Comparative evaluation of the diagnostic performance of four serological assays for bovine brucellosis in African buffalo (*Syncerus caffer*)

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1. SUMMARY

The diagnostic performance of four serological assays for bovine brucellosis in African buffaloes, namely Rose-Bengal test (RBT), complement fixation test (CFT), indirect enzyme-linked immunosorbant assay (iELISA) and fluorescence polarisation assay (FPA) were evaluated and compared in a case-control study. The study followed the OIE assay validation pathway for validation of diagnostic tests applicable to wildlife species where there is a validated test available in a taxonomically closely related species. Two uninfected and four infected herds were recruited and an uninfected composite reference panel of 107 sera and infected composite reference panel of 93 were selected using composite reference standards. Diagnostic sensitivity (DSe) and diagnostic specificity (DSp) were calculated for individual tests and for different combinations of two tests in series and in parallel. Cut-off points were adjusted using receiver operating characteristics (ROC) analysis. Using these cut-off values, the index tests performed as follows: RBT DSe of 98.9% (95% CI 96.83% - 100%) and DSp of 98.1% (95% CI 95.6% - 100%), iELISA (cut-off >40.5%) DSe 98.9% (95% CI 94.2% - 100%) and DSp 100% (95% CI 96.6% - 100%), CFT (cut-off >0 iU/ml) DSe 74.2% (95% CI 64.1% - 82.7%) and DSp 100% (95% CI 96.6% - 100%) and FPA (cut-off >16ΔmP) DSe 97.9% (95% CI 94.2% - 99.7%) and DSp 100% (95% CI 96.6% - 100%). Based on performance index and area under the ROC curve, the iELISA performed best (198.9% and 1.0), followed closely by the FPA (197.9% and 0.989) and the RBT (197.0%). The CFT's lower performance (174.2%, and 0.871) was due to low DSe. Kappa values for test agreement between the index tests was above 0 for all combinations, and varied from unweighted Kappa of 0.685 (95% CI 0.608 – 0.762) between FPA and iELISA to 0.26 (0.136-0.383b) between CFT and RBT. Consideration of the indices for positive and negative test agreement between the index tests supported the differential specificity of tests for different immunoglobulin classes and higher in line with the findings in cattle. Positive predictive value in herd C and E were 100% for the iELISA, CFT and FPA, 97.3% in herd C and 98.4% in herd E for the RBT. Negative predictive values in herd C ranged from 89% for the CFT to 99.2% for the RBT and in herd E 73.1% for the CFT to 98.7% for the RBT. Overall repeatability was satisfactory, except for the FPA, which was considered the result of sample quality related to prolonged storage in a freezer. The index tests were all found fit for use to detect or confirm brucellosis in populations and individual animals. The values for DSe and DSp that were estimated will be of use in the interpretation of serological results and determination of diagnostic strategies in different circumstances. Different combinations of tests in series and parallel increased the DSp and DSe. Using the RBT in combination with the CFT/FPA/iELISA interpreted in series or in parallel in relation to the epidemiological setting and objective of testing is recommended.

2   INTRODUCTION

Bovine brucellosis is a zoonotic disease controlled in South Africa according to the Animal Diseases Act (South African Government 1984). The disease is also included in the World Organisation for Animal Health (OIE) list of diseases (OIE 2014). In the Pan African Animal Health Yearbook of 2010, brucellosis is mentioned with rabies, tuberculosis and anthrax as one of the most important zoonotic diseases that occurred in 2010 (African Union Interafrican Bureau for Animal Resources 2011).

Bovine brucellosis is not only a disease of cattle but also wildlife, with the African buffalo (*Syncerus caffer*) in the Kruger National Park (KNP) of South Africa endemically infected (Michel & Bengis 2012, Bengis 1998).

Historically, serological surveys for bovine brucellosis in African buffalo depended on the use of tests developed for cattle. The conventional tests, e.g. Rose-Bengal (RBT) -, serum agglutination (SAT) - and complement fixation (CFT) tests were initially used in African buffalo surveys (Wolhuter, Bengis, Reilly et al. 2009, Chaparro, Lawrence, Bengis et al. 1990, Paling, Waghela, Macowan et al. 1988, Herr, Marshall 1981, de Vos & van Niekerk 1969, Roth 1967, Rollinson 1962) and later also enzyme linked immunosorbant assays (ELISA) (Alexander, Blackburn, Vandewalle et al. 2012, Fyumagwa, Wambura, Mellau et al. 2009, Hamblin, Anderson, Jago et al. 1990). Only the recent ecological study of bovine brucellosis in African buffaloes in the KNP is supported by diagnostic performance data of the serological tests used (RBT, CFT and indirect ELISA (Gorsich, Ezenwa, Cross et al. 2015, Gorsich, Bengis, Ezenwa et al. 2015).

Movement of African buffaloes in South Africa is subject to control according to the Animal Diseases Act (Department of Agriculture, Forestry and Fisheries 2006, SA Govt 1984). Movement of buffaloes is only approved if test results for bovine brucellosis based on the RBT, SAT and/or CFT support the negative infection status of the animal/s (Maja 2013, DAFF 2006). It is therefore essential and of utmost importance that all diagnostic tests used are validated and confirmed fit for the purpose of detecting bovine brucellosis infection in African buffaloes. All currently used tests lack validation in target species other than domestic cattle (Michel & Bengis 2012, OIE 2009a).

2.1   Hypothesis

Null hypothesis: The diagnostic performance characteristics of four serological tests (RBT, CFT, indirect ELISA and FPA) for the detection of *Brucella abortus* antibodies in African buffalo (*Syncerus caffer*) from South Africa are not comparable (diagnostic sensitivity and specificity at recommended cut-off value differ more than 5%) to those in cattle.

Alternate hypothesis: The diagnostic performance characteristics of four serological tests (RBT, CFT, indirect ELISA and FPA) for the detection of *Brucella abortus* antibodies in African buffalo (*Syncerus caffer*) from South Africa are comparable to those in cattle (diagnostic sensitivity and specificity at recommended cut-off value differ no more than 5%).

2.2   Benefits arising from the project

The diagnostic performance characteristics of the tests will contribute to data necessary for acceptance of the test by the OIE as validated and the data will be useful for the design and interpretation of sero-epidemiological studies and testing requirements for the certification of African buffaloes as free from infection with regard to bovine brucellosis (OIE 2014).

2.3   Objectives

The main objective of the study were to determine and compare the diagnostic performance characteristics of four serological tests for bovine brucellosis in African buffalo, namely Rose Bengal test (RBT), an indirect ELISA IDEXX Brucellosis Serum X2 *Brucella abortus* antibody test (iELISA), the complement fixation test (CFT) and the Diachemix Brucella Abortus FPA Test (FPA).

a) To determine performance characteristics in terms of the diagnostic sensitivity (DSe), diagnostic specificity (DSp), performance index and area under the receiver-operator characteristic curve.
b) To determine test agreement between tests in terms of unweighted Kappa and indices of positive and negative agreement.
c) To determine the effect of using two tests in combination on the DSe and DSp.

The study also aimed to provide data towards provisional recognition of the four serological tests by the OIE to be used for international movement of animals.

3   LITERATURE REVIEW

3.1   Aetiological agent of brucellosis

Brucellosis is caused by bacteria of the genus *Brucella,* of which *Brucella melitensis* is the type of - species (Osterman 2010).

Brucella spp. are facultative intracellular and gram-negative cocco-bacilli and belong to the super kingdom Eubacteria, phylum Protobacteria, class Alphaproteobacteria and order Rhizobiales (Guerrero, Peralta, Aguilar et al. 2005).   The class Alphaproteobacteria include other mammalian pathogens of the genera *Bartonella, Ehrlichia* and *Rickettsia.* In the same order are plant symbionts and pathogens such as *Rhizobium*, of which many are studied for their nitrogen fixing ability (Ficht 2011).   Other genera in the family Brucellaceae are: *Daeguia, Mycoplana, Ochrobactrum, Paenochrobactrum, Pseudochrobactrum* (National Center for Biotechnology Information n.d.) *Brucella* is thus closely-related to soil dwelling organisms (Triplett, Breil & Splitter 1994) and it should not be surprising that *Brucella microti* was isolated out of soil (Ficht 2011, Scholz, Nöckler, Göllner et al. 2010).

Deoxyribonucleic acid hybridization studies showed that *Brucella* is a monospecific genus (Verger, Grimont, Grimont et al. 1985), but the Subcommittee on the Taxonomy of *Brucella* of the International Committee on Systematics of Prokaryotes decided in 2003 to return to the six classical *Brucella* nomenspecies with their biovars (Osterman 2010).

Currently, ten *Brucella* species are recognised with three species divided into biovars (Osterman 2010).   There are the six classical species: *Brucella abortus, Brucella melitensis, Brucella ovis, Brucella canis, Brucella suis* and *Brucella neotomae*.   The other four species added more recently are: *Brucella. ceti, Brucella pinnipedalis, Brucella microti* and *Brucella inopinata* (Osterman 2010) (Table 3-1).

Other isolates belonging/potentially belonging to the genus are being described, and with the use of molecular techniques, organisms previously identified incorrectly may now be identified correctly as *Brucellae* (Scholz, Vergnaud 2013*)*.   A potentially new *Brucella* sp. was isolated out of two baboons (*Pappio* sp.) originating from Tanzania in a laboratory in the USA following stillbirths (Schlabritz-Loutsevitch, Whatmore, Quance et al. 2009).   Isolates from wild rodents and a case of human pneumonia may be added to *B.* inopinata in future (Audic, Lescot, Claverie et al. 2011) and *Brucella inopinata*–like bacterium was isolated from abscesses in a big-eyed tree frog (*Leptopelis vermiculatus*) in Germany (Fischer, Lorenz, Heuser et al. 2012) and African bull frogs caught wild in Tanzania (Eisenberg, Hamann, Kaim et al. 2012).

Table 3-1 *Brucella* species and biovars

Species	Biovar	Host preferences	Public Health Risk	LPS
Brucella melitensis	1			
	2	Goats and sheep	serious	smooth
	3			
Brucella abortus	1			
	2			
	3			
	4	Cattle	serious	smooth
	5			
	6			
	9			
Brucella suis	1	pig	serious	
	2	wild boar, hare	low	
	3	pig	serious	smooth
	4	reindeer, caribou	serious	
	5	rodent	absent	
Brucella ovis		sheep	absent	rough
Brucella neotomae		desert wood rat	moderate	smooth
Brucella canis		dog	low	rough
Brucella ceti		dolphins	possible	smooth
Brucella pinnipedalis		seals	possible	smooth
Brucella microti		common vole, red fox	unknown	smooth
Brucella inopinata		human	unknown (one isolate out of human breast implant)	smooth

Source: (Audic, Lescot, Claverie et al. 2011, Ficht 2011, Godfroid, Nielsen & Saegerman 2010, Osterman 2010, Scholz, Nöckler, Göllner et al. 2010, Xavier, Paixã, den Hartigh et al. 2010)

This division of *Brucella* into species and biovars is based on differentiation by cultural, phenotypical, biochemical, phage typing and serological differences and host preferences (Verger, Grimont, Grimont et al. 1985). According to Corbel (1991:1066), the biovars of *B. melitensis* should rather be seen as serovars (Corbel 1991).

Guzmán-Verri, Gonzalez-Barrientos, Hernandez-Mora et al (2012:8) compared the dispersion of *Brucella* spp. against their preferred hosts. They made the proposal that *Brucella* crossed species barriers and jumped to a mammal host of a different order, where the clone persisted and became a novel species (Guzman-Verri, Gonzalez-Barrientos, Hernandez-Mora et al. 2012).

3.1.1   Genetic material

Brucella has two circular chromosomes sized 2.05 and 1.14Mb (Cardoso, Macedo, Azevedo et al. 2006, Michaux-Charachon, Bourg, Jumas-Bilak et al. 1997), except for *B. suis* biovar 3 that has only one (Ficht 2011). Plasmids are absent (Martirosyan, Moreno & Gorvel 2011).

There is greater than 90% homology between *Brucella* species (Sankarasubramanian, Vishnu, Sridhar et al. 2015, Ficht 2011). The pan-genome for the *Brucella* genus was published in 2015, and the genus divided into five clades: the *B. abortus* clade, the *B. melitensis* clade, the *B. ovis* clade, the *B. suis and B. canis* clade and the *B. microti* and *B. pinnipedalis* clade (Sankarasubramanian, Vishnu, Sridhar et al. 2015). An earlier phylogenetic tree reflects the speciation (Michaux-Charachon, Bourg, Jumas-Bilak et al. 1997).

Genetic exchanges have not been demonstrated and the 16S RNA genes are highly conserved, probably because it mainly survives in the host (Sankarasubramanian, Vishnu, Sridhar et al. 2015, Vizcaino, Cloeckaert, Verger et al. 2000).

3.1.2   Cell envelope

The cell envelope of gram negative bacteria consists of the inner membrane that is in contact with the cytoplasm, on its outside the periplasmic space with the thin cell wall of peptidoglycans responsible for the gram negative staining, and on the outside is the outer membrane (Salton & Kim 1996).

The outer membrane consists of a double phospholipid layer, ornithine lipids, lipoproteins and lipopolysaccharides (LPS) (Salton & Kim 1996). The LPS is a complex macromolecule consisting of lipid A that is embedded in the phospholipid layer, an oligosaccharide core and carbohydrate or O-side chains (Cardoso, Macedo, Azevedo et al. 2006, Salton & Kim 1996). Lipopolysaccharides forms the outermost layer of the bacterial envelope and is an important factor in virulence, a major antigen and responsible for the smooth appearance of certain bacteria (Cardoso, Macedo, Azevedo et al. 2006, Salton & Kim 1996).

Lipid A is largely responsible for the endotoxic properties of the LPS of gram negative bacteria (Salton & Kim 1996). Lipid A is a potent pathogen associated molecular pattern (PAMP) and binds with pathogen pattern recognition receptors (PPR), most notably toll like receptor 4 (TLR4), triggering pro-inflammatory responses (Mogensen 2009).

The long aliphatic hydrocarbon side chains contain the O-antigens of gram negative bacteria (Salton & Kim 1996). LPS occurs as a smooth or rough LPS based on the structure of the presence (smooth) or absence (rough) of side chains (Porte, Naroeni, Ouahrani-Bettache et al. 2003).

The cell envelope of *Brucella* spp. have a typical structure gram negative structure (Raybould, Beesley & Chantler 1981), but do not have pili, fimbria, capsules and flagella, except for an

exceptional observation of a flagellum in *B. melitensis* (Martirosyan, Moreno & Gorvel 2011). The LPS of *Brucella* are not typical and give rise to higher hydrophobicity (Martirosyan, Moreno & Gorvel 2011). It is considered to have an important role in the resistance of *Brucella* against the host's antimicrobial mechanisms in both the intra- and extracellular space (Xavier, Paixã, den Hartigh et al. 2010).

3.1.3   Survival in the environment

The organisms can survive for extended periods outside the host, depending on the temperature, humidity and pH (Wray 1975). Wet and muddy conditions favour survival, with survival in wet soil two to three months, dry soil one to two months, four months in faeces and up to eight months in liquid manure. It can survive up to eight months in foetuses in the shade and more than 200 day in uterine exudate (Godfroid, Bosman, Herr et al. 2004, Wray 1975).

3.2   African buffalo (*Syncerus caffer*) as a host species for *Brucella*

3.2.1   Classification

The African buffalo (*Syncerus caffer)* is a ruminant that belongs to the sub family Bovinae, family Bovidae, order Cetartiodactyla, class Mammalia (International Union for Conservation of Nature 2011). The tribe Bovini within the sub-family Bovinae consists of thirteen cattle and buffalo species, including: *Syncerus caffer (*African buffalo), *Bubalus bubalis* (water buffalo) *Bison bison* (American bison) and *Bison bonasus* (European bison). Domestic cattle (*Bos taurus* and *B. indicus*) are considered a subspecies of their ancestor, the *Bos gaurus* (gaur). The tribe Bovini is divided into two sub tribes, Bovina (*Bos* spp., including *B. bison* and *B. bonasus*) and Bubalina (*Bubalus* spp. and *Syncerus caffer*) (eds. Melletti & Burton 2014). There are four African buffalo subspecies: the forest buffalo (*S. caffer nanus*); West African savannah buffalo (*S. caffer brachyceros*); Central African savannah buffalo (*S. caffer aequinoctialis*); and Southern savannah or Cape buffalo (*S. caffer caffer*) (eds. Melletti & Burton 2014, IUCN 2011).

They are considered as least concern by the International Union for Conservation of Nature and Natural Resources (IUCN 2011).

3.2.2   Distribution

The African buffalo, *Syncerus caffer,* is widespread in Africa, with nearly 900 000 animals in their natural range throughout sub-Saharan Africa (IUCN 2011). In South Africa there are large populations of the Southern Savannah buffalo in the Kruger National Park and neighbouring game reserves in Mpumalanga and Limpopo, the Hluhluwe-iMfolozi Park in KwaZulu Natal, Addo National Park in Eastern Cape and many private reserves throughout the country (eds. Melletti & Burton 2014, eds. Skinner & Smithers 1990).

3.2.3   Habitat

The African buffalo is a grazer and adapted to digesting fibrous food, and will feed on old grass. They are sensitive to heat. The savannah buffalo of South Africa require a habitat of grass, shade and water (eds. Skinner & Smithers 1990).

3.2.4   Behaviour

The African buffalo is a gregarious animal, with larger and smaller herds that can combine and separate over time and move seasonally (Ryan, Knechtel & Getz 2007, eds. Skinner & Smithers 1990). Herd sizes are from about 200 to 1 200 with individuals within a herd a few meters apart (Cross, Lloyd-Smith & Getz 2005). Different herds are usually not within visual contact of each other with 1 to 40 km separating them (Cross, Lloyd-Smith & Getz 2005). There appears to be a loose female hierarchy, and females and their offspring tend to move together (Ryan, Knechtel & Getz 2007). Bachelor groups form when young bulls break away from the herd, and old bulls tend to remain solitary (eds. Skinner & Smithers 1990).

3.2.5   Reproduction

The mean age of first ovulation is 3.2 years and the cow may have her first calf at four to five years old. Gestation is about eleven months long (Ryan, Knechtel & Getz 2007).

The calving seasons are long (Ryan, Knechtel & Getz 2007), and in the wetter part of the year when protein levels in grass are rising (eds. Skinner & Smithers 1990). In captivity, calves were born from September to June (Skinner, Dott, Matthee et al. 2006).

During calving, the cow remains with the herd, but if the herd moves on before the calf is strong enough to follow, the cow may stay temporarily behind with the calf. While the cow grazes, new borne calves often shelter in thick undergrowth. The calf suckles until the next calf is born or up to 15 or even 18 months of age. Intercalving period varies, with a yearly calf possible under favourable conditions to 509 days to every two years under suboptimal conditions (Ryan, Knechtel & Getz 2007).

3.2.6   Ecological and socio-economical role

The African buffalo, together with other large herbivores have an important role in maintaining biodiversity, and their impact on birds, rodents and plants have been demonstrated (Ogada, Gadd, Ostfeld et al. 2008, Smart, Hatton & Spence 1985). If there is excessive high buffalo density, this effect may be negative (Chardonnet, des Clers, Fischer et al. 2002).

The African buffalo is host to infectious diseases of ecological, economic, trade and zoonotic importance due to their ability to spread to domestic animals, humans and other wildlife. Examples are foot and mouth disease, Corridor disease, bovine tuberculosis, bovine brucellosis, Rift Valley fever and anthrax (Michel & Bengis 2012).

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As a member of the "Big-five" it also plays an important role in the African economy through wildlife-tourism and breeding (Skinner, Dott, Matthee et al. 2006, Chardonnet, des Clers, Fischer et al. 2002), and animals free of the controlled diseases (foot and mouth disease, corridor diseases, bovine tuberculosis and bovine brucellosis) are scarce and fetch high prices (Skinner, Dott, Matthee et al. 2006). Disease free breeding projects were implemented due to the economic value of disease free African buffaloes and the need to reintroduce the African buffalo into their historical ranges. (Laubscher & Hoffman 2012, Hofmeyr 2006).

The African buffalo is also a source of protein for humans, predators and other scavenger animals and scavenger birds in Africa (eds. Melletti & Burton 2014, Chardonnet, des Clers, Fischer et al. 2002).

3.3   Epidemiology of brucellosis

Brucella species are multihost pathogens, but with differing host preferences (Nicoletti 2010). Domestic livestock are the preferred hosts for the main pathogenic species worldwide, namely cattle for *B. abortus,* sheep and goats for *B. melitensis* and pigs for *B. suis (*Godfroid 2002*).*

Bovine brucellosis in cattle is mainly caused by *Brucella abortus* while *B. melitensis* can play a role where there is contact with infected goats and sheep, and infection with *B. suis* has been reported without abortion, but contact with infected pigs (Godfroid, Bosman, Herr et al. 2004).

Horizontal transmission between cattle occurs mainly after abortion or parturition when *Brucella* organisms are excreted in the milk, uterine fluids and uterine discharges, and are also present in placenta and foetus (Xavier, Paixã, den Hartigh et al. 2010, Godfroid, Bosman, Herr et al. 2004). Transmission of the disease is through direct and indirect contact and environmental contamination (Godfroid, Bosman, Herr et al. 2004). *Brucella* can also be present in the semen of bulls but do not play a significant role in the transmission, except possibly with artificial insemination (Carvalho Neta, Mol, Xavier et al. 2010). Venereal transmission occurs naturally with *B. ovis, B. canis* and *B. suis* (Xavier, Paixã, den Hartigh et al. 2010, Godfroid, Bosman, Herr et al. 2004). Ingestion of other infected mammals is a possible route of infection for marine mammals (Xavier, Paixã, den Hartigh et al. 2010). Dogs can act as mechanical and biological vectors of brucellosis (Burriel, Bisias, Butsini et al. 2002, Rezaei-Sadaghiani, Zowghi, Marhemati-Khamene et al. 1996, Moegle, Heizmann, Katz et al. 1985, Hirchert, Lange & Leonhardt 1967, Aleandri, Mannini 1962). Face and biting flies also play a role in transmission (Bengis 1998).

