal; OMEG – Bat-eared fox; BOV - Bovine					