Vertical transmission to calves occur intra-uterine and through the ingestion of milk (Carvalho Neta, Mol, Xavier et al. 2010).

Spread of the disease between populations is usually by introduction of an infected animal and the successful establishment of the disease will depend on successful transmission. Behaviour of wildlife

and livestock play an important role in the successful transmission between wildlife and livestock and within a wildlife population (Godfroid, Garin-Bastuji, Saegerman et al. 2013).

The disease impact on the health, reproduction and production of livestock, wildlife and humans and has national and international trade implications (McDermott & Arimi 2002).

3.3.1   Global distribution

Brucellosis occurs worldwide. Some countries eradicated *B. abortus,* including Australia, Canada, Cyprus, Denmark, Finland, the Netherlands, New Zealand, Norway, Sweden and the United Kingdom. Countries affected most are the Mediterranean countries of Europe, northern and eastern Africa, Near East countries, India, Central Asia, Mexico and Central and South America (ed. Robinson 2003).

3.3.1.1   Wildlife

Brucellosis occurs in terrestrial mammals on all the continents and in marine mammals in nearly all the seas (Godfroid, Garin-Bastuji, Saegerman et al. 2013, Godfroid 2002). Most of the evidence is not substantiated by bacterial isolation of the organisms (Godfroid, DeBolle, Roop et al. 2014, Godfroid 2002). Wildlife may be spill over hosts infected by domestic animals, amplifying hosts or sustain infection and re-infect domestic animals and wildlife (Godfroid, DeBolle, Roop et al. 2014, Godfroid, Garin-Bastuji, Saegerman et al. 2013).

Bovine brucellosis is only sustainable in some terrestrial wildlife species, with ungulates from the tribe Bovini maintaining infection at higher levels than other ungulate species (Cross, Maichak, Brennan et al. 2013). *B. abortus* infection is maintained in bison and elk in North America and African buffalo in Africa (Michel & Bengis 2012, Godfroid 2002), but only elk and African buffalo are considered reservoirs (Michel & Bengis 2012), and can maintain the infection in the absence of infection in domestic livestock (Godfroid 2002). In Europe *B. suis* biovar 2 infections is sustained in the European wild boar and also the European hare is a possible reservoir (Godfroid 2002). In the Arctic region, Siberia, Canada and Alaska, *B. suis* biovar 4 infects reindeer, caribou, moose and occasionally carnivores (Godfroid 2002). *B. melitensis* does not appear to have a wildlife reservoir. Spill over infections are seen in France and Italy in e.g. chamois and ibex, in the Middle East in nomadic one-humped camels in contact with sheep and goats and in South America in llamas other small camelids (Godfroid 2002).

3.3.2   African livestock

Brucellosis was present in East Africa in domestic and wild ruminants before the introduction of cattle from other continents, while importation of infected cattle from Europe led to its introduction into southern Africa (Bigalke 1994).

Brucellosis was considered as one of the most important zoonotic diseases reported in African livestock in 2010 in terms of number of outbreaks and number of countries affected, with rabies,

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tuberculosis and anthrax (African Union Interafrican Bureau for Animal Resources 2011). During 2010, eighteen African countries reported 1 090 outbreaks, with 394 in South Africa (AU IBAR 2011). Extensive and agro-pastoralists systems for cattle, sheep, goats and camels, are affected most by losses due to brucellosis (McDermott & Arimi 2002). The true prevalence of brucellosis in livestock in Africa is not well known (Marcotty, Matthys, Godfroid et al. 2009, McDermott & Arimi 2002). There is evidence of infection of cattle with *B. abortus* as well as *B. melitensis* (Godfroid, Al Dahouk, Pappas et al. 2013).

B. abortus is considered the main agent and widespread in cattle in Africa *B. abortus* biovar 1 is likely to be the predominant cause of brucellosis in both commercial and smallholder cattle farms in Zimbabwe (Matope, Bhebhe, Muma et al. 2009).

B. melitensis is prevalent in sheep and goats, with camels also becoming infected, in countries on the Mediterranean Sea (Blasco & Molina-Flores 2011). There is also evidence of infection with *B. melitensis* of cattle in North Africa (Hassan Samaha, Meshref Al-Rowaily, Khoudair et al. 2008) and Kenya (Muendo, Mbatha, Macharia et al. 2012). It probably has a low prevalence in Sub-Saharan Africa, and may be absent in Mozambique (Marcotty, Matthys, Godfroid et al. 2009), but present in Swaziland (Emslie & Nel 2002) and Namibia (OIE 2011).

Brucella suis was reported in Mozambique from 1996 to 1998 with the last occurrence in 2009, but very little is known about its occurrence in Africa (OIE 2011, McDermott & Arimi 2002).

3.3.2.1   South Africa

Brucellosis is a controlled disease according to the Animal Diseases Act (Act 35 of 84), and a disease scheme exists to eradicate of bovine brucellosis and promote human health (SA Govt 1988).

Bovine brucellosis has been reported in all the provinces in the country, but accurate current prevalence data are not available. An exception is the prevalence study done by U. Hesterberg of the serological prevalence in cattle in rural areas of Kwa Zulu Natal (Hesterberg 2007). The disease is more prevalent in the northern provinces of the country (Figure 3-1).

B. abortus biovar 1 and 2 are present in the cattle with biovar 1 predominating (Livestock Production Research 1977, Directorate Veterinary Services, Subdirectorate Animal Health 1976).
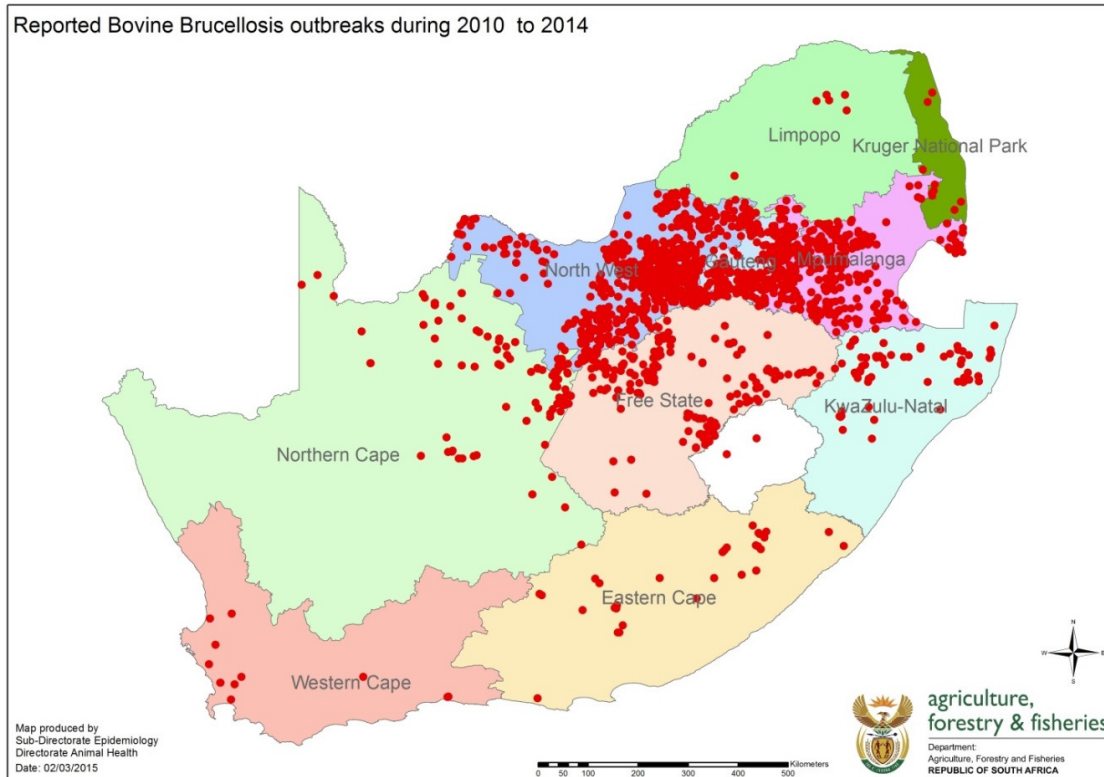
Figure 3-1 Map of bovine brucellosis outbreaks reported from 2010 to 2014

Brucella melitensis is present in South Africa, but probably not at a high prevalence. It is presumed to have been eradicated from KwaZulu Natal goats in 2000 (Emslie & Nel 2002). In December 2010 it caused an outbreak of abortions in domestic goats in Gauteng (Outbreak Response Unit NHLS-NICD, Department of Agriculture, Forestry and Fisheries & Department of Health 2011). *B. melitensis* was isolated out of cattle in 1976/77 (Livestock Production Research 1977). It appears to be an isolated incident.

B. suis has not been reported in South Africa (OIE 2011).

3.3.3   African wildlife

The true prevalence of brucellosis in wildlife in Africa is not well known (Marcotty, Simpson & Godfroid 2011, McDermott & Arimi 2002). Many reports of surveys for brucellosis in sub-Saharan African wildlife are available, mostly based on serological assays developed for cattle (Alexander, Blackburn, Vandewalle et al. 2012, Wolhuter, Bengis, Reilly et al. 2009, Chaparro, Lawrence, Bengis et al. 1990, Paling, Waghela, Macowan et al. 1988, Hamblin, Burnett & Hedger 1986, Herr, Marshall 1981, Kaliner, Staak & Daliner 1973, de Vos & van Niekerk 1969, Roth 1967, Rollinson 1962) and some combined with direct testing for the organism (Gradwell, Schutte, Van Niekerk et al. 1977, Kaliner, Staak & Daliner 1973, Roth 1967). Lack of information of the diagnostic performance of the

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diagnostic assays and the design of the surveys does not allow for accurate information on the prevalence of brucellosis in any of the species tested (Alexander, Blackburn, Vandewalle et al. 2012).

Positive serological results were reported in the following species other than the African buffalo: giraffe (Roth 1967), hippopotamus (Rollinson 1962), impala (de Vos & van Niekerk 1969), waterbuck (Rollinson 1962) , eland (Paling, Waghela, Macowan et al. 1988, Keep & Bishop 1985, Condy & Vickers 1972, Roth 1967, Rollinson 1962), zebra and Burchell's zebra (Rollinson 1962), tsessebe (Cooper & Carmichael 1974 cited by Chaparro et al. 1990), bushbuck, duiker, grysbok, kudu (Condy & Vickers 1972), nyala (Chaparro, Lawrence, Bengis et al. 1990, Keep & Bishop 1985, Condy & Vickers 1972), beisa oryx (Paling, Waghela, Macowan et al. 1988), black backed jackal (Roth 1967), spotted hyena (Sachs, Staak & Groocock 1968) cited by (Chaparro, Lawrence, Bengis et al. 1990, Paling, Waghela, Macowan et al. 1988), blue wildebeest, Kafue lechwe (Muma, Munyeme, Matope et al. 2011, Muma, Lund, Siamudaala et al. 2010, Roth 1967), sable (Condy, Vickers 1969) and black rhinoceros (Madsen & Anderson 1995, Chaparro, Lawrence, Bengis et al. 1990, Sachs 1966) (Table 3-2 Sub-Sahara African wildlife (excluding African buffalo) for which positive serologic findings were reported.

Table 3-2 Sub-Sahara African wildlife (excluding African buffalo) for which positive serologic findings were reported.

Species		Country	Source
beisa oryx	*Oryx beisa*	Kenya	Condy & Vickers 1972
black backed jackal	*Canis mesomelas*	Tanzania Zimbabwe	Sachs et al. 1968 cited by Chaparro, Lawrence, Bengis et al. 1990 Roth 1967
black rhinoceros	*Diceros bicornis*	Serengeti	Madsen & Anderson 1995 Chaparro, Lawrence, Bengis et al. 1990 Sachs & Staak 1966
blue wildebeest	*Connochaetes taurinus*	Tanzania Kenya Zambia	(Fyumagwa, Wambura, Mellau et al. 2009) Waghela & Karstad 1986 Rottcher 1978 cited by Muma, Samui, Oloya et al. 2007 Sachs et al. 1968 and Sach & Staak 1966 cited by Chaparro, Lawrence, Bengis et al. 1990
bushbuck	*Tragelaphus scriptus*	Zimbabwe Zambia	Condy & Vickers 1972 Rottcher 1978 cited by Muma, Samui, Oloya et al. 2007

Species		Country	Source
eland	*Taurotragus oryx*	Zimbabwe, Kenya, East Africa Zambia	Rottcher 1978 cited by Muma, Samui, Oloya et al. 2007 Condy & Vickers 1972 de Vos & van Niekerk 1969
giraffe	(not mentioned)	Botswana, Zimbabwe	Alexander, Blackburn, Vandewalle et al. 2012 Paling, Waghela, Macowan et al. 1988 Roth 1967
hippopotamus	*Hippopotamus amphibicus*	South Africa Zimbabwe East Africa	Condy & Vickers 1972 de Vos & van Niekerk 1969 Rollinson 1962
impala	*Aepyceros melampus*	South Africa Zimbabwe Zambia	Motsi, Tichiwangana, Matope et al. 2013 de Vos & van Niekerk 1969 Rottcher 1978 cited by Motsi, Tichiwangana, Matope et al. 2013 Condy & Vickers 1972
Kafue lechwe	*Kobus leche kafuensis*	Zambia	Muma, Munyeme, Matope et al. 2011 Muma, Lund, Siamudaala et al. 2010 Roth 1967
nyala	*Tragelaphus angasi*	Natal	Condy & Vickers 1972
sable	*Hippotragus niger*	South Africa	Roth 1967
spotted hyena	*Crocuta crocuta*	Tanzania	Sachs et al. 1968 cited by Chaparro, Lawrence, Bengis et al. 1990
tsessebe	*Damaliscus lunatus*	Botswana	Chaparro, Lawrence, Bengis et al. 1990 Cooper & Carmichael 1974
waterbuck	*Kobus ellipsiprymnus*	South Africa Zimbabwe East Africa	Gomo, Garine-Wichatitsky, Caron et al. 2012 Madsen & Anderson 1995 de Vos &van Niekerk 1969 Rollinson 1962
zebra Burchell's zebra	*Equus burcehelli*	East Africa Zimbabwe Zambia	Rottcher 1978 cited by Muma, Samui, Oloya et al. 2007 Condy &Vickers 1972 Rollinson 1962
rodents	*Mastomys natalensis, Arvicanthis nilothicus, Aethomyces kaiseri, Tatera robusta*	Kenya	Heisch, Cooke, Harvey et al. 1963

Species		Country	Source
puku antelope	*Kobus vardonii*	Zambia	Rottcher 1978 cited by (Muma, Samui, Oloya et al. 2007
duiker	Sylvicarpa grimmia	Zimbabwe	Gradwell, Schutte, Van Niekerk et al. 1977
kudu	*Tragelaphus strepsiceros*	Zimbabwe	Gradwell, Schutte, Van Niekerk et al. 1977

There are only a few reports of isolation of *Brucella* spp. from wildlife in Africa. The following were reported for wildlife other than African buffaloes: *B. melitensis* biovar 3 from sable (OIE 2005) and *B. abortus* biovar 1 from an eland (E. Dyason pers. comm. 16 August 2012) in South Africa, *B. abortus* biovar 1 from waterbuck and eland in Zimbabwe (Gomo, Garine-Wichatitsky, Caron et al. 2012, Madsen & Anderson 1995, Condy, Vickers 1969, Rollinson 1962), *Brucella* spp. with characteristics of *B. melitensis* from Kafue lechwe in Zambia (Muma, Lund, Siamudaala et al. 2010), *B. melitensis* from one impala in the Serengeti (Schiemann, Staak 1971) and *B. suis* biovar 3 from rodents in Kenya (Heisch, Cooke, Harvey et al. 1963) (Table 3-3).

Table 3-3 Reports of *Brucella* isolated form African wildlife other than African buffaloes.

Species		Country	*Brucella* sp.	Source
waterbuck	*Kobus ellipsiprymnus*	Zimbabwe	*B. abortus* biovar 1	Condy & Vickers 1969
eland	*Taurotragus oryx*	Zimbabwe South Africa	*B. abortus* biovar 1	E. Dyason pers. comm. 16 August 2012 Condy & Vickers 1972
impala	*Aepyceros melampus*	Serengeti	*B. melitensis* biovar 1	Schiemann & Staak 1971
sable	*Hippotragus niger*	South Africa	*B. melitensis*	OIE 2005
Kafue lechwe	*Kobus leche kafuensis*	Zambia	*Brucella* spp, possibly *melitensis*	Muma, Lund, Siamudaala et al. 2010
rodents	*Mastomys natalensis, Arvicanthis nilothicus*	Kenya	*B. suis* biovar 3	Heisch, Cooke, Harvey et al. 1963

3.3.3.1   African buffaloes

African buffalo can be considered a reservoir for *B. abortus* (Godfroid 2002). It has not yet been shown conclusively whether the disease is indigenous to African buffalo or whether it was introduced by cattle. Serological evidence from East Africa indicates that brucellosis was present in Africa before introduction of exotic cattle (Bigalke 1994).

Bacteriological evidence shows that brucellosis in African buffalo is associated with *Brucella abortus* biovar 1 in the Kruger National Park, South Africa (Directorate of Veterinary Services, Subdirectorate Animal Health 1987, Gradwell, Schutte, Van Niekerk et al. 1977), and biovar 3 in Tanzania (Staak, Sachs & Groocock 1968).

Reports of positive serological findings in African buffaloes were published for East Africa (Rollinson 1962), Tanzania (Hamblin, Anderson, Jago et al. 1990, Kaliner, Staak & Daliner 1973), the Serengeti (Fyumagwa, Wambura, Mellau et al. 2009) and Uganda (Kalema-Zikusoka, Bengis, Michel et al. 2005). Serological surveys for brucellosis in African buffaloes in South Africa and neighbouring countries, Botswana and Zimbabwe, indicate sero-prevalence ranging from 0% in central Botswana, 17% in Zimbabwe to 37.8% in cows in the Kruger National Park (Motsi, Tichiwangana, Matope et al. 2013, Alexander, Blackburn, Vandewalle et al. 2012, Herr, Marshall 1981). A recent study in Mozambique confirmed the presence of seropositive buffaloes in the Limpopo National Park (Tanner, Inlameia, Michel et al. 2014).

Samples for most studies surveys were acquired conveniently from other studies, activities or archives (Alexander, Blackburn, Vandewalle et al. 2012, Gomo, Garine-Wichatitsky, Caron et al. 2012, Wolhuter, Bengis, Reilly et al. 2009, Chaparro, Lawrence, Bengis et al. 1990, Herr, Marshall 1981, Gradwell, Schutte, Van Niekerk et al. 1977, Condy & Vickers 1972, de Vos & van Niekerk 1969). Only one study on seroprevalence in the Kruger National Park (KNP) was supported by diagnostic performance data in African buffaloes for the serological tests used (Gorsich, Ezenwa, Cross et al. 2015, Gorsich, Bengis, Ezenwa et al. 2015). (Table 3-4)

Table 3-4 Brucellosis serological studies in African buffaloes from South Africa and neighbouring countries

Year specimens collected	Country	District/Area	Tests	Serological prevalence	Source of samples	Source
1966-1971	Zimbabwe		SAT	14.7%	Anti-tsetse fly game control operations, culling, cropping	Condy & Vickers 1972
1967	Zimbabwe		SAT	1 positive, 1 suspicious out of unknown number		Roth 1967
published 1969	South Africa	KNP	SAT	14.2% (11%)	randomised, cropping	de Vos & van Niekerk 1969
Published 1977	South Africa	KNP	Bacteriology	5,9% pregnant cows bacteriological positive, 7.4% if smear positive cows are included.	slaughter	Gradwell, Schutte, Van Niekerk et al. 1977
published 1981	South Africa	KNP	RBT, SAT and confirmed by CFT (cut-off 30 iU/ml)	37.8% of cows 28,5% of bulls 25% heifers 7,7% male juveniles 0% calves	culling	Herr & Marshall 1981
published 1990	South Africa	KNP	Screened by RBT and confirmed by CFT (cut-off 30 iU/ml)	12,6% adult female 10,7% sub-adult females 3% juvenile females 15,1% adult males 10,6% sub-adult male 5,3% juvenile males	culling	Chaparro, Lawrence, Bengis et al. 1990

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Year specimens collected	Country	District/Area	Tests	Serological prevalence	Source of samples	Source
1995	Botswana	Chobe	Screened RBT, confirmed FPA	4% (0-14%)	archive	Alexander, Blackburn, Vandewalle et al. 2012
1996	Botswana	Chobe Ngamiland	Screened RBT, confirmed FPA	13% (0-41%) 7% (0-19%)	archive archive	Alexander, Blackburn, Vandewalle et al. 2012
1998	Botswana	Chobe Ngamiland	Screened RBT, confirmed FPA	5% (0-15%) 17 (0-62%)	archive archive	Alexander, Blackburn, Vandewalle et al. 2012
1999	Botswana	Chobe Central	Screened RBT, confirmed FPA	7% (0-22%) 0%	archive archive	Alexander, Blackburn, Vandewalle et al. 2012
2000	Botswana	Chobe	Screened RBT, confirmed FPA	3% (0-10%)	archive	Alexander, Blackburn, Vandewalle et al. 2012
1990, 1991	Zimbabwe		RBT, SAT and CFT	6,5%	Not mentioned	Madsen & Anderson 1995
2003 – 2004	South Africa	KNP	SAT, CFT	Not mentioned.	Study on demodectic mange risk factors and concomitant infections.	Wolhuter, Bengis, Reilly et al. 2009
2001 to 2007	South Africa	KNP	Screened by RBT, confirmed by CFT and SAT in parallel	South 26.8% Central 15.6% North: 15%	Cross sectional serological survey.	Gorsich, Ezenwa, Cross et al. 2015
July 2007 to October 2009	Zimbabwe	Gonarezhou National Park	Screened RBT, confirmation by cELISA	negative (interpreted)	Cross sectional, convenience sampling (slaughter, hunter kills, translocation, specific for purpose of the study)	Gomo, Garine-Wichatitsky, Caron et al. 2012

Year specimens collected	Country	District/Area	Tests	Serological prevalence	Source of samples	Source
2009 to 2012	Zimbabwe	Gonarezhou and Hwange, safari areas and private wildlife sanctuaries	RBT and CFT (cut-off 20 iU/ml) in series	17%	Routine disease surveillance	Motsi, Tichiwangana, Matope et al. 2013
Published 2014	Mozambique	Limpopo National Park	RBT and blocking ELISA parallel	27.4%	Survey for brucellosis and tuberculosis.	Tanner, Inlameia, Michel et al. 2014

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Seroprevalence increases with age until they are adult (> six years old), and decrease after nine years of age, and sex does not appear to be a risk factor for infection (Gorsich, Ezenwa, Cross et al. 2015, Motsi, Tichiwangana, Matope et al. 2013)

The impact of the disease on African buffaloes is not known, and in the KNP, there is no readily apparent effect on the African buffalo population (Bengis 1998, Madsen & Anderson 1995). A recent longitudinal study suggests that it may lead to lower population growth rates which can only be confirmed by further longitudinal studies (Gorsich, Ezenwa, Cross et al. 2015, Bengis 1998). Spatial analysis revealed that the highest prevalence of brucellosis is in the south-west of the park with granite soils and unpalatable grasses, and the lowest prevalence on granite soils of the central area of the park (Gorsich, Ezenwa, Cross et al. 2015). Buffaloes decreased in condition after sero-converting to brucellosis and infected animals had higher mortality, though no association between fecundity and brucellosis status was demonstrated (Gorsich, Ezenwa, Cross et al. 2015).

3.3.3.2 Buffalo/cattle interface

African buffaloes and cattle share diseases such as foot and mouth disease, Corridor disease, bovine tuberculosis and bovine brucellosis (Michel & Bengis 2012, Bengis, Kock, Thomson et al. 2004). Due to the economic importance of these diseases, cattle and African buffaloes have to be separated in South Africa (DAFF 2006, Bengis, Kock, Thomson et al. 2004). All owners of African buffaloes in South Africa have to register the property for the keeping of African buffaloes with the National Director of Animal Health. Buffaloes must be contained by buffalo proof fences and those infected with foot and mouth - and Corridor disease may not be kept with cloven-hoofed livestock (DAFF 2006). This is however not always successful, as can be seen e.g. in the outbreaks of foot and mouth disease in Mpumalanga 2009, 2011 and 2013, and regular reports on stray buffaloes in Mpumalanga and Limpopo in areas neighbouring the Kruger National Park. Contact between cattle and African buffaloes were reported in Limpopo, with African buffaloes leaving the Kruger National Park and cattle entering the park (Brahmbhatt, Fosgate, Dyason et al. 2012).

Transmission of brucellosis between cattle and African buffaloes has not yet been demonstrated. Gomo Garine-Wichatitsky, Caron et al (2012:83) studied the seroprevalence of brucellosis in cattle on the Zimbabwean side of the Greater Limpopo Transfrontier Conservation Area and found that cattle grazing in the national park had a higher chance of testing positive for brucellosis than others (Gomo, Garine-Wichatitsky, Caron et al. 2012). Motsi Tichiwangana, Matope et al (2013:3) studied seroprevalence in African buffalo in the same area, and found seropositivity where there is no known history of contact between cattle and buffaloes (Motsi, Tichiwangana, Matope et al. 2013). Their evidence suggested low rates of transmission between wildlife and livestock, and that the prevalence in wildlife is low (Motsi, Tichiwangana, Matope et al. 2013).

There is no molecular evidence to proof whether African buffaloes and domestic cattle share the same strains, and if they do, what is the direction of transmission (Tanner, Inlameia, Michel et al.

2014, Motsi, Tichiwangana, Matope et al. 2013). Cattle were suspected to be the source of infection for farmed African buffaloes in the Northwest province after they became seropositive and *B. abortus* biovar 1 was isolated, following brucellosis with abortions in cattle on a neighbouring farm (J. Steyl pers. comm. 10 November 2014). The source of infection for recently infected African buffaloes in the Freestate was not confirmed (I. Fick pers. comm. 30 June 2014).

3.4   Clinical signs and lesions

Brucellosis shows similar pathogenesis and clinical manifestation between hosts and what is known about the disease in domestic cattle is used as a model for the disease in cattle (Rhyan 2013), though susceptibility may be different, as was demonstrated with American bison (Olsen & Johnson 2011).

3.4.1   Domestic cattle

The disease can be divided into three phases: (1) incubation period, (2) acute phase when clinical signs are initially seen and the (3) chronic phase, which varies according to the specific *Brucella* and host (Martirosyan, Moreno & Gorvel 2011).

Brucellosis in cattle is mainly caused by *Brucella abortus* and is mostly a disease of cows (Xavier, Paixã, den Hartigh et al. 2010). Late abortions at five to seven months of gestation are the most important sign, but usually only occurs once, or in some infected cows not at all. The placenta shows necro-haemorrhagic placentitis (Xavier, Paixã, den Hartigh et al. 2010). Foetuses may show lesions due to bacterial infection, e.g. fibrinous pericarditis and pleuritis and pneumonia (Xavier, Paixã, den Hartigh et al. 2010). In subsequent gestations the cows give birth to either weak or healthy calves (Godfroid, Bosman, Herr et al. 2004). Clinical mastitis is not detected, but there may be mild to moderate interstitial mastitis (Xavier, Paixã, den Hartigh et al. 2010). Chronically infected animals may show hygromas, mostly of carpal joints.

On a herd level, outbreaks in dairies present with abortions and post-partum metritis associated with retained placentas, decreased milk production and increased somatic cell counts (Godfroid, Bosman, Herr et al. 2004).

Bulls can develop uni- or bilateral, acute or chronic orchitis with or without seminal vesiculitis and epididymitis, leading to temporary or permanent infertility (Xavier, Paixã, den Hartigh et al. 2010, Godfroid, Bosman, Herr et al. 2004).

3.4.2   African buffalo

De Vos and van Niekerk (1969:333) described clinical signs in 8 out of 36 buffalo that tested positively with the serum agglutination test, namely: hygroma on the carpus of six, bilateral orchitis and epididymitis in one and purulent endometritis in one. Bacterial isolation was unsuccessful (de Vos & van Niekerk 1969). Waghela & Karstad (1986:191) described seeing hygromata in African buffaloes in Kenya in 1986, where serological reactions were found, but no bacterial isolation was attempted

(Waghela, Karstad 1986). Gradwell et al (1977:41) found that in one of four pregnant cows that was bacteriologically positive for *Brucella abortus* the foetus died in utero before the cow was killed, with signs suggestive of placentitis: cloudy brownish-red allantoic fluid and dull greyish allanto-chorionic membrane, with cotyledons separating easily from caruncles (Gradwell, Schutte, Van Niekerk et al. 1977, Cooper, Carmichael 1974) Bengis 1998:178 reported late abortions (Bengis 1998). *B. abortus* was isolated out of the placenta from a cow that aborted, and placentitis, with masses of intracellular bacteria were seen miscroscopically (D. Janse van Rensburg pers. comm 10 November 2014).

The clinical course of the disease in African buffaloes therefore appears to be similar to the clinical course in cattle.

3.5 Pathogenesis

The pathogenesis of brucellosis at a cellular level and type of lesions produced are considered similar across host and *Brucella* species (Rhyan 2013), yet differences were described in the pathogenesis and immunological responses between cattle, elk *Cervus elaphus nelsoni* and bison (*Bison bison)* (Olsen & Johnson 2011, Olsen, Fach, Palmer et al. 2006, Olsen, Christie, Grainger et al. 2006). It can therefore not be accepted that the disease in African buffaloes will mirror that of cattle exactly.

The success of infection and course of the disease will depend on the *Brucella* species and in certain instances the biovar, host species, age, sex, stage of reproduction and resistance (Carvalho Neta, Mol, Xavier et al. 2010, Godfroid, Bosman, Herr et al. 2004).

The pathogenesis of brucellosis is different from many other bacterial diseases. Although it is a gram negative organism, endotoxins does not play a significant role in the pathogenesis. Unlike other intracellular bacteria, which are mostly gram positive, it also does not produce exotoxins (Cardoso, Macedo, Azevedo et al. 2006). *Brucella* also has no capsule to protect it against phagocytosis (Cardoso, Macedo, Azevedo et al. 2006). Being able to enter the host cells and survive and replicate inside, is key to the pathogenesis and the development of chronic infections (Cardoso, Macedo, Azevedo et al. 2006). The discussion focuses on the disease in cattle due to lack of information on the disease in African buffaloes.

Brucella enters the body across mucous membranes of the gastro-intestinal tract (pharynx of importance in cattle), respiratory tract, urinary tract and genital system (Godfroid, Bosman, Herr et al. 2004). In the gastro-intestinal tract it is through M cell epithelial transmigration, or in intra-epithelial phagocytes (Xavier, Paixã, den Hartigh et al. 2010). *Brucella* organisms are then phagocytosed in the lamina propria and carried to the draining regional lymph nodes intracellular in macrophages and neutrophils (Martirosyan, Moreno & Gorvel 2011, Godfroid, Bosman, Herr et al. 2004). Here it multiplies and induce a lymphadenitis (Godfroid, Bosman, Herr et al. 2004). The organisms leave the lymph node through efferent lymphatics and enter the blood stream and are disseminated through the body (Carvalho Neta, Mol, Xavier et al. 2010). In the blood, they may be intracellular in neutrophils

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and macrophages or free in the plasma (Godfroid, Bosman, Herr et al. 2004). This may be months after colonising the lymph nodes and the bacteraemia can be of short duration to several months long, or in 5 to 10 percent of animal be recurrent over years (Godfroid, Bosman, Herr et al. 2004).

The organisms can then establish themselves in the reticulo-endothelial system, uterus, udder, synovial structures, testes and male accessory glands (Martirosyan, Moreno & Gorvel 2011, Godfroid, Bosman, Herr et al. 2004). This is the time acute clinical signs may be seen and the organisms can be found in microgranulomas in infected tissues (Xavier, Paixã, den Hartigh et al. 2010, Godfroid, Bosman, Herr et al. 2004).

In the gravid uterus, the organisms enter the trophoblasts, where they can survive with limited degenerative changes to the cell (Meador & Deyoe 1989). At about four and a half months of gestation, the brucellae begin to increase its replication, and usually cause abortion in the last trimester (Samartino & Enright 1993). The high levels of erythritol and steroid hormones during this period play a role in enhancing replication of *Brucella*. The infected placenta's integrity is compromised, and there is also hormonal changes mimicking the changes of parturition. Abortion or the birth of weak calves results from the infection of placenta and foetus (Xavier, Paixã, den Hartigh et al. 2010). This leads to variable incubation period in cows, where it can be as short as 50 days when infected at seven months of gestation, 225 days when infected at insemination and eighteen months in congenitally infected heifers (Godfroid, Bosman, Herr et al. 2004)..

Persistent infection is established in the lymphoid tissues (Xavier, Paixã, den Hartigh et al. 2010). Chronic infections occur in ruminants, though some animals may recover but due to the longevity of the antibody response they cannot be identified

3.5.1   Host cell interactions with Brucella

The main host cells are macrophages, dendritic cells and non-phagocytic cells such as trophoblasts.

Brucella enters macrophages in three ways. They get opsonised and enter through FC or complement receptors, or non-opsonised bacteria enter through lectin and fibronectin receptors or lipid rafts (Xavier, Paixã, den Hartigh et al. 2010). They then enter the lysosome, where the majority of bacteria are destroyed, especially where there were opsonisation of the bacteria or IFNγ-activation of the macrophage (Xavier, Paixã, den Hartigh et al. 2010). The *Brucella* containing vacuole acidifies rapidly, a process possibly associated with the lipid rafts. Acidification induces virulence genes and ensures the survival and replication of the organisms (Porte, Naroeni, Ouahrani-Bettache et al. 2003). Fusion with lysosomes is inhibited and when it happens is temporary. The LPS-O side chain plays a role in this inhibition (Martirosyan, Moreno & Gorvel 2011, Godfroid, Bosman, Herr et al. 2004). The survivors actively exclude lysosomal proteins. The *Brucella* containing vacuole then fuses with the endoplasmic reticulum where the surviving organisms can multiply safely (Martirosyan, Moreno & Gorvel 2011, Godfroid, Bosman, Herr et al. 2004).

The survival and replication of *Brucella* in dendritic cells is similar to that in macrophages, except that dendritic cells are infected more efficiently (Xavier, Paixã, den Hartigh et al. 2010). *Brucella* does not survive in neutrophils (Carvalho Neta, Mol, Xavier et al. 2010). *Brucella* enters epithelial cells through the involvement of the cytoskeleton. The intracellular trafficking is similar to that in phagocytes (Xavier, Paixã, den Hartigh et al. 2010). In trophoblasts the organisms multiply in the rough endoplasmic reticulum (Samartino & Enright 1993). The *Brucella* enters the cell with minimal changes to the structure of the cell and in fact promotes survival in its presence (Martirosyan, Moreno & Gorvel 2011, Godfroid, Bosman, Herr et al. 2004). *Brucella* can multiply intracellular without damaging the host cell. The extensive replication in the gravid uterus does lead to necrosis and subsequent inflammation (Godfroid, Bosman, Herr et al. 2004).

The survival of *Brucella* in the cell is strongly linked to the cell envelope that resists the cidal action of complement, lysozyme, phospholipases and lactoferrin. It also does not bind complement, microbicidal defensins, bactenecins, cathelicidins and catioinic bactericidal molecules (Martirosyan, Moreno & Gorvel 2011, Godfroid, Bosman, Herr et al. 2004).

3.5.2 Immune response to Brucella

3.5.2.1 Innate immunity

The innate immune response is responsible for the early recognition of pathogens with resulting pro-inflammatory and antimicrobial responses and is more intimately connected to the adaptive immune response than previously recognised (Mogensen 2009). One of the pathways is through Toll-like receptors that combine with the relevant pathogen associated molecular patterns (PAMP), triggering signalling pathways and the expression of genes (Mogensen 2009).

Brucellae are weak inducers of the innate immune system, and in the early phases of infection (incubation period), there is a lack of pro-inflammatory response (Martirosyan, Moreno & Gorvel 2011, Godfroid, Bosman, Herr et al. 2004). Contributing to this is the fact that the LPS do not have a marked PAMP and flagellums are absent (Palacios-Chaves, Conde-Alvarez, Gil-Ramirez et al. 2011). Internal molecules such as DNA and peptidoglycans with PAMP are also not presented to pathogen recognition receptors due to the high resistance of smooth brucellae to complement and bactericidal peptides (Palacios-Chaves, Conde-Alvarez, Gil-Ramirez et al. 2011). Inhibition also plays a role, since *B. melitensis* has been shown to secrete inhibitory homologues of the Toll/interleukin-1 receptor (TLR) domain (Cirl, Wieser, Yadav et al. 2008).

Brucella organisms establish themselves within phagocytic cells in this period, and only later, following stimulation of the innate immunity, does the adaptive immune system respond (Palacios-Chaves, Conde-Alvarez, Gil-Ramirez et al. 2011). The role of specific Toll-like receptors (TLR) in the

response to brucellae are still not clarified with certainty, but TLR 9 with bacterial DNA as a ligand, is considered to play the most important role (Oliveira, de Oliveira, Macedo et al. 2008).

3.5.2.2   Adaptive immunity

The adaptive immune response begins when TLR mediates maturation of dendritic cells and presentation of the antigen in association with major histocompatibility complexes to T-lymphocytes and the activation of pathogen specific T-lymphocytes (Mogensen 2009). The adaptive immune response is delayed, which could be related to the finding in *Brucella suis* in vivo that it inhibits the maturation of human dendritic cells and the expression of major histocompatibility complex classes I and II and impairs antigen presentation to T cells (Billard, Dornand & Gross 2007).

CD4+ and CD8+ T cells have been shown to play a role in the adaptive immune response to *Brucella*. Cellular immunity, mediated by CD8+/cytotoxic T cells are important for eliminating intracellular bacteria, while humoral immunity with production of antibodies by plasma cells, mediated by CD4+/T helper cells, are less important (ed. Parham 2009). T helper cell 1 is the most important cell, with the chief cytokine produced IFNγ (Carvalho Neta, Mol, Xavier et al. 2010).

By staying intracellular and compromising the function of CD8+ T cells, brucellae prevent destruction of the host cells by cytotoxic cells and it leads to fewer and less effective memory cells (Durward, Radhakrishnan, Harms et al. 2012).

B cells become antibody producing plasma cells after activation by pathogens in the presence of T helper cells (ed. Parham 2009). As time goes by, cells producing antibodies that bind more tightly to the pathogen are favoured. The class of immunoglobulin produced also change, from IgM that is produced in the initial phases to IgG in the lymph nodes and spleen and IgA in the Peyer's patches of intestinal mucosa and other mucosal associated lymphoid (ed. Parham 2009). This ensures that the antibody reaches the space where it is most needed, the blood stream for IgG and lumen of organs form IgA (ed. Parham 2009). The protective effect of antibodies against *Brucella* infection is questionable, but the diagnosis of brucellosis depends strongly on detection of antibodies (Carvalho Neta, Mol, Xavier et al. 2010).

The stimulation of the adaptive immune system relates to the time that clinical signs appear and delayed type hypersensitivity reactions are present during the chronic phase of infection (Martirosyan, Moreno & Gorvel 2011). This is why antibodies are only detected after abortion in 15% of the cases, or during the peri-parturient period for latently infected heifers born to infected mothers (Nicoletti 1980).

Most host species respond to LPS. Xin, Yang, Wang et al et al (2013:1415) found that there is a host and bacterial species variation in the development of antibodies against the 26-kDa cytosoluble protein (BP26) which is only expressed by *Brucella* in infections. They ascribed this to the difference

in pathogenicity of the bacteria and processing in the macrophages leading to immune response directed to surface and/or internal proteins (Xin, Yang, Wang et al. 2013). Some individuals fail to produce antibodies against LPS despite infection (Xin, Yang, Wang et al. 2013).

3.6 Diagnosis

The diagnosis of bovine brucellosis relies on laboratory testing. Direct tests demonstrate the presence of the organism through bacterial culture (golden standard) (Nielsen & Yu 2010) or polymerase chain reaction (PCR). Indirect tests measure levels of anti-*Brucella* antibodies or delayed type hypersensitivity (Gall & Nielsen 2004). The antibody tests commonly used detect antibodies against the smooth-LPS, more particularly the O side chain (Cardoso, Macedo, Azevedo et al. 2006).

The choice of a particular diagnostic assay depends on the objective, e.g. clinical diagnosis versus prevalence survey, the epidemiological situation and the availability of laboratory expertise (Godfroid, Nielsen & Saegerman 2010).

3.6.1   Serological diagnosis

Serological diagnosis of brucellosis is challenged by the inability of the commonly used serological tests to distinguish between the different smooth *Brucella* sp., the latent period and the longevity of the antibody reaction after recovery (Godfroid, Al Dahouk, Pappas et al. 2013, Treanor, Geremia, Crowley et al. 2011). Serological diagnosis should therefore rather be done on herd basis than on individual animals (Racloz, Schelling, Chitnis et al. 2013).

Most hosts have been shown to develop antibodies to LPS and therefore tests to detect LPS is widely utilised for brucellosis diagnostics (Xin, Yang, Wang et al. 2013).

Serological tests used can be divided in to conventional tests and primary binding tests. Conventional tests involve a secondary function in addition to the binding of the antibody to its antigen (Nielsen & Yu 2010). Conventional tests used are the serum agglutination test (SAT) with different modifications, milk ring test (MRT), Rose Bengal test (RBT) with modifications, agar gel immunodiffusion, radial immunodiffusion and complement fixation tests (CFT) with modifications (Nielsen & Yu 2010). The conventional tests are based on subjective interpretation of the reaction and are therefore more variable than the primary binding tests (Nielsen & Yu 2010). Primary binding assays used are radioimmunoassay, fluorescence immunoassay, particle counting fluorescence immunoassay, indirect immunoassay, competitive enzyme immunoassay, fluorescence polarization assay (Nielsen & Yu 2010). The brucellin skin test and gamma interferon production in response to antigenic stimulation of sensitised peripheral lymphocytes can be used to assess hypersensitivity (Nielsen & Yu 2010, OIE 2009a).

Tests prescribed by the OIE for use in international trade are the buffered *Brucella* antigen test (BBAT), complement fixation test (CFT), enzyme-linked immunosorbant assay (ELISA) and

fluorescence polarization assay (FPA) (OIE 2009a). The tests used in South Africa for control purposes in cattle is the RBT with or without SAT as a screening test and the CFT as a confirmatory test, while MRT is used to monitor dairies using bulk milk tank (Brucellosis Advisory Group 2013).

Gall and Nielsen (2004:989) reviewed literature on serological diagnosis of brucellosis and compared sensitivity, specificity and performance indexes for serological tests for bovine brucellosis. They found the conventional tests to be more variable than the primary binding tests, although the CFT showed improvement from 1995 onwards, which could be ascribed to implementation of quality assurance. Primary binding tests were found to be more sensitive and specific. Of all the tests evaluated, the FPA had the highest performance index, the diagnostic accuracy was as good as or better than the indirect and competitive ELISAs, is cheaper and easier, can be automated and quality assurance can be applied easily. It can be used in the field and laboratory. The FPA and competitive ELISA were reported to be able to distinguish between infection with field strain or vaccine strain (Gall & Nielsen 2004).

3.6.1.1   Antibody effect

The class of antibody detected by an assay must be considered. IgM is produced early in the infection, followed by IgG1, with IgG2 and IgA produced later. IgM is also the main antibody formed in response to the O-antigen. Several other gram negative bacteria share epitopes of the O-antigen of *Brucella* spp. Tests detecting IgM are therefore more sensitive, but also more prone to showing cross reactions. IgM is the main antibody responsible for agglutination. The agglutination tests can be modified in several ways to inhibit IgM activity and reduce false positives. IgG1 is considered to be the best antibody to target for testing. The RBT (buffered to final pH of 3.65 and CFT targets IgG1 (Nielsen & Yu 2010). Different ELISA assays target IgG or specific IgG sub-classes (Godfroid, Nielsen & Saegerman 2010). Godfroid, Nielsen Saegerman et al. (2010:302) illustrated the different outcomes possible with the SAT and IgG ELISA tests at different during the course of infection, with the SAT positive and ELISA negative early (three weeks) in the infection, going over to SAT and ELISA positive at four weeks, SAT negative and ELISA becoming negative after more than a year (Godfroid, Nielsen & Saegerman 2010).

3.6.1.2   Antigen effect

The antigen used in the assay is also important. Whole cells or LPS of *B. abortus* is generally used for serological tests in cattle which cross reacts with other smooth brucellae such as *B. melitensis, B. suis* and vaccine strains (Nielsen & Yu 2010). The O side chain is immunodominant and use of smooth strains or LPS will also detect antibodies against other micro-organisms that share epitopes (Nielsen & Yu 2010). Other options are using more *Brucella* specific proteins or rough LPS, but it is not applied commonly (Nielsen & Yu 2010).

3.6.1.3   False positives

False positive reactions remain a problem in bovine brucellosis serological testing and can be ascribed to vaccination with smooth strains, e.g. S19, cross reactions with other gram negative

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bacteria, e.g. *Yersinia enterocolitica, Salmonella, E. coli*, and unknown environmental factors (Nielsen & Yu 2010). There is no vaccine available for use in the African buffalo, so false positive results as a result of vaccination is not of concern (Godfroid, Nielsen & Saegerman 2010).

Solid phase tests are also more prone to false positive reactions due to non-specific binding, which can be overcome by the liquid-phase ELISA and FPA (Jolley & Nielsen 1999).

3.6.1.4   False negatives

False negative results can be ascribed to prozone reactions (e.g. RBT and CFT) and low levels of antibodies (Nielsen & Yu 2010). Infected bulls have been shown to be negative for antibodies in serum yet have antibodies in seminal plasma as demonstrated by a seminal plasma agglutination test (Lima-Ribeiro, Júnior, Moraes et al. 2008). Some hosts may fail to respond to the LPS antigen during an infection (Adone & Pasquali 2013).

3.6.1.5   Principle of the Rose-Bengal test

The Rose Bengal test is a conventional agglutination test using a whole cell *B. abortus* antigen stained with Rose Bengal and stored in an acid buffer (Nielsen & Yu 2010), (OIE 2009a). The test can be performed in a WHO haemagglutination plate and equal volumes (25-30µl) of the stained antigen and test serum are rocked at room temperature for two minutes and any sign of agglutination is recorded as positive. There is no quantification of the result.

Acidification reduces the false positive reactions (OIE 2009a, Rose, Roepke 1957) and encourages IgG1 activity (Nielsen & Yu 2010). The RBT detects IgM and IgG, especially IgG1 (AU IBAR 2013).

D. Gall and K Nielsen (2004:993) reported a mean sensitivity of the RBT of 81,2% and mean specificity 86,3%, and the mean performance index 167,6 with a standard deviation of 24,8% (Gall & Nielsen 2004). The lack of standardisation of the antigen for the RBT and its susceptibility to deterioration with repeated change in temperatures contributes to the variability of results with the test (Gall & Nielsen 2004). False positive results still occur with vaccination and cross reactions, and false negative results can be seen as a result of pro-zoning (Nielsen & Yu 2010).

3.6.1.6   Principle of the complement fixation test (CFT)

The complement fixation test (CFT) is another conventional test widely used for the confirmation of brucellosis (OIE 2009a). The test is based on the principle that activation of the complement system by antigen-antibody complexes in the presence of red blood cells will lead to haemolysis of the red blood cells, which can be appraised visually. Reagents are titrated to according to standard specifications, and serial dilutions are tested, making it possible to calculate the titre. Complement in the test serum is heat inactivated before the addition of the whole cell *Brucella* CFT antigen and incubation – cold or warm – to allow the complement cascade to occur if anti-*Brucella* antibodies are present – so-called complement fixation. The haemolytic system – usually a suspension of sheep red

blood cells and anti-sheep red blood cell serum is added. Complement that were not fixed during incubation of the test serum with *Brucella* CFT antigen, will be activated and cause haemolysis of the red blood cells. Remaining red blood cells sink to the bottom of the wells. U-shaped wills are used and it forms a button on the bottom off the well. The degree of haemolysis are assessed visually by the naked eye and scored by the analyst (AU IBAR 2013, Nielsen & Yu 2010, Godfroid, Nielsen & Saegerman 2010, OIE 2009a).

IgM and IgG1 fix complement, but the heat inactivation step destroys the IgM component (AU IBAR 2013, Godfroid, Nielsen & Saegerman 2010, Nielsen & Yu 2010). Prozone reactions occur when there is high concentration of IgG2 that interferes with the binding of IgG1 with the antigen (Nielsen & Yu 2010). Anti-complementary reactions occur when there are non-specific factors in the test serum that fix the complement, and an anti-complementary control without the *Brucella* CFT antigen needs to be included for every test serum (OIE 2009a).

The CFT is therefore a complicated, labour intensive test that is not easily standardised (Godfroid, Nielsen & Saegerman 2010, Nielsen & Yu 2010).

D. Gall and K Nielsen (2004:993) reported a mean sensitivity of the CFT of 89% and mean specificity 83,5%, and the mean performance index 172,5 with a standard deviation of 24,3% (Gall & Nielsen 2004). Others reported higher diagnostic specificity of 99.7% - 99.9%, and the CFT is used widely for the confirmation of brucellosis (Godfroid, Nielsen & Saegerman 2010).

3.6.1.7 Principle of the indirect enzyme linked immunosorbant assay (iELISA)

The iELISA is a primary binding assay that detects and quantifies antibodies. It is a rapid test that can be automated. A 96 well plate is coated with the antigen. Serum samples are added and antibodies if present will bind with the antigens. The plate is washed and a conjugate consisting of an antibody against the immunoglobulins of the relevant species labelled with an enzyme. After another wash step the substrate (chromogen) is added and if enzyme is present, it will cause a colour reaction which can be measured and quantified (IDEXX n.d.).

Gall and Nielsen (2004: 993) reported a mean sensitivity of the ELISA for bovine brucellosis in cattle as 96.0% and mean specificity 93.8% and the mean performance index 189.8% with a standard deviation of 18.1% (Gall & Nielsen 2004). False positive reactions occur with vaccination with S19 and cross reactions. Commercial kits are available for testing cattle serum and milk (Nielsen & Yu 2010).

With the iELISA antibody against the immunoglobulins of the relevant species can be replaced with protein A, protein G or recombinant protein A/G and labelled with an enzyme as detection reagent and be used in multiple species (Nielsen, Smith, Yu et al. 2005), for example arctic wildlife, which includes cetaceans, carnivores and ungulates (Nymo, Godfroid, Asbakk et al 2013). Species differ in

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their affinity for binding of the Fc region of antibodies to protein A, G or A/G (Nymo, Godfroid, Asbakk et al 2013

3.6.1.8   Principle of the fluorescence polarisation assay (FPA)

The FPA is also a primary binding assay for detection and quantification of *Brucella* antibodies. Molecules in solution rotate, and the larger the molecule, the slower the rotation will be. The degree of depolarisation of polarised light by rotating fluorescing molecules, changes with the speed of rotation. In a FPA, antibody levels are quantified by attaching a fluorescing molecule to an antigen. When the labelled antigen attaches to antibody in the test sample, the complex will rotate slower proportionally to the amount of antigen present. The resultant change in degree of depolarisation is measured (Nielsen & Yu 2010, OIE 2009a).

The antigen used in FPA for bovine brucellosis is prepared from *B.* abortus S1119.*3* hydrolysed to an average molecular weight of 22 kd, purified and conjugated with fluorescein isothiocyanate (Nielsen, Gall, Smith et al. 2001, Jolley & Nielsen 1999).

The FPA do not involve a solid phase contributing to high specificity (Jolley & Nielsen 1999).

The assay is simple to do, and 96 well plate or single tube devices for field use can be used. No washing is necessary, and incubation time is a minimum of 2 minutes for blood or serum and maximum of 15 seconds for blood (Nielsen & Yu 2010, OIE 2009a). The sample is diluted by a buffer and the degree of depolarisation of polarised light determined. The conjugated antigen is added and after the incubation period a second reading taken and the difference calculated. The results are expressed in millipolarization units (Nielsen, Gall, Smith et al. 2001). Bulk milk tank samples can also be tested by FPA (Godfroid, Nielsen & Saegerman 2010, Gall & Nielsen 2004), but with slightly lower sensitivity (Godfroid, Nielsen & Saegerman 2010).

False positive reactions as result of vaccination with S19 can occur (Diachemix 2010) which can be addressed by adjusting the cut-off value (Nielsen & Yu 2010). Cross reactions can also be distinguished from infection (Nielsen & Yu 2010).

Gall and Nielsen (2004: 993) reported a mean sensitivity for bovine brucellosis of FPA on serum as 97.5% and mean specificity 98.9%, and the mean performance index of 196,4, with a standard deviation of 4,4% (Gall & Nielsen 2004). The mean performance index of 196.4 is the highest of all tests reviewed (Gall & Nielsen 2004). Expensive equipment is required, but it can be used for many tests (Nielsen & Yu 2010) and it was found to be the least expensive (Gall & Nielsen 2004). Commercial kits are available and can be automated (Nielsen & Yu 2010). These characteristics makes the FPA a test of choice and it has been developed/evaluated/validated for several species, e.g.: cattle (Dajer, Luna-Martinez, Zapata et al. 1999, Nielsen, Gall, Jolley et al. 1996), bison (Gall,

Nielsen, Forbes et al. 2000), goats (Nicola, Elena, Alonso et al. 2010), caribou, elk, red deer, reindeer (Gall, Nielsen, Forbes et al. 2001), sheep and pigs (Nielsen, Gall 2001).

3.6.2   Bacterial culture and identification

Bacterial culture and identification remains the gold standard (Nielsen & Yu 2010, OIE 2009a). The success of bacterial culture depends on the correct handling and storage of samples, prevention of contamination, sampling the correct sites in adequate numbers, having an adequate size of innoculum, culture techniques used and the experience of the laboratory personnel (Gall & Nielsen 2004). In clinical cases, the specimens will be according to the signs: foetal stomach contents, spleen, lung and membranes, vaginal secretions, milk, semen, joint or hygroma fluid (OIE 2009a). Corner et al. (1987:243) found that in cattle, 7 specimens have to be cultured to detect all infected cows, and 9 specimens in heifers post mortem. In cows these are: mammary tissue, parotid, mandibular, and subiliac lymph nodes (Corner, Alton & Iyer 1987). A more recent study demonstrated the usefulness of culturing paired samples of the retropharyngeal, supramammary and internal iliac in cows from herds with active brucellosis culture in slaughtered animals, satisfactorily identifying animals that are in early seronegative and later seropositive stages of infection satisfactorily (O'Grady, Byrne, Kelleher et al. 2014).

Stamp's staining, which is based on *Brucella* organisms' ability to resist decolourisation by weak acids, is commonly used as a preliminary tests on specimens, but lack sensitivity and specificity (OIE 2009a).

Milk can be centrifuged and tissues must be thoroughly minced and macerated to increase the sensitivity of isolation (OIE 2009a, Ewalt 1989).

Culture and isolation of *Brucella* organisms are done on solid media, or biphasic media for blood, body fluids and milk. Serum needs to be added to ensure growth of *B. abortus* biovar 2. Media can be prepared to be more selective by the addition of antibiotics, but this may at the same time decrease the sensitivity for certain *B. abortus* and *B. melitensis* strains (OIE 2009a). Use of two to four different selective media is advocated (OIE 2009a, Ewalt 1989). Enrichment media should be used for specimens where low numbers of organisms are suspected such as milk, colostrums and tissue samples other than abortion material (OIE 2009a).

Incubation is done in an atmosphere of 5-10% CO_2 at 37º C. *B. abortus* is dependent on CO_2 for growth with few exceptions, but *B. melitensis* is not. *Brucella* grows slowly and colonies can be seen after 3-5 days. The OIE allows discarding of cultures after eight to ten days, but some growth may only become visible after 28 days (OIE 2009a, Ewalt 1989).

Colonies are examined morphologically and can be stained with crystal violet to detect dissociation into rough forms, which may affect the outcome of further tests. The staining characteristics with

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Gram and or Stamp's stain and organism morphology, biochemical tests (urease, oxidase and catalase tests) and serological tests (slide agglutination with anti-*Brucella* polyclonal serum) can identify it as *Brucella* spp. Further identification to species and biovar level requires further biochemical testing (CO_2 requirement for growth, production of H_2S, growth in presence of basic fuchsin and thionin), serological testing and phage lysis. Nucleic acid recognition methods can also be used on culture to identify the species and biovar (OIE 2009a).

Contamination is a serious limiting factor for successful isolation and correct specimen handling is critical (OIE 2009a, Ewalt 1989) and sensitivity can be improved by inoculating more plates per specimen (O'Grady, Byrne, Kelleher et al. 2014).

3.6.3 Nucleic acid recognition methods

Several nucleic acid recognition methods have been developed for *Brucella* spp. and can be used to identify *Brucella* to species level, biovar level to some extent, and distinguish wild strains from vaccine strains. These are PCR, PCR restriction fragment length polymorphism, pulse-field gel electrophoreses and multilocus analysis of variable tandem repeats (MLVA) (OIE 2009a, Ewalt 1989). These methods, especially MLVA, can, and will in future more and more, contribute to epidemiological analysis of the disease (Godfroid, Nielsen & Saegerman 2010, OIE 2009a, Mnisi, Michel, Godfroid et al. 2008).

PCR can be used for primary diagnosis, but there is limited validation and the performance index is low (Gall & Nielsen 2004), and is more useful in identification of cultured bacteria (Mnisi, Michel, Godfroid et al. 2008). The specificity is close to 100% (Godfroid, Nielsen & Saegerman 2010). It has however been shown to be that PCR on semen can be more sensitive than bacterial culture (Kanani, Lata Jain, Patel et al. 2008).

Sample handling, storage and contamination will affect the outcome of PCR tests (Gall & Nielsen 2004).

The AMOS PCR assay is an assay that can be used to identify and differentiate *Brucella abortus* (biovars 1, 2 and 4), *B. melitensis* (biovars 1, 2 and 3), *B. ovis* and *B. suis* (biovar 1). From this the *Brucella abortus* species–specific polymerase chain reaction assay was developed for use in cattle to differentiate *Brucella abortus* (biovars 1, 2 and 4) and S19 and RB51 vaccine strains from other *Brucella* sp. and non *Brucella* bacteria (Mayer-Scholl, Draeger, Goellner et al. 2010, Bricker, Ewalt, Olsen et al. 2003). There is also the multiplex "Bruce ladder" PCR test that can differentiate all the known *Brucella* species, including vaccine strains (Mayer-Scholl, Draeger, Goellner et al. 2010, LóPez-Goñi, García-Yoldi, Marín et al. 2008).

3.6.4   Tests for cell-mediated immunity

The brucellin skin test, that assess delayed type hypersensitivity in the skin and interferon-gamma tests for detection of lymphocyte response in blood can be used, especially where false positive serological reactions are important, but are not recognised by the OIE (OIE 2009a, Bercovich, Muskens 1999, Kittelberger, Bundesen, Cloeckaert et al. 1998).

3.6.5   Diagnosis of brucellosis in African buffalo

3.6.5.1   Serological diagnosis

The same antigens used for livestock are used for serological testing of wildlife for brucellosis to distinguish between exposed and non-exposed populations or individuals, due to the cross reaction between species which contain smooth LPS (Godfroid, Nielsen & Saegerman 2010)(Godfroid, Nielsen & Saegerman 2010).

Serological surveys for bovine brucellosis in African buffaloes are based on serological tests developed for cattle.   Initially mainly the conventional tests were used surveys (Paling, Waghela, Macowan et al. 1988, Herr, Marshall 1981, de Vos & van Niekerk 1969, Staak, Groocock 1968, Roth 1967, Rollinson 1962) and later also enzyme linked immunosorbant assays (Fyumagwa, Wambura, Mellau et al. 2009, Hamblin, Anderson, Jago et al. 1990).   None of these tests have been fully validated in African buffalo but demonstrate that African buffaloes do develop antibodies against LPS and that the antibodies are detected with the tests developed for cattle.

Diagnostic sensitivity and specificity of serological tests differ between different domestic and wildlife species (Bengis, Kock & Fischer 2002, Madsen & Anderson 1995) and it is possible that the sensitivity and specificity of African buffaloes will not be the same than that of cattle.

3.6.5.2   Bacterial isolation of *Brucella abortus*

Procedures recommended for livestock was used for the isolation of *B abortus* biovar 1 out of cotyledons and carpal hygromas and *B. abortus* biovar 3 from testis (Directorate of Veterinary Services, Subdirectorate Animal Health 1987, Gradwell, Schutte, Van Niekerk et al. 1977, Directorate Veterinary Services, Subdirectorate Animal Health 1976).

3.6.5.3   Differential diagnosis

Rift Valley Fever (Reininghaus 2009) and listeriosis (Chukwu, Muhammad, Nwankpa et al. 2006) were reported as causes of abortion in African buffaloes. Abortions seen in African buffaloes when they are handled repeatedly have been ascribed to stress (I. Fick pers. comm. 30 June 2014).

3.7 Diagnostic assay development and validation

The development and validation of diagnostic assays should ideally be according to host species and causative agent (Hueffer, Gende & O'Hara 2013, Meegan, Field, Sidor et al. 2010, OIE 2009a).

The development and validation of diagnostic assays are continuous processes, and continue as long as the assay is used (OIE 2010). A properly developed assay is an assay that is: fit for the intended purpose; optimised to ensure that the performance characteristics is best suited to the intended application; standardised by calibration to standard reagents and robust in the face of small variations in method parameters (OIE 2010).

Once the assay has been developed, the assay is validated through determining the analytical and diagnostic performance and reproducibility and then the assay can be considered as validated for the original intended purpose(s) and can be implemented (OIE 2010).

The analytical test characteristics are defined as follows in the OIE Terrestrial Manual 2010: Repeatability: the level of agreement between results of replicates of a sample both within and between runs of the same test method in a given laboratory. Analytical specificity is the degree to which the assay distinguishes between the target analyte and other components that may be detected in the assay. Limit of detection, or analytical sensitivity, is the smallest amount of analyte in a sample that can be detected by a direct detection assay in at least 50% of the replicates for each dilution, in a dilution series (OIE 2010).

Diagnostic performance of an assay is indicated by the diagnostic sensitivity and specificity, which is defined in the OIE Terrestrial Manual as follows: Diagnostic sensitivity is the proportion of samples from known infected reference animals that test positive in an assay. Diagnostic specificity is the proportion of samples from known uninfected reference animals that test negative in an assay (OIE 2010).

Diagnostic performance of an assay is relative to the population under study, the conditions under which the assay is performed, and interpretations made. The sensitivity in the individual animal may be influenced by the stage of infection or immunity of the host, and the mix of cases tested may impact on the sensitivity (Greiner, Gardner 2000). Other factors that have been shown to have an impact on sensitivity are whether the disease is endemic or epidemic, the prevalence of the disease in the herd, time of sampling (Greiner, Gardner 2000). Diagnostic specificity in serology may be affected by components in the serum other than the analyte targeted, such as antibodies of cross-reacting pathogens and rheumatoid factors. Diagnostic specificity may be affected by presence of maternal antibodies, persistence of antibodies after recovery or vaccination, depending on the purpose of the testing. Different exposure of different populations to these factors will lead to differences in the performance of the assay between populations. Time of sampling during an epidemic may also affect

specificity, and an increase can occur with the concurrent removal of false- and true positive animals, and a decrease if antibodies persist but infectious agent no longer present (Greiner, Gardner 2000).

Several methods can be employed to determine the diagnostic performance of an assay. Standardised sensitivity and specificity can be determined using national or international serum panels, but operational test parameters determined within an epidemiological perspective are more applicable to animal populations. Internal validity (unbiased for the animal population under study) must be considered when sampling strategy is planned, blinding should be applied and retesting of animals with unexpected results should not be done unless for technical reasons. Extrapolation of the diagnostic sensitivity and specificity must be carefully considered as the external validity may be affected by factors such as prevalence in the population (Greiner, Gardner 2000).

Typically, the diagnostic sensitivity and specificity is determined by testing samples from animals with known status. The status can be completely or partially verified with a "gold standard." The samples are categorised and the results tabulated in a 2X2 table (OIE 2010, Greiner, Gardner 2000). The sensitivity can be estimated as the true positives, divided by the sum of true positives and false negatives. The specificity can be estimated as the true negatives divided by the sum of the true negatives and false positives. Corrections for bias need to be done with partial verification of status (Greiner, Gardner 2000).

Sampling can be cross-sectional, with or without stratification, or convenience sampling can be used. Convenience sampling includes experimental studies and makes use of e.g. serum samples submitted to laboratories for testing, or obtained from serum banks. With experimental studies, care must be exercised with extrapolation to populations. Submission based sampling may be representative of the population but not necessarily (Greiner, Gardner 2000). Sample size needs to be adequate to obtain adequate confidence in the results. Formula for calculation size is provided by Greiner and Gardner, or can be read from a table in the OIE Terrestrial Manual 2010 (OIE 2010, Greiner, Gardner 2000). Due to the constraints with validation studies in wildlife, the OIE accepts smaller numbers of reference samples for preliminary validation (OIE 2014).

Clae Enøe (2000:63) and other describe methods (latent class models) to estimate diagnostic sensitivity and specificity in the absence of knowledge about the true disease status, which necessitates the use of complicated statistical concepts and formula. It relies on the use of two (or in some cases more) tests, where the diagnostic sensitivity and specificity of one test is imperfect but known, or the sensitivity and specificity of both tests are unknown (Enoe, Georgiadis & Johnson 2000).

Where the accuracy of one test is known, the assumption is made that the classification errors with the two tests are independent and this is very seldom the case (Enoe, Georgiadis & Johnson 2000).

Where the accuracy of both tests is unknown, populations with different disease prevalence must be tested, and apart from the assumption that there is conditional independence between the tests, the assumption is also made that the accuracy of each test will be the same in both populations (Enoe, Georgiadis & Johnson 2000).

An example of the estimation of serological tests in the absence of a gold standard by a Bayesian approach and assuming conditional independence of tests, is the study by G. T. Fosgate and others 2002 where two water buffaloes and two cattle herds known to be *Brucella* infected and two water buffaloes and 2 cattle herds considered brucellosis free were tested using the standard plate agglutination, card, buffered plate agglutination and standard tube agglutination tests (Fosgate, Adesiyun, Hird et al. 2002).

The diagnostic sensitivity and specificity relates to the cut-off values used when categorising animals as positive/infected or negative/not infected, and it must be reported as such. Receiver-operator characteristics (ROC) analysis can be used to assist with the selection of cut-off values. Sensitivity and specificity plotted as a function of the cut-off values demonstrate the relationship visually. Factors to take into account when deciding on a cut-off point are e.g. the purpose of the test, epidemiological data such as the prevalence of disease, and the impact of misclassification. An intermediate category, where the test result is considered neither negative nor positive can be included (Greiner, Pfeiffer & Smith 2000).

The OIE may acknowledge a validated assay as a prescribed or alternate test for trade purposes based on their usefulness on national, regional or international basis. The assay can also be included in the OIE register after being certified as fit for a specific purpose(s). A Standard Operating Procedure (SOP) for OIE Validation and Certification of Diagnostic Assays is available on the website of the (OIE 2009b).

After implementation, the validation status of an assay is maintained during the application of that assay in different laboratories or field conditions through the continuous monitoring by e.g. quality control, proficiency testing, re-evaluations after modification or replacement of agents (OIE 2010).

3.7.1   Use and validation of diagnostic assays for use in wildlife

Diagnostic performance of assays may differ between livestock and wildlife due to possible differences in the prevalent pathogen strain, the response of the wildlife host to infection and the presence of cross-reaction organisms infecting wildlife species (Gardiner, Hietala & Boyce 1996).

There are several ways that assays can be developed to overcome the problem of species-specificity. Monoclonal antibodies against immunoglobulins can cross react with immunoglobulins of other species, and the closer the phylogenetic relationship between species, the higher the degree of cross-reaction (Henning, Nielsen 1992, Jefferis, Lowe, Ling et al. 1982). Examples of cross reactions

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between ruminant species demonstrated are between: cattle and bison (Henning, Nielsen 1992), deer and cattle (Chintu Ravishankar, Nandana, John et al. 2012), small ruminants and cattle (Alonso-Urmeneta, Marin, Aragon et al. 1998), cattle and camels (Gwida, El-Gohary, Melzer et al. 2011) and cattle and water buffalo (Chand 2003). Applying ELISA with anti-bovine IgG peroxidase conjugate to water buffalo may lead to inaccurate (lower) results (Chand 2003).

Another method enabling the development of multispecies ELISA test is the use of protein A ,G or AG that interact immunoglobulin G of various mammals. The chimeric protein AG is effective in African buffalo (Kelly, Tagwira, Matthewman et al. 1993).

Competitive or blocking ELISAs are not species specific.

Validation is required in every species, with adaptation of tests where necessary, due to differences between species which does not appear to follow phylogeny (Kramsky, Manning & Collins 2003).

The OIE requires that serological tests for brucellosis should be validated in every species they are used, but in many cases this are not done (OIE 2010, OIE 2009a). They recognised the challenges associated with validation of diagnostic assays in wildlife and will therefore provisionally recognise diagnostic tests for use on a regional or international basis validated with smaller numbers of samples than what is required for domestic animals (OIE 2014). Where there is no validated test in a related species, determination of diagnostic sensitivity and specificity and determination of cut-off values must be based on a minimum of 30 positive and 30 negative reference samples. Where there is a validated test available in a related species, only 10 positive and 10 negative reference samples are required (OIE 2014).

3.7.2 Use of serological assays for antibody detection developed for cattle in African buffalo

An evaluation of the CFT, RBT and commercial cattle iELISA on the KNP population of African buffaloes by Bayesian inference and latent class analysis was published recently, which found these tests suitable for use (Gorsich, Bengis, Ezenwa et al. 2015).

There is prior evidence that anti-ruminant IgG will bind with African buffalo IgG in ELISAs:
- Anti-sheep IgG produced in a rabbit was used successfully in an iELISA to detect antibodies against Rift Valley fever virus in African buffaloes (Paweska, Vuren, Kemp et al. 2008).
- Hamblin, Burnett and Hedger (1990:586) did a serological survey in several game species in Tanzania, using serum collected for rinderpest surveillance. He used an iELISA for *B. abortus* with rabbit anti-bovine IgG (Hamblin, Anderson, Jago et al. 1990, Hamblin, Burnett & Hedger 1986).
- Dewals, Gillet, Gerdes et al. (2005:212) used in-house developed immunofluorescence assays to detect antibody against bovine herpes virus 4 in cattle and African buffalo using rabbit anti-cow immunoglobulin (Dewals, Gillet, Gerdes et al. 2005).

There is also evidence that the conventional serological tests for brucellosis will detect anti-*Brucella* antibodies in African buffaloes:

- Condy and Vickers (1972:175) infected buffalo, duiker, impala, kudu and wildebeest with *Brucella abortus* biovar 1, and tested them with the serum agglutination test at monthly interval. In the two buffalo infected, they found that the SAT titre rise after infection for a short period, and then falls below what is considered as positive in cattle. They recorded any reaction as positive (Condy & Vickers 1972).

- Kaliner, Staak and Daliner (1973:151) found CFT tires was 1:100 and the serum agglutination content 31.25i.u. in a buffalo from which *Brucella abortus* biovar 3 was isolated (Kaliner, Staak & Daliner 1973).

- Waghela and Karstad (1986:190) tested 17 buffalo with the CFT, Rose Bengal plate test (RBPT), and SAT and found seventeen positive with the CFT, with five of them also positive with the RBPT (Waghela, Karstad 1986).

- Madsen et al. (1995) used the RBT, SAT and CFT for bovine brucellosis in African buffalo in Zimbabwe and considered the performance of the test to be apparently similar to that in cattle, with higher numbers of positive results in the RBT and SAT than the CFT (Madsen & Anderson 1995).

- Fyumagwa, Wambura, Mellau et al. (2009:64) tested African buffalo serially with the RBT and cELISA (Fyumagwa, Wambura, Mellau et al. 2009).

- Wolhuter, Bengis, Reilly et al. (2009:503) used the RBT and SAT for bovine brucellosis, but does not describe interpretation of results (Wolhuter, Bengis, Reilly et al. 2009).

- Gomo, Garine-Wichatitsky, Caron et al. (2012:78) used RBT and cELISA on African buffalo. RBT scores of 2 and 3 were considered as positive. They did not report reactions to the RBT test, although RBT positives were followed up with cELISA, and none out of 47 were classified as positive. The cELISA used was interpreted according to manufacturer's instructions (Gomo, Garine-Wichatitsky, Caron et al. 2012).

False positive serological reactions due to vaccination, is a constraint for the use of serological tests in cattle. A vaccine is not available for African buffaloes and cross reactions with vaccine strain is not of importance (Godfroid, Nielsen & Saegerman 2010).

4   MATERIALS AND METHODS

4.1   Study and experimental design

The diagnostic performance of four serological assays for bovine brucellosis in African buffaloes, namely the Rose-Bengal test (RBT), complement fixation test (CFT), indirect enzyme-linked immunosorbant assay (iELISA) and fluorescence polarisation assay (FPA) were evaluated individually, and compared in a case-control study of infected and uninfected composite reference serum panels. The study followed stage two of the OIE assay validation pathway for validation of diagnostic tests applicable to wildlife where there is a validated test in a related species (OIE 2014, OIE 2013) (Figure 4-1).

Sampling was based (Greiner, Gardner 2000). All sera received from four infected and two uninfected herds were analysed by the four index tests. Sera were excluded based on predetermined exclusion criteria and the infected and uninfected composite reference panels selected out of the remaining sera through the application of composite reference standards (Reitsma, Rutjes, Khan et al. 2009, Alonzo & Pepe 1999, Jacobson 1998). Diagnostic performance characteristics, based on cut-off values for cattle, were determined using 2X2 tables and OIE definitions for diagnostic sensitivity and specificity (OIE 2013). Receiver-operating characteristics (ROC) analyses for diagnostic tests were performed and cut-off values adjusted (Greiner, Pfeiffer & Smith 2000) to increase sensitivity or specificity. Fitness for purpose was determined according to recommendations by the OIE (OIE 2014, OIE 2013). Diagnostic performance of different test combinations, in series and parallel, were determined.

4.2   Recruitment of herds

South African veterinarians and researchers in the KNP were requested to submit specimens from African buffaloes, particularly from herds with known brucellosis status. This was done in person, by direct e-mail, or via the electronic discussion groups RuralVet and Wildlife VetNet,

Four state veterinarians, one veterinary pathologist from the Faculty of Veterinary Science, University of Pretoria (UP), one private wildlife veterinarian and one research team studying disease ecology in the KNP responded to the call for specimens. All specimens they provided were included in the study. The specimens from the seven contributors came from five different buffalo herds. Populations that were located in the same environment (epidemiological unit) were considered as one herd for the purpose of this study, e.g. the Greater Kruger National Park and the Hluhluwe-iMfolozi Park.

African buffalo sera from the Hluhluwe-iMfolozi Park, collected in 2010, were retrieved from the serum bank of the Department Veterinary Tropical Diseases, Faculty of Veterinary Sciences, UP.

In total sera were received from 353 animals in the six herds, as well as specimens for bacterial isolation from 85 animals from two herds. One hundred and fifty one animals were adult, 58 sub-adult, 26 juveniles, 2 calves and 118 of unknown age, where adults are ≥ six years, sub-adults > two to , six years, juveniles one to two years and calves under one year old. Fifty one were female, 59 male and 243 of unknown sex (Table 4-1).

```
┌─────────────────────┐      ┌─────────────────────┐
│   Infected herds    │      │  Uninfected herds   │
└─────────────────────┘      └─────────────────────┘
           │                            │
           ▼                            ▼
┌────────────────────────────────────────────────────┐
│        Tests:  RBT, iELISA, CFT, FPA               │
└────────────────────────────────────────────────────┘
                        ⇩
┌────────────────────────────────────────────────────┐
│                  Raw test data                      │
└────────────────────────────────────────────────────┘
           │                            │
           ▼                            ▼
┌─────────────────────┐      ┌─────────────────────┐
│  Infected composite │      │ Uninfected reference│
│   reference panel   │      │        panel        │
└─────────────────────┘      └─────────────────────┘
           │                            │
           ▼                            ▼
┌────────────────────────────────────────────────────┐
│  1.  Determine diagnostic test performance at      │
│      approved cut-off values for cattle.           │
└────────────────────────────────────────────────────┘
                        ▼
┌────────────────────────────────────────────────────┐
│  2. Apply alternative cut-offs to                   │
│     a. increase sensitivity                         │
│     b. increase specificity                         │
└────────────────────────────────────────────────────┘
                        ▼
┌────────────────────────────────────────────────────┐
│           3. Determine fitness for purpose          │
└────────────────────────────────────────────────────┘
                        ▼
┌────────────────────────────────────────────────────┐
│ 4. Determine performance for different test combinations │
└────────────────────────────────────────────────────┘
```

Figure 4-1 Flow-diagram of experimental design

Table 4-1 Origin, age and sex of animals from which sera were received

Herd	Age	Sex	Number
A	unknown	unknown	101
B	juvenile	male	10
C	all	male and female	175
	adult	male and female	134
		female	9
		male	11
		unknown	114
	sub-adult	male and female	29
		female	4
		male	10
		unknown	15
	juvenile	male and female	4
		female	2
		male	2
	calf	female	2
	unknown	unknown	6
D	unknown	unknown	4
E	all	male and female	57
	adult	female	11
	sub-adult	male and female	29
		female	12
		male	17
	juvenile	male and female	12
		female	6
		male	6
	unknown	male and female	5
		female	2
		male	3
F	all	all	7
	adult	male and female	5
		female	3
		unknown	1
	unknown	unknown	2
Total	calf		2
	juvenile		26
	sub-adult	male and female	58
	adult		149
	unknown		118
		female	51
	all ages	male	59
		unknown	243
		Grand Total	**353**

4.2.1 Uninfected herds

4.2.1.1 Herd A

Herd A was from the Hluhluwe-iMfolozi Park (HiP), a provincial nature reserve in the Hlabisa Local Municipality of KwaZuluNatal, geographic co-ordinates -28.2694365°S, 31.8359991°E. The African buffalo population estimate in 2010 was 5 500 (Cooper 2015). All sub-populations of African buffaloes in the HiP were considered to be free of brucellosis based on serological surveillance from 1997 to 2012. A total of 7 007 buffalo sera were tested in this period, and only one was positive with the RBT, but negative to the CFT (Cooper 2014). One hundred and ten archived sera collected randomly during the 2010 annual bovine tuberculosis testing were accessed for this study. No animal information was available for the sera.

4.2.1.2 Herd B

Herd B was located on a private farm in the Thaba Chweu Local Municipality of Mpumalanga, geographic co-ordinates of nearest town 25.108743°S, 30.451095°E. The herd was established in 2010, when 49 African buffaloes were bought from disease free breeding projects. From 2011 to 2014, a total of 24 African buffaloes from the herd underwent pre-movement testing. RBT, serum agglutination test and CFT were done by the ARC-OVI and no reactions were seen. Other evidence of freedom from brucellosis was absence of clinical signs and no detection of bovine brucellosis on neighbouring cattle farms with regular testing (J. Engelbrecht pers. comm. 10 December 2014). Sera were accessed from ten buffaloes that were tested before movement in January 2013 (M. Malan, pers. comm.).

4.2.2 Infected Herds

4.2.2.1 Herd C

Herd C was from the Greater Kruger National Park Complex (GKNPC), which includes the national nature reserve Kruger National Park (KNP) and private nature reserves to the west, with free movement between them. The African buffalo population estimate for the Kruger National Park for 2010/11 was 37 130 (SanParks n.d.) African buffalo herds in the Kruger National Park are known to be infected with *B abortus* and the disease is considered to have reached endemic stability (Bengis 1998).

Specimens were accessed from African buffaloes that were culled in a longitudinal, cross-sectional study (E. Gorsich pers. comm. 2 August 2012): One hundred and thirty four sera were from buffaloes culled in 2012 in the south of the KNP (31.3483105°E 24.4186941°S) near Lower Sabie and Crocodile Bridge (Gorsich 2015), and 40 sera were from animals culled in 2013 in the central (31.60163°E 24.97427°S and 31.73365°E 24.83683°S) and northern (31.41286°E 23.07122°S and

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31.33006°E 23.23694°S) KNP (L. M. De Klerk-Lorist pers. comm. 8 August 2013). Specimens for bacterial isolation were received from 83 animals and were submitted to the ARC-OVI for isolation. The specimens were collected on a non-random basis, with some selected based on previous positive results for brucellosis on RBT, CFT or iELISA (Gorsich 2012).

One serum sample was from an African buffalo that was trophy hunted in 2013 in a private reserve of the GKNPC in Mpumalanga, geographic co-ordinates 31.3583548°E and 24.3619335°S. It had hygromas from which *B. abortus* biovar 1 was isolated by the ARC-OVI (B. Reininghaus pers. comm. 25 July 2013).

4.2.2.2   Herd D
Herd D was located in the Emnambithi Ladysmit Local Municipality in KwaZuluNatal, geographic co-ordinates of nearest town 29.77028°E 28.78722°S. Bovine brucellosis was suspected in this herd of fourteen following the detection of two seropositive cows during pre-movement testing in 2012 (McKernan 2012). The confirmation of *B. abortus* infection was through the isolation of *B. abortus* biovar 1 by the Allerton Provincial Veterinary Laboratory from one seropositive cow that was culled (S. McKernann pers. comm 5 May 2013). Four sera, one from the serologically positive cow culled in March 2013, and three collected in April 2013 during whole herd sampling, were accessed from the Allerton Provincial Veterinary Laboratory (M. Lukubisa pers. comm. 5 June 2013 and S. Chisi pers. comm. 6 June 2013).

Bacteriology results were accessed from Allerton Provincial Veterinary Laboratory for the cow culled in March 2013. *B. abortus* biovar 1 was isolated from eight lymph nodes (Chisi 2014).

4.2.2.3   Herd E
Herd E was from the Moqhaka Local Municipality in the Free State, geographical co-ordinates of nearest town, 27.0681421°E 27.0681421°S. The herd was established in 2008/09 and consisted of 57 animals in June 2014. Brucellosis was first suspected in December 2013 when an abortion occurred. In May 2014 another four cows showed evidence of abortion or perinatal death of a calf, and five animals were tested for diagnostic purposes (Owner pers. comm. 30 June 2014, van Zijl 2014). Three cows, together with one's male calf, were found serologically positive with RBT and CFT with *B. abortus* biovar 1 was isolated by the Kroonstad Veterinary Laboratory from the vaginal swab of one of the seropositive cows that had a haemorrhagic vaginal discharge (Van Zijl 2014). In June and early July 2014 there were another two abortions, with isolation of *B. abortus* biovar 1 from one of the placentas (Owner pers. com 30 June 2014, D. Janse van Rensburg pers. comm. 11 November 2014). Fifty seven sera were accessed from whole herd sampling for diagnostic purposes on 30 June 2014, and bacterial specimens from two animals were submitted to the ARC-OVI for isolation of *B. abortus.*

4.2.2.4   Herd F

Herd F was from the Madibeng Local Municipality (27.7839662°E 25.633104°S) in North West. Brucellosis was first suspected when there were positive RBT results with pre-movement tests (Steyl 2014). Abortions due to *B. abortus* were diagnosed in cattle on a neighbouring farm previously (J. Steyl pers. comm. 10 November 2014). *B. abortus* biovar 1 infection was confirmed in herd F when it was isolated out of specimens from two of the seropositive cows culled in March 2014 (Steyl 2014). Sera were accessed from six of these cows. Bacteriological results were available for four of them, and *B. abortus* biovar 1 was isolated from the lymph nodes of one (J. Steyl pers. comm. 10 November 2014).

4.3   Sample collection and management

Blood was collected through venepuncture from immobilised African buffalo or exsanguination during culling, into evacuated serum tubes without any additives. The blood was allowed to clot at room temperature. The clot and serum was separated and serum was stored at -20°C until use.

Before analysis, the samples received were thawed, mixed and dispensed into 1 ml tubes in sets of 96. Eight tubes per 96 micro-tube storage rack were left empty to allow for placement of positive and negative controls. Sets of 96 were numbered I to V, and specimens according to rows A to H and columns 1 to 12.

Specimens for bacteriology were collected aseptically and cooled to 2°C to 8°C or frozen at -20°C until use.

4.4   Bacteriological testing

Bacterial culture for *B. abortus* was done at the Bacteriology Laboratory of the Agricultural Research Council-Onderstepoort Veterinary Institute (ARC-OVI) according to the method prescribed by the OIE (OIE 2009a). Solid tissues were macerated and specimens inoculated onto a solid *Brucella* selective medium containing Farrel's medium with six antibiotics (OIE 2009a). The agar plates were incubated at 37°C (±1°C) in 5-10% CO_2 atmosphere. Specimens were examined every second day until growth was observed for a maximum of two weeks. If no growth was observed the culture was classified as negative. Identification of *Brucella* species and biovar was based on phenotypical characteristics of colony morphology and growth characteristics, Gram-negative staining, partial acid-fast staining with Stamp's stain, biochemical reactions and positive agglutination reaction when mixed with a polyclonal serum against *Brucella* (OIE 2009a).

4.5 Serological testing

4.5.1 Rose Bengal test (RBT)

The Rose Bengal Test was performed according to the procedure prescribed by the OIE (OIE 2009a).

- Equal volumes (25 µL) of serum and whole cell acidified antigen Rose Bengal Ag Pourquier (Lot 381-100) (https://www.idexx.com/livestock-poultry/ruminant/b-abortus.html viewed 24 February 2015) were dispensed into wells of a WHO haemagglutination plate.
- Positive (BRUCELLA ABORTUS POS SERUM from Onderstepoort Biological Products, Expiry date 1/3/2015) and negative (ARC-OVI in-house bovine negative sera no: 9462-8) control sera were included on every plate.
- The test serum and antigen were mixed by tapping the plate by hand, and then agitated for 4 minutes on a rocker at room temperature.
- Immediately after agitation, the plate was placed on an X-ray light box, and examined visually (by naked eye) for agglutination.
- Any evidence of agglutination was recorded as positive on the Brucellosis Submission Form (CA5).
- Data from the CA5 were entered into a Microsoft XCell spreadsheet.

4.5.2 Indirect enzyme-linked immunosorbant assay (iELISA)

The iELISA was performed using the commercial IDEXX Brucellosis Serum X2 *Brucella abortus* antibody test kit. The kit contained ten 96 well micro titre plates coated with S-LPS antigen of *Brucella.* Manufacturer's instructions were followed (IDEXX n.d.).

- Duplicate samples of 10 µL serum (sample and controls) were diluted 1:10 with 90 µL wash solution in the micro titre plate.
- The micro titre plate was subsequently incubated for 60 minutes at 37℃ ±2℃.
- The micro titre plate was washed manually three times with 100µL wash solution.
- 100 µL horse–radish peroxidase-labelled anti-ruminant IgG conjugate was added to each well.
- The micro titre plate incubated again for 60 minutes at 37℃ ±2℃.
- The plate was washed manually three times with 100µL wash solution.
- 100µL of the chromogenic substrate, 3,3',5,5' tetramethylbenzidine was added to each well and the micro titre plates covered.
- Incubated for 15 minutes on the desk at 18 to 26℃.
- The reaction was stopped with 100µL stop solution in each well.
- The colour reactions in wells were read in a photometer (Bio-Tek ELx800) at a wavelength of 450nm.

- The calculated optical density (OD) readings were printed out.
- The data were entered into a Microsoft XCell spreadsheet.

Validity of the assay was confirmed by checking that the OD of the negative control did not exceed 0.500 and the difference between the OD of the positive and negative controls were ≥ 0.300.

The sample: positive ratio (S/P ratio) was calculated in a Microsoft XCell spreadsheet as follows:
- The OD values of the duplicate samples were averaged.
- Correction of the average OD of the positive control and samples by subtracting the average OD of the negative control.
- S/P ratio for the sample: S/P% = 100 x (average sample corrected OD/average positive control corrected OD). Equation 1.

Equation 1 S/P ratio

$$\frac{S/P}{ratio} = 100 \times \frac{(OD\ sample\ 1 + OD\ sample\ 2)/2 - (OD\ negative\ control\ 1 + OD\ negative\ control\ 2)/2\text{-}}{(OD\ positive\ control\ 1 + OD\ negative\ positive\ control\ 2)/2\text{-}- (OD\ negative\ control\ 1 + OD\ negative\ control\ 2)/2}\ \%$$

4.5.3   Complement fixation test (CFT)

The CFT was performed by the Bacteriology Serology Laboratory of the ARC-OVI according to the OIE standard, and the SAVLSF Harmonised Serology SOP for *Brucella abortus* Complement Fixation Test (CFT) (OIE 2009a, Potts, Human, Theron et al. 2014). The procedure was as follows:
- Test and control sera were heat inactivated at 58°C ±2°C for 30 minutes.
- Positive (BRUCELLA ABORTUS POS SERUM from Onderstepoort Biological Products, and in-house negative control sera were included in every plate.
- 25 µL Veronal buffer was pipetted into the wells of a U-bottom 96 well micro titre plate.
- Serial dilutions were done using an 8 channel micropipette. It started with a 1/2 dilution of 25 µL serum and 25 µL CFT buffer (Veronal buffer), by picking up 25 µL of diluted serum and dispensing it into the next row of wells filled with 25 µL Veronal buffer. The highest dilution was 1/128.
- 50 µL guinea-pig complement for CFT and CFT buffer is dispensed into the row with1:2 dilutions of the samples.
- 50 µL guinea-pig complement for CFT and the whole cell antigen, Onderstepoort Biological Products *Brucella abortus* complement fixation antigen diluted 1/10 is dispensed into the rest of the rows.
- Test sera and reagents were mixed by tapping the plates gently.
- Plates were incubated at 37°C ±2°C for 30 minutes.

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- 50 µL of the haemolytic system consisting of sheep red blood cells and Amboceptor (antibodies against sheep red blood cells) diluted in the CFT buffer were then added to all the wells with diluted serum samples.

- Plates were incubated at 37°C ±2°C for 30 minutes on a shaker.

- The plates were centrifuged for 1 minute at 1000 g.

- The plates were placed over a magnifying mirror, and the degree of haemolysis in the last dilution where there is haemolysis assessed. Complete haemolysis (no sediment) was regarded as negative.

- The endpoint of the CFT reactions was converted to IU ml^{-1} from a table, on a scale where 50% haemolysis in a 1/220 serum dilution is equivalent to 1000 IU ml$^{-1.}$

- Where the well without antigen was not negative, the result was recorded as ant-complementary.

- Results were recorded on the Brucellosis Submission Form (CA5).

- Data on the CA5 were entered into a Microsoft XCell Spreadsheet.

4.5.4   Fluorescence polarisation assay (FPA)

The FPA was performed in duplicate with the commercial Brucella S antibody test kit, Brucella FPA, Diachemix, batch number 123, expiry date May 2015. The kit is United States Department of Agriculture (USDA) approved and licensed for detection of brucellosis in cattle (Diachemix 2010). The antigen is the O side chain of the LPS of *Brucella abortus* conjugated with a fluorescent dye (Diachemix 2010). The procedure was as follows:

- 20 µL of the positive and negative controls included in the kit, and test samples were pipetted into the wells of a black 96 well micro titre plate, in duplicate. Three negative and one positive control was included for every 44 test samples.

- 180 µL of the reaction buffer was dispensed into all the wells.

- Sera and buffer were mixed carefully.

- The plate was incubated at room temperature for 4 minutes, on a shaker for the first three minutes, and standing still for 1 minute.

- All the wells in the plate were read automatically in a FPA reader (BMG).

- 10 µL of the FPA conjugate was dispensed into all the wells.

- A second reading was obtained.

- The readings were captured electronically by Diachemix software, positive and negative control values were assessed to be within the expected range, and the change in millipolarisation units (ΔmP) was calculated.

- The results were printed out.

- Results were entered into a Microsoft XCell spreadsheet.

Sera were selected for repeated analysis in duplicate on the following criteria:
- High variation between duplicate readouts

- high readouts (≥173ΔmP)

- low readouts (≤5ΔmP)

- low positive readouts (23 to 83ΔmP) according to interpretation recommended by manufacturer

- suspect readouts (10 to 20ΔmP) according to interpretation recommended by manufacturer.

4.6 Selection of composite reference panels

A total of 354 sera were accessed from the six herds, of which eleven were excluded from the selection of reference panels and further analyses. An uninfected composite reference panel of 107 sera and infected composite reference panel of 93 sera were selected (Table 4-2).

4.6.1 Exclusion criteria and data cleaning

Sera which were too little to allow for all four index tests to be performed on the specimen were excluded as well as sera that tested anti-complementary with the CFT. As an exception, sera were included in cases where only the CFT could not be performed and CFT results on duplicate specimens from a different veterinary laboratory, following the same basic standard operational procedure, were available.

For sera where a positive and a negative result were reported on repeated analyses with the RBT test, the test result was considered positive.

iELISA was performed in duplicate according to manufacturer's instructions, and the averages of the two readings were used as the final result for analysis.

To determine the FPA value for further analyses extreme readings considered to be artefact were removed and the remaining readings per serum averaged. The difference between all combinations of two readings were calculated and expressed as a fraction of the lowest reading. Where the lowest reading was 0, it was substituted by 0.1. Where the absolute value of the fraction was > 2.5, the higher value was discarded as artefact. The remaining values were averaged and rounded off to the nearest integer and used as the FPA result for analysis. For example: The following four readings were available for the serum in box II, micro tube G1: 5, 6, 436 and 6. 436 were discarded as artefact. The average of 5, 6 and 6 is 5.67. The value of 6 was used for further analysis (Figure 4-2).
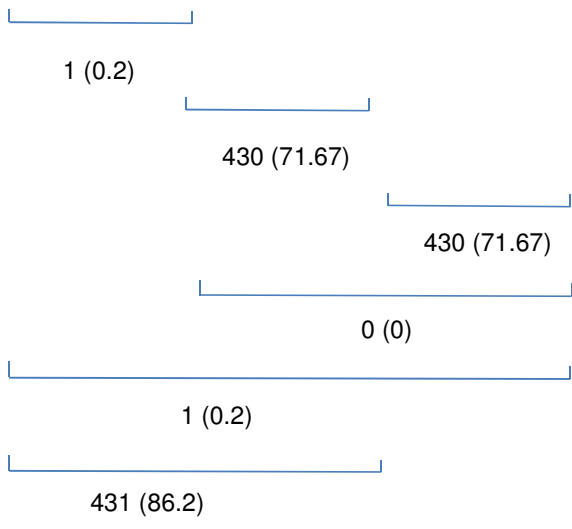
FPA reading 1	FPA reading 2	FPA reading 3	FPA reading 4
5	6	436	6

1 (0.2)

430 (71.67)

430 (71.67)

0 (0)

1 (0.2)

431 (86.2)

_____

Above: Example identification of artefact FPA readings was identified as artefact. The blue lines indicate the two readings used for the calculation. The first value under the line is the difference between the two values, and the second value in brackets the difference divided by the absolute value of the lower of the two readings. FPA reading 3 was discarded.

Figure 4-2 Identification of artefact FPA readings

Four of the 101 sera received from herd A were excluded: two because of inadequate sample volume and two due to anti-complementary result with the CFT.

Four of the 157 sera from herd C were excluded: three because of inadequate sample volume, and one due to anti-complementary result with the CFT.

Two of the 57 sera from herd E were excluded because of inadequate sample volume.

One of the seven sera from herd F was excluded from the study, because it was a duplicate specimen, and only one set of results was used.

4.6.2   Inclusion criteria: uninfected reference panel

Bacteriology is highly specific but not sufficiently sensitive to correctly identify animals as uninfected (Gall & Nielsen 2004). A composite reference standard was applied. The strict criteria set by Jacobson 1998:447 for verification of uninfected status would not allow any South African African buffaloes to be verified as uninfected. E.g. there are no areas that can claim freedom from endemic infection. The following criteria were applied to include serum in the uninfected reference panel:

- Herds are closed to African buffaloes infected with bovine brucellosis and not tested serologically and found negative prior to introduction into the herd.
- No confirmed evidence of antibody against *Brucella* present in the herd with repeated testing over two or more years. Isolated case/s of RBT positivity without evidence of spread, positivity on CFT, or bacterial isolation of *Brucella* acceptable.
- No history of clinical signs consistent with bovine brucellosis.

4.6.3   Inclusion criteria: infected reference panel

Bacteriology is highly specific, but due to the low sensitivity will only identify a proportion of infected animals as infected (Gall & Nielsen 2004). A composite reference standard was applied to select the infected (positive) reference panel (Reitsma, Rutjes, Khan et al. 2009, Alonzo & Pepe 1999, Jacobson 1998). The inclusion criteria were as follows:

- I.   direct detection of *B. abortus* through bacterial isolation, or
- II. an animal from a bacteriologically confirmed infected herd with clinical signs and/or pathology consistent with bovine brucellosis, or
- III. at least three of the four index tests yielded results which were classified as positive either by the OIE or manufacturer's criteria (Bisoffi, Buonfrate, Sequi et al. 2014). That included:

  - any agglutination reaction with RBT (OIE 2009a),
  - iELISA S/P ratio of ≥ 80% (IDEXX)
  - CFT titre of ≥ 20 iU/ml (OIE 2009a), and
  - FPA read out of > 20 ∆mP (Diachemix n.d.).

4.6.4 Composite infected and uninfected reference panels

4.6.4.1   Uninfected reference panel

One hundred and seven sera from the known uninfected herds A and B were selected for the uninfected reference panel. Ninety seven sera were from herd A and 10 from herd B. The sera from herd A were from animals of unknown sex and age and herd B were juvenile males.

4.6.4.2 Infected reference panel

Ninety three sera from infected herds met the composite reference standard for the infected reference panel. *B. abortus* was detected directly in five animals, of which seven showed clinical signs and/or pathology of bovine brucellosis and 92 sera was classified as positive according to the OIE or kit manufacturer's instructions by three or more of the index tests (Table 4-2).

Sixty sera were from herd C, two from herd D, 25 from herd E and six from herd F. Age was known for 42 animals: 21 were adult, 14 sub-adult and seven juvenile. Sex was known for 43 animals: 27 was female and 16 male (Table 4-3).

Table 4-2 Selection of composite reference panels

Herd	A	B	C	D	E	F	Total
Total sera received	101	10	175	4	57	6	**353**
Analysed: RBT, iELISA, CFT, FPA							
Sera excluded	4	0	4	0	2	0	**10**
Applied criteria for uninfected reference panel							
Uninfected reference panel	**97**	**10**	**0**	**0**	**0**	**0**	**107**
Applied criteria for infected reference panel							
Direct detection *B. abortus*	0	0	1	1	2	1	**5**
Clinical signs and/or pathology	0	0	1	0	6	0	**7**
3 out of 4 index tests positive	0	0	60	2	24	6	**92**
Infected reference panel	**0**	**0**	**60**	**2**	**25**	**6**	**93**

Table 4-3 Infected reference panel age and sex distribution

	adult	sub-adult	juvenile	unknown	all ages
female	17	8	1	1	27
male	2	6	6	2	16
unknown	2	0	0	48	50
male and female	21	14	7	51	93

4.7 Data analyses

4.7.1 Distribution analysis

The distribution of results of all sera recruited were analysed using the free statistical package R to draw box and whiskers plots and histograms with and without fitting of normal distribution curves (R Core Team 2014)
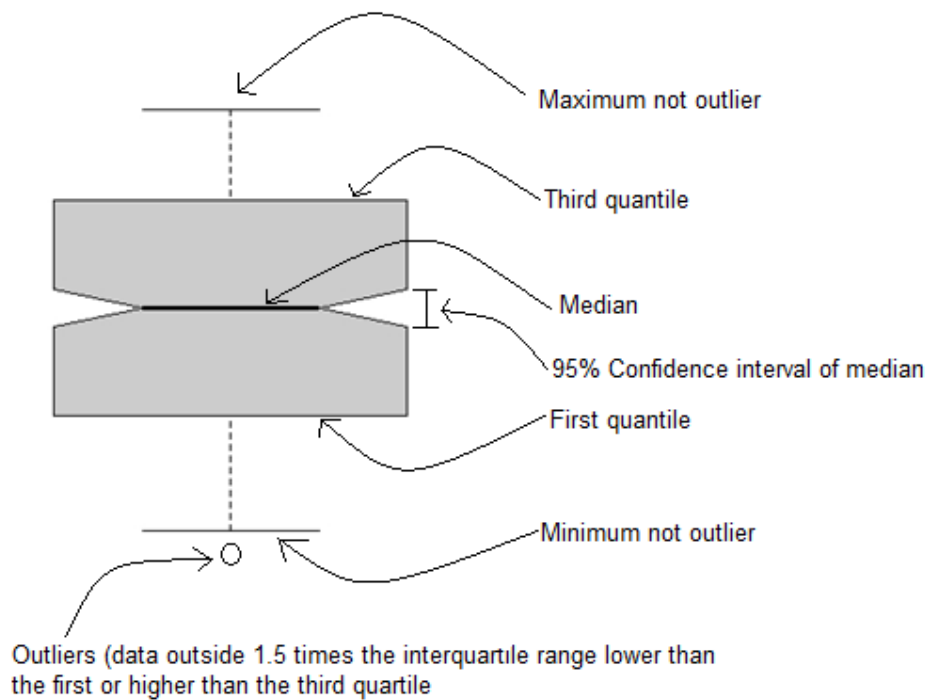
Figure 4-3 Explanation of the components of the box and whiskers plot

4.7.2 Frequency distribution analysis

Frequency distribution tables and graphs were compiled using the FREQUENCY function and chart tools of Microsoft Excel 2010 (Microsoft Corporation 2010).

4.7.3 Two by two tables for diagnostic sensitivity and specificity

Diagnostic sensitivity (DSe) and diagnostic specificity (DSp) were determined according to OIE formulas. Test results of the reference panels were categorised and cross tabulated in a 2 X 2 table with cells for true positive (TN) , false positive (FP), false negative (FN) and true negative (TN) (OIE 2013) (Table 4-4).

Table 4-4 Format of 2x2 table for diagnostic sensitivity and specificity (OIE 2013)

	Known positive	Known negative	
Test result positive	TP	FP	
Test result negative	FN	TN	
	Diagnostic sensitivity TP/(TP+FN)	**Diagnostic specificity** TN/(TN+FP)	

Statistical uncertainty was determined by calculating the 95% confidence interval (OIE 2014), using an online calculator of the Centre for Clinical Research and Biostatistics of the Chinese University of Hong Kong (Centre for Clinical Research & Biostatistics, 2014).

4.7.4 Receiver operator characteristics analyses

Receiver operator characteristics analyses (OIE 2014) were done with MedCalc Statistical Software (MedCalc 2015) and receiver operator curves produced for the FPA, iELISA and CFT. The area under the curve as an indicator of diagnostic accuracy was interpreted as follows:

- 0.9 – 1.0 excellent
- 0.8 - 0.9 very good
- 0.7 - 0.8 good
- 0.6 - 0.7 sufficient
- 0.5 - 0.6 bad
- < 0.5 test not useful

4.7.5 Performance of tests used in combination

Sera were categorised as positive or negative, based on different combinations of the four index tests in series and parallel (Larson 2008) and diagnostic sensitivity and specificity calculated.

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4.7.6 Test agreement

Test agreement were calculated between categorised results of the index tests and true status (reference panels) (Gardner, Holmes 1993) and between the categorised results of the index tests for all sera received (Lynch, Duignan, Taylor et al. 2011, The TDR Diagnostics Evaluation Expert Panel, 2010, Bajani, Ashford 2003). An online calculator was used for the simple unweighted Kappa coefficient (Lowry, n.d.). Indices of positive (P_{pos}) and negative (P_{neg}) agreement between the index tests were calculated in Microsoft Excel 2010 (Microsoft Corporation 2010) using a "two by two" contingency table (Table 3-1) and formulas below (Sanogo, Thys, Achi et al 2013, The TDR Diagnostics Evaluation Expert Panel 2010). Confidence intervals were calculated using Microsoft Excel 2010 (Microsoft Corporation 2010).

Table 4-5 Contingency table for calculation of indices for positive and negative agreement between two diagnositc tests

	Test 1 positive	Test 1 negative
Test 2 positive	number (a)	number (b)
Test 2 negative	number (c)	Number (d)

$P_{pos} = 2a/(2a+b+c)$

$P_{neg} = 2d/(2d+b+c)$

The Kappa coefficients were interpreted as follows:
- 0 – 0.1 = poor,
- 0.1 – 0.20 = slight,
- 0.21 – 0.40 = fair,
- 0.41 – 0.60 = moderate,
- 0.61 – 0.80 = substantial, and
- 0.81 – 1 = almost perfect (Sim, Wright 2005).

4.7.7 Test repeatability

The following statistical tools were used to determine test repeatability
- Pearson's correlation coefficient for the iELISA and FPA. The interpretation was as follows:
  - 0.00-0.19 = very weak
  - 0.20-0.39 weak
  - 0.40-0.59 moderate
  - 0.60-0.70 strong, and.

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- 0.8 to 1.0 very strong (ed. Evans 1996)
- Unweighted Kappa coefficient for the RBT. See paragraph 3.6.8 for interpretation.
- Scatter graph for the iELISA and FPA.
- Frequency distribution of differences between duplicate measurements and standard deviation for the iELISA and FPA.
- Bland-Altman plots for the FPA and iELISA.

4.7.7.1   Pearson's correlation coefficient

Pearson's correlation coefficient was calculated to assess repeatability of duplicate runs of the iELISA and FPA (OIE 2014, Vaz, Falkmer, Passmore et al. 2013), using the CORREL function of Microsoft Excel 2010 (Microsoft Corporation 2010).

4.7.7.2   Unweighted Kappa coefficient

The repeatability of duplicate runs, and reproducibility of the RBT and repeatability of categorised FPA results were assessed by calculating the Kappa value (OIE 2014) using the online calculator for Kappa (http://vassarstats.net/kappa.html).

4.7.7.3   Scatter graph

A scatter graph of the duplicate analyses was compiled and the linear trend, calculated with the least squares method, and line of equality inserted for calculated O/D values measured by iELISA results and ΔmP by FPA (Bland & Altman 2003) using Microsoft Excel 2010 (Microsoft Corporation 2010).

4.7.7.4   Frequency distribution of differences between duplicate measurements and standard deviation

The frequency distribution of the differences between measurements of duplicate analyses were illustrated for continuous data (iELISA and FPA)) (Bland & Altman 2003) using the FREQUENCY and chart functions of Microsoft Excel 2010 (Microsoft Corporation 2010).

The standard deviation of the differences between measurements of duplicate analyses were calculated for continuous data (iELISA and FPA), and from the standard deviation the repeatability coefficient (1.96 X the standard deviation). The difference will only exceed the repeatability coefficient in 5% of duplicate measures (Bland & Altman 2003).

4.7.7.5   Bland-Altman plots

The difference between the duplicate analyses were plotted against the mean of the duplicate measurement in a scatter plot and lines of 2 standard deviations above and below the mean inserted for continuous data (iELISA and FPA) (Vaz, Falkmer, Passmore et al. 2013, Bland & Altman 2003) using MedCalc Statistical Software (MedCalc 2015).

4.7.8 True prevalence and predictive values

The true prevalence and positive and negative predictive values were calculated using the formulas of Fegan (2000:30) (Fegan 2000).

Equation 2 True Prevalence

$$\textbf{True prevalence (TP)} \quad = \quad \frac{\text{apparent prevalence} + (DSp - 1)}{DSp + (DSe-1)}$$

Equation 3 Positive predictive value

$$\textbf{Positive predictive value} \quad = \quad \frac{(TP \times DSe)}{(TP \times DSe + (1-TP) \times (1-DSp))}$$

Equation 4 Negative predictive value

$$\textbf{Negative predictive value} \quad = \quad \frac{((1-TP) \times DSp)}{(1-TP) \times DSp + TP \times (1-DSe)}$$

5 RESULTS

Three hundred and fifty three sera were analysed with the four index tests and specimens from 85 animals were analysed bacteriologically for *B. abortus.* DSe and DSp, performance index and test agreement with reference panels were calculated for the four index tests. Test agreement between tests was determined. Test repeatability was assessed for the RBT, iELISA and FPA. DSe and DSp for all combinations of two tests in series and parallel were determined. Tests were applied to herds C and E and true prevalence and positive and negative predictive values calculated.

5.1 Bacteriology results

The specimens from 83 animals in infected herd C and two animals in infected herd E all tested negative for *Brucella* by bacterial isolation.

5.2 Serology results

Diagnostic specificity was ≥ 98% for all four index tests and DSe ≥ 97.9% for the FPA, iELISA and RBT. The DSe of the CFT was 74.2% and consequently the CFT had the lowest performance index (174.2%) of the four index tests. The repeatability between duplicate runs as indicated by the Pearson's correlation-coefficients was 0.97 for the iELISA and 0.17 for the FPA, while duplicate RBT tests had unweighted Kappa coefficient of 07855. Test agreement of categorised results of index tests with inclusion in infected and uninfected reference panels was the highest for the iELISA (unweighted Kappa = 0.9799) and lowest for the CFT (unweighted Kappa = 0.7547). Test agreement between the index tests was the lowest between the CFT and RBT (unweighted Kappa = 0.26, P_{pos} = 44.3% and P_{neg} = 80.8%) and the highest between the FPA and iELISA (unweighted Kappa = 0.685, P_{pos} = 83.8% and P_{neg} = 84.78%). Use of all combinations of two tests in series increased the DSp to 100%, and in parallel increased the DSp to 100%. Positive predictive values calculated for herds C and E were 100% for the iELISA, CFT and FPA and for the RBT (97.3%) in herd C and (98.4%) in herd E. The RBT showed the highest negative predictive value (99.2% in herd C and 98.7% in herd E) and the CFT the lowest (89% in herd C and 73.1% in herd E).

5.2.1 Rose Bengal Test (RBT)

One hundred and four of the 343 sera received tested RBT positive, two out of 107 sera from the two uninfected herds (uninfected reference panel), and 102 out of 236 sera from infected herds, with the rest negative (
Table 5-1).

Table 5-1 RBT results for sera received

	Herd	Total number sera	Number RBT positive
Uninfected herds	A	97	2
	B	10	0
	Total	**107**	**2**
Infected herds	C	171	64
	D	4	2
	E	55	30
	F	6	6
	Total	**236**	**102**
Infected plus uninfected herds		**343**	**104**

In the infected reference panel one of the 93 sera tested RBT positive.

5.2.2 Indirect enzyme-linked immunosorbant assay (iELISA)

The mean S/P ratios on 343 sera ranged from -74.9% to 132 with a median of 34.8%, mean of 30.6%, first quartile -18.4%, third quartile 87,8% (Figure 5-1).

iELISA results all sera
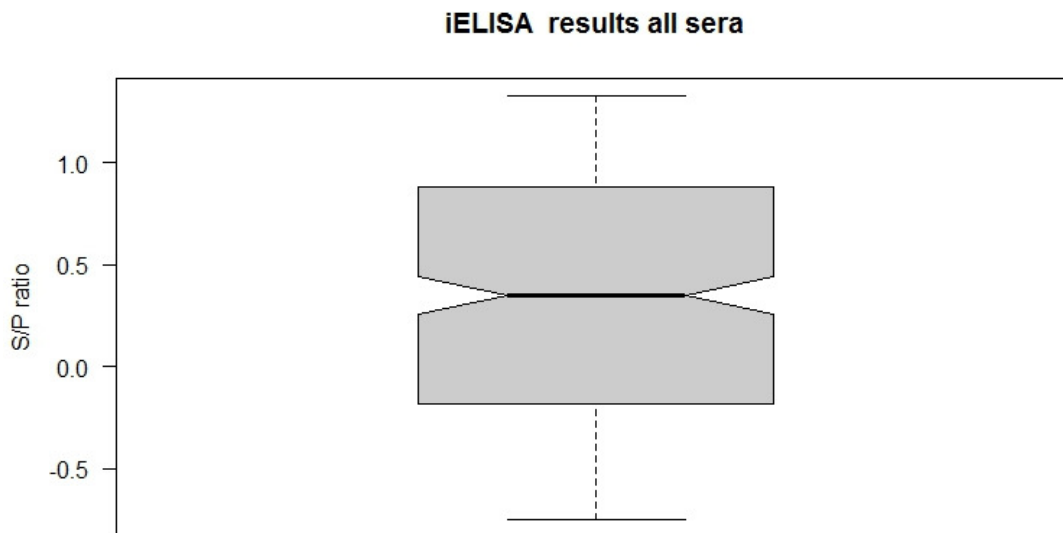
Figure 5-1 Box and whiskers plot illustrating distribution of all iELISA results

In the two uninfected herds (uninfected reference panel) the range was from -74.9% to 40.5%, the median -55%, mean -44.4%, with the first quartile -64.9% and the third quartile -20%, and in the infected herds the range was -11.8% to 132.7%, the median 74.5%, mean 64.6%, the first quartile 26.7% and the third quartile 93.9% (Figure 5-2).

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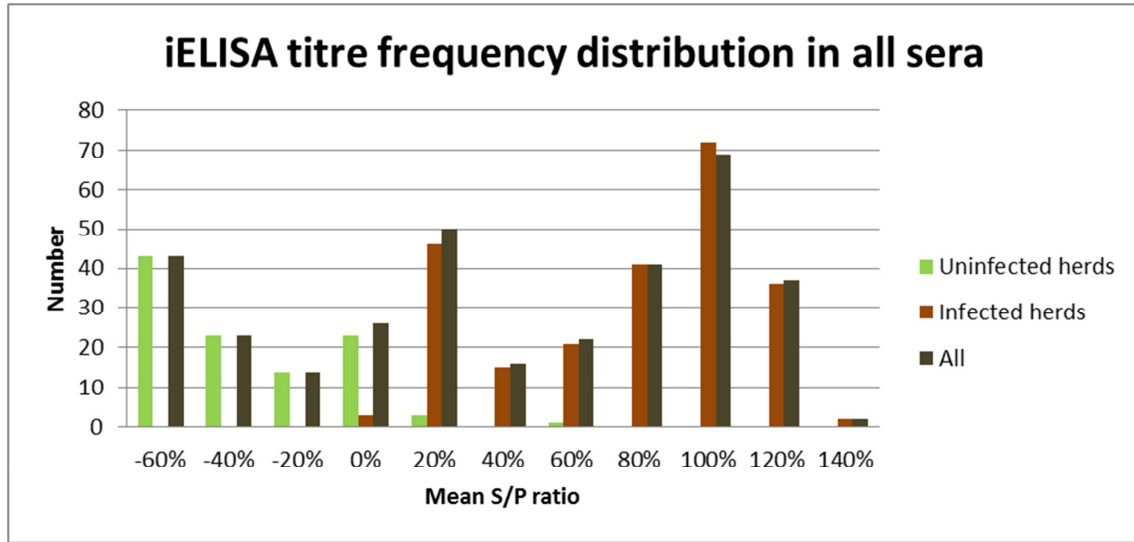
Figure 5-2 iELISA frequency distribution in 343 sera received

The range of iELISA titres in the infected reference panel was from 6.1% to 132.7%, median of 97.4%, mean of 94%, first quartile of 86.6% and third quartile of 105.3%. The median for sera in the infected reference panel in herd C was 99.1% and in herd E 83.4% (Figure 5-3).
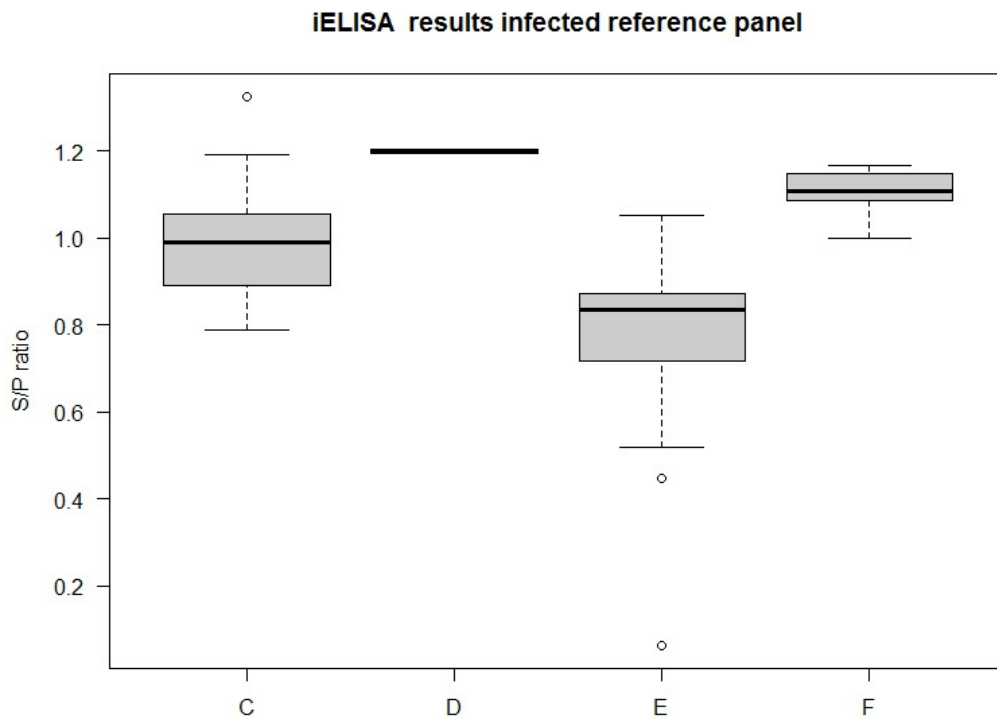
Figure 5-3 Box and whiskers plot iELISA results infected reference panel per herd

5.2.3   Complement fixation test (CFT)

The CFT titres ranged from 0 iU/ml to 784 iU/ml (end point), the median, first and third quartiles were 0 iU/ml, and the mean 47.4 iU/ml (Figure 5-4).

CFT results all sera
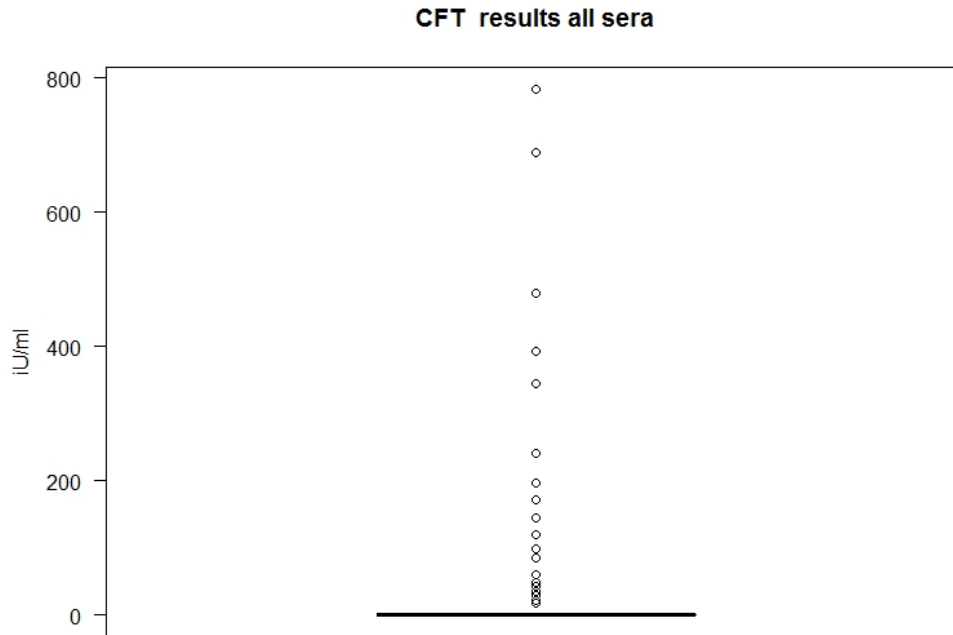
Figure 5-4 Box and whiskers plot for all CFT titres
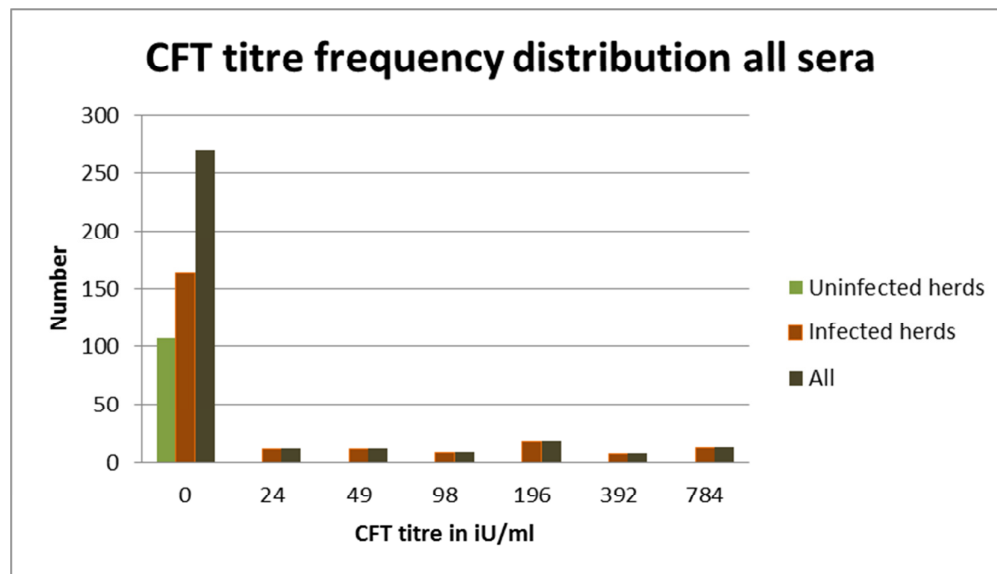
Figure 5-5 CFT titre frequency distribution for all sera received

All sera from uninfected herds (uninfected reference panel) had a titre of 0 iU/ml, as well as 164 sera from the infected herds (Figure 5-5).

In the infected herds, the titres ranged from 0 iU/ml to 784 iU/ml, with the first quartile and median o iU/ml, third quartile 30 iU/ml and the mean 68.84 iU/ml.

In the infected reference panel, titres ranged from 0 iU/ml to 784 iU/ml, with median of 49 iU/ml, mean of 165.4 iU/ml first quartile 0 iU/ml and third quartile of 196 iU/ml. The median for infected reference sera from herd C was 25.5 iU/ml and herd E 12 iU/ml (Figure 5-6).

CFT results infected reference panel
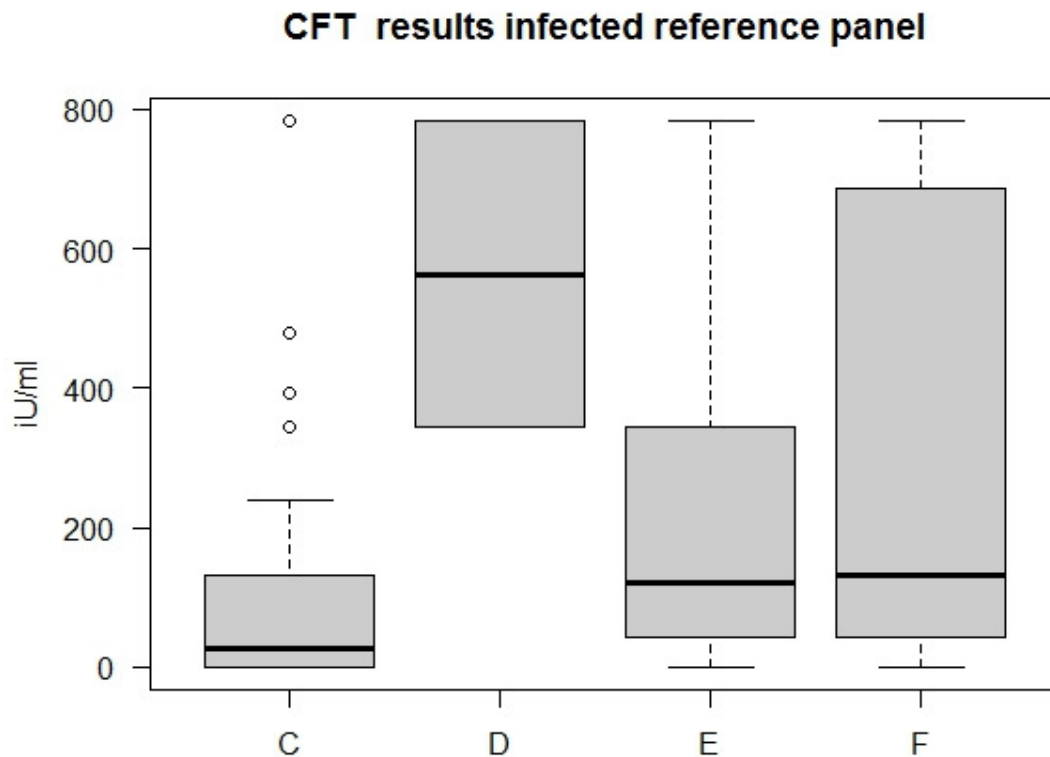
Figure 5-6 Box and whiskers plots illustrating distribution of CFT titres in infected reference panel per herd

5.2.4   Fluorescence polarisation assay (FPA)

FPA readings before exclusion of sera and removal of readings considered artefact ranged from -2 092 ΔmP to 4 905 ΔmP, median of 17 ΔmP and mean of 58.05 ΔmP, first quartile 2 ΔmP and third quartile 61 ΔmP. A large number of outliers were observed in the box and whiskers plot (Figure 5-7). Box and whiskers plots per herd showed that herds A and C had the most outliers, while there were no outliers in herd E (Figure 5-8).

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FPA readings before removal of artefact
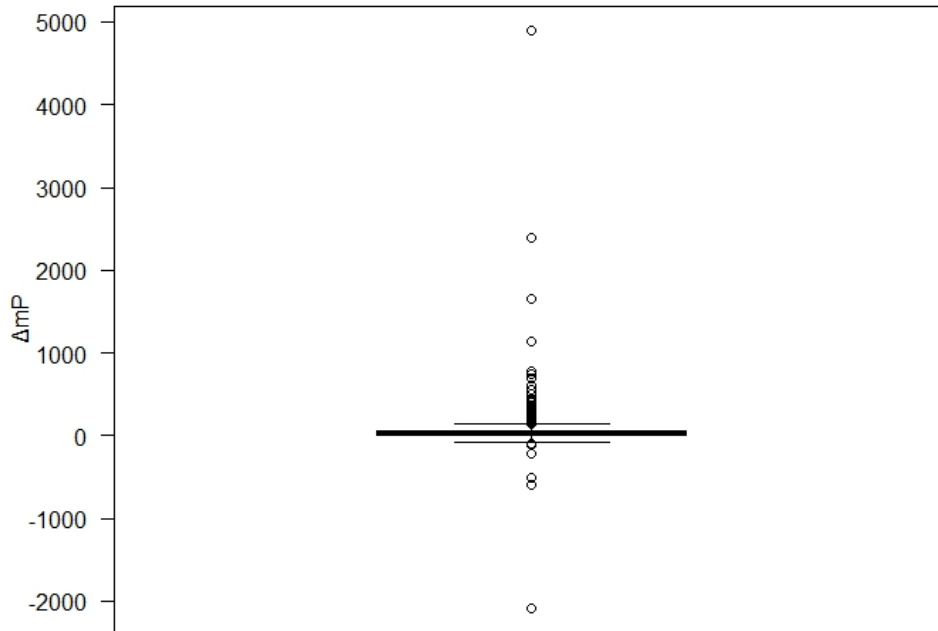
Figure 5-7 Box and whiskers plot illustrating distribution of all FPA readings before removal of readings considered artefact.

FPA readings according to herd, before removal of artefact
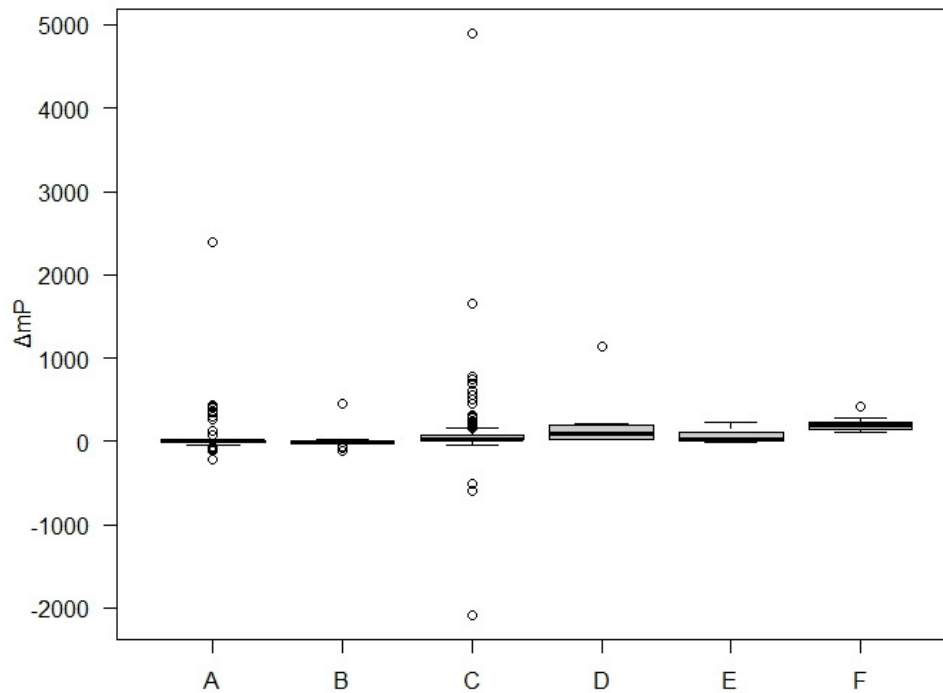
Figure 5-8 Box and whiskers plot for all FPA readings per herd

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The FPA titres calculated after exclusion of sera and removal of readings regarded as artefact ranged from -1 026 ΔmP to 272.75 ΔmP, median 16 ΔmP, mean 30.21 ΔmP, first quartile -1 ΔmP and third quartile 45.75 ΔmP (Figure 5-9).

FPA results all sera
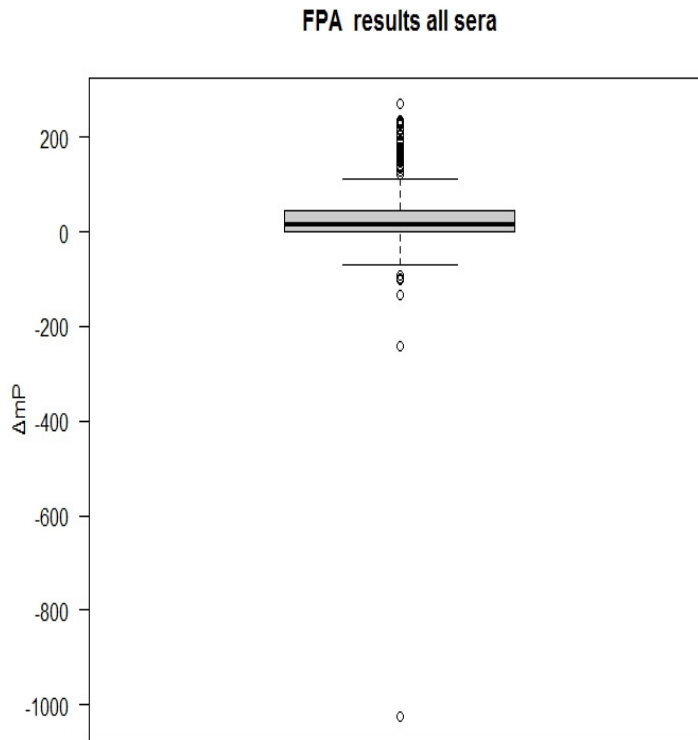
Figure 5-9 FPA results calculated for all sera

The range of FPA results in the uninfected herds (uninfected reference panel) -101 ΔmP to 16 ΔmP (Figure 5-10), with the median 16 ΔmP, the mean -13.04 ΔmP, first quartile -19.5 ΔmP and third quartile 2.5 ΔmP. In the infected herds, the range was – 1 026 ΔmP to 273 ΔmP, with a median of 24.5 ΔmP, mean of 49.77 ΔmP, first quartile of 15 ΔmP and third quartile of 82.5 ΔmP.
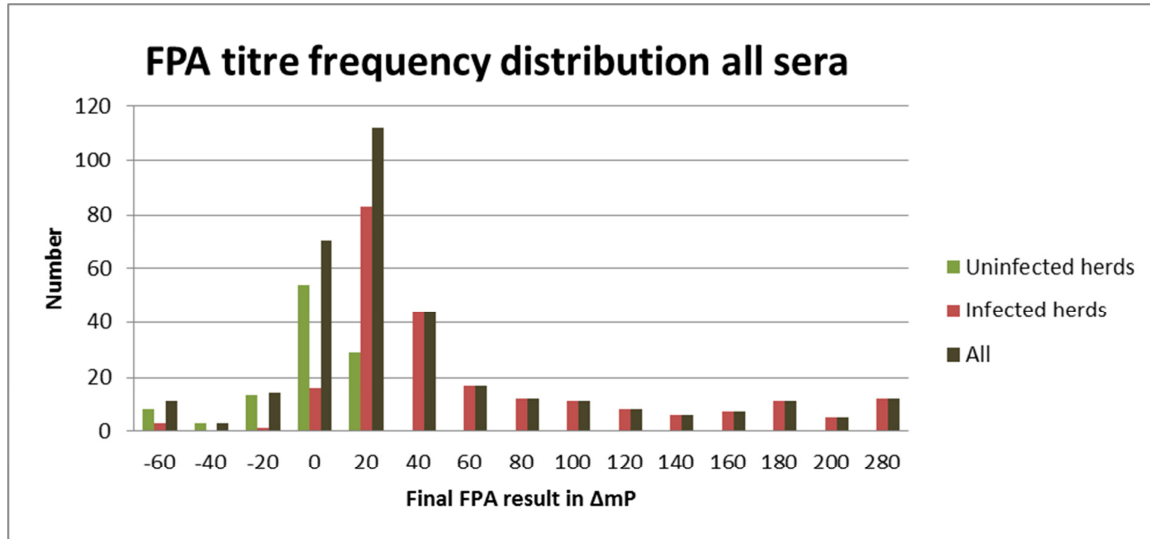
Figure 5-10 FPA titre frequency distribution for 343 sera received

In the infected reference panel, the range was – 1026 ΔmP to 273 ΔmP, with a median of 102 ΔmP and mean of 103.8 ΔmP, first quartile 59 ΔmP and third quartile 166 ΔmP (Figure 5-11). The median for herd C was 87 ΔmP and herd E 121.5 ΔmP (Figure 5-12).
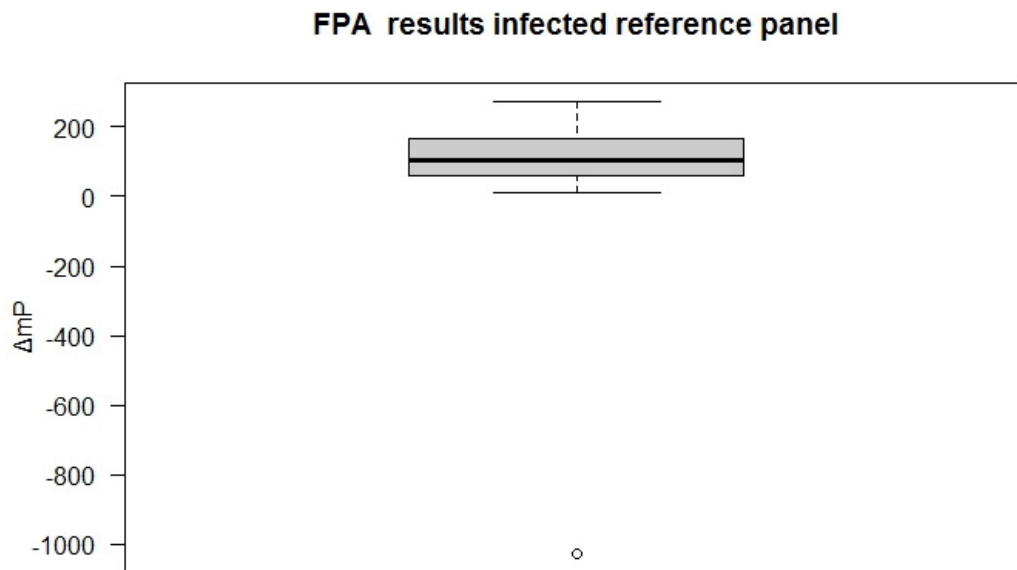
Figure 5-11 Box and whiskers plot illustrating distribution of FPA titres of infected reference panel

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FPA results infected reference panel per herd
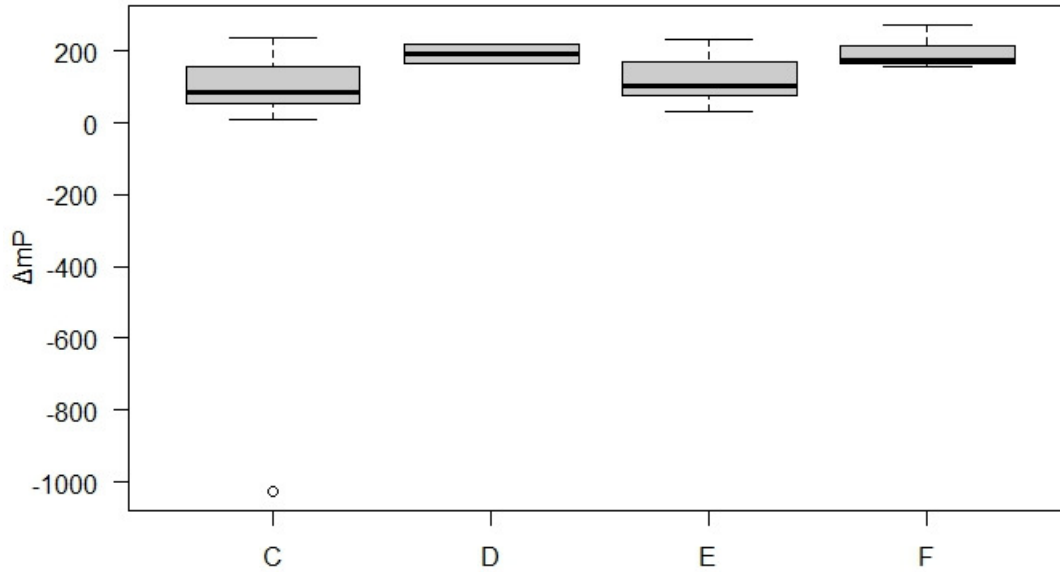
Figure 5-12 Box and whiskers plot illustrating distribution of FPA titres of infected reference panel

5.2.5 Diagnostic test performance

5.2.5.1 Rose Bengal Test (RBT)

In the uninfected reference panel, 2 out of the 107 sera tested positive. In the infected reference panel, 92 out of the 93 sera tested positive (Table 5-2).

Table 5-2 Two by two table for calculation of diagnostic sensitivity and specificity of the RBT

RBT	Known infected	Known uninfected	Total
Test positive	92	2	94
Test negative	1	105	106
Total	93	107	200
	Diagnostic sensitivity	**Diagnostic specificity**	
	98.9%	98.1%	
	Lower 95% CI	Lower 95% CI	
	96.83%	95.57%	
	Upper 95% CI	Upper 95% CI	
	100.0%	100.0%	

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Diagnostic sensitivity (DSe) was calculated as 98.9% (95% CI 96.83% - 100.0%) and diagnostic specificity (DSp) as 98.1% (95% CI 95.57% - 100.0%).

Test agreement, as evaluated by the unweighted Kappa coefficient, was 0.9699 (95%CI 0.93561-1).

The repeatability of the RBT was evaluated by calculation of the unweighted Kappa coefficient. One hundred and sixty seven sera were tested in triplicate by the RBT. Two of the runs by the same person showed 100% concordance. The reproducibility between these results and a third run by a different person were assessed, and the unweighted Kappa coefficient calculated as 07855 (95%CI 0.658 – 0.913).

5.2.5.2   Indirect enzyme-linked immunosorbant assay (iELISA)

Diagnostic sensitivity (DSe) was calculated as 86.0% (95% CI 79.0% - 93.1%) and diagnostic specificity as 100.0% (95% CI 100.0% - 100.0%) at the cut-off value of 80% (Table 5-3).

Table 5-3 iELISA 2x2 table for DSe and DSp at 80% cut-off value

iELISA (80%)	Known infected	Known uninfected	Total
Test positive	80	0	80
Test negative	13	107	120
Total	93	107	200
	Diagnostic sensitivity	**Diagnostic specificity**	
	86.0%	100.0%	
	Lower 95% CI	Lower 95% CI	
	79.0%	100.0%	
	Upper 95% CI	Upper 95% CI	
	93.1%	100.0%	

The area under the ROC curve (AUC) for the iELISA was 1.000 (95% CI of 0.981 to 1.000). DSe at cut-off value > 40.5% was 98.92% (95% CI 94.2% - 100%) and DSp 100% (95%CI 96.6% - 100%) and the performance index (sum of values of DSe plus DSp) (Gall & Nielsen 2004) was 198.92%. This was the maximum value possible for performance index. The cut-off value of 40.5% was recommended for the iELISA (Figure 5-13).
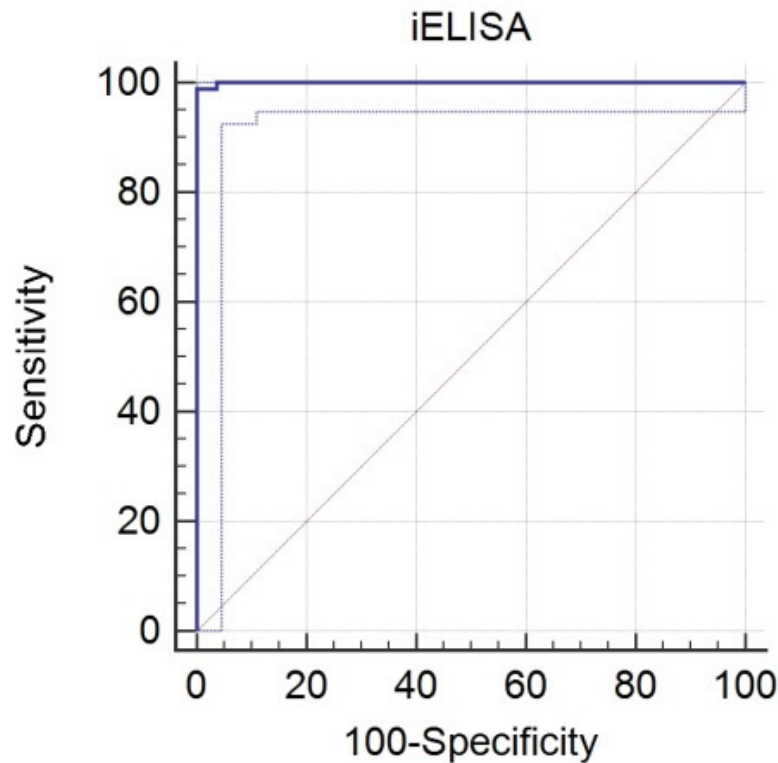
Figure 5-13 Receiver-operator characteristics (ROC) curve iELISA

Test agreement between categorised result (cut-off value >40.5%) and infected and uninfected reference panel, as evaluated by the unweighted Kappa coefficient was 0.9899 (95%CI 0.9702-1).

All sera were analysed in duplicate by iELISA, providing 354 sets of duplicate calculated O/D values. The Pearson's correlation coefficient was 0.97. The points on the scatter graph were distributed on both sides of the line of equality, with more points to the right and below the line of equality (Bland & Altman 2003) (Figure 5-14).

The mean of the difference between the duplicate tests was 0,011, close to zero, as is expected with repeat measurements (Bland & Altman 2003). The standard deviation was 0.117 and the repeatability coefficient (1.96 x standard deviation) was 0.229. The frequency distribution curve of the differences is bell-shaped, with 87.6% of measurements within one standard deviation of the mean, 98.0% within two standard deviations and 98.9% within three standard deviations (Figure 5-15).

The standard deviation appeared uniform trough out the range as illustrated in the Bland-Altman plot (Figure 5-16).
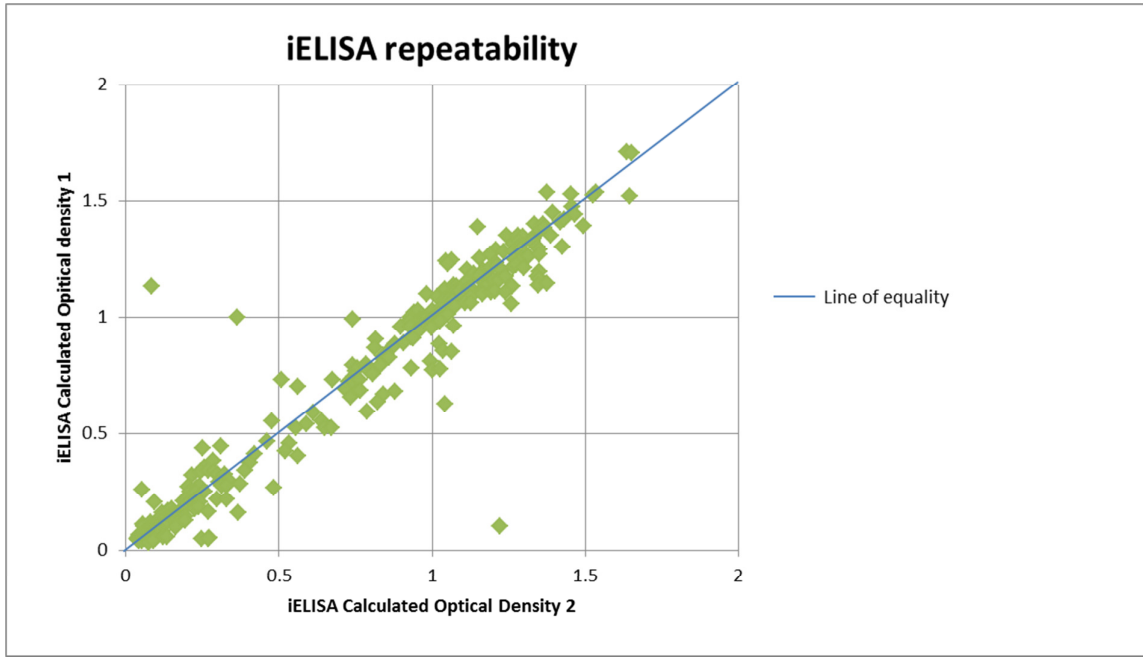
Figure 5-14 Scatter graph of duplicate calculated optical density measurements by iELISA
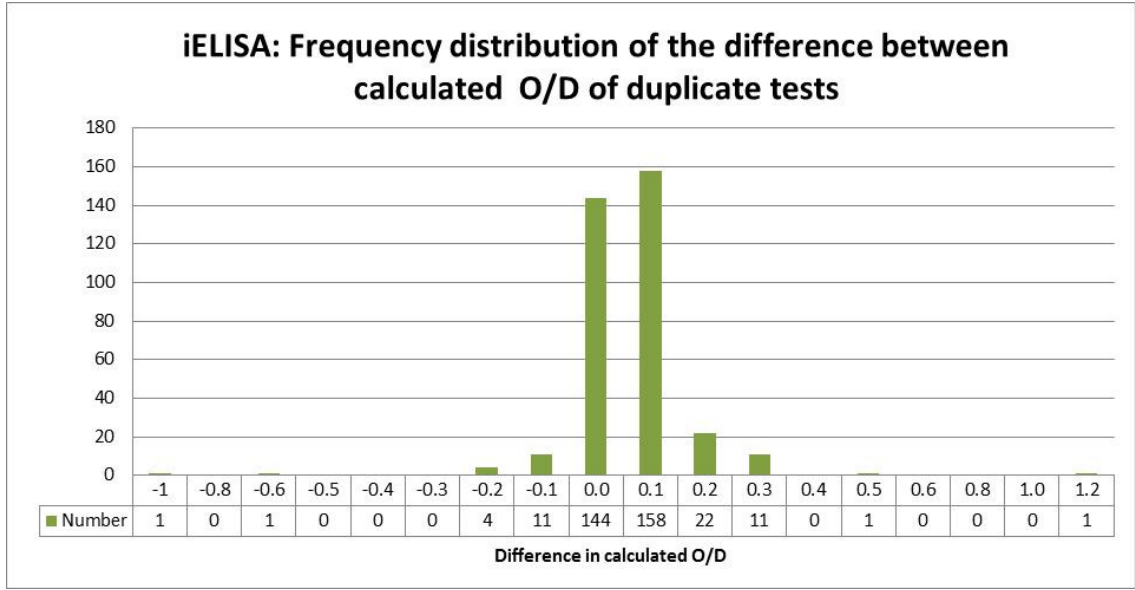
Figure 5-15 Histogram of differences between duplicate O/D measurements with iELISA.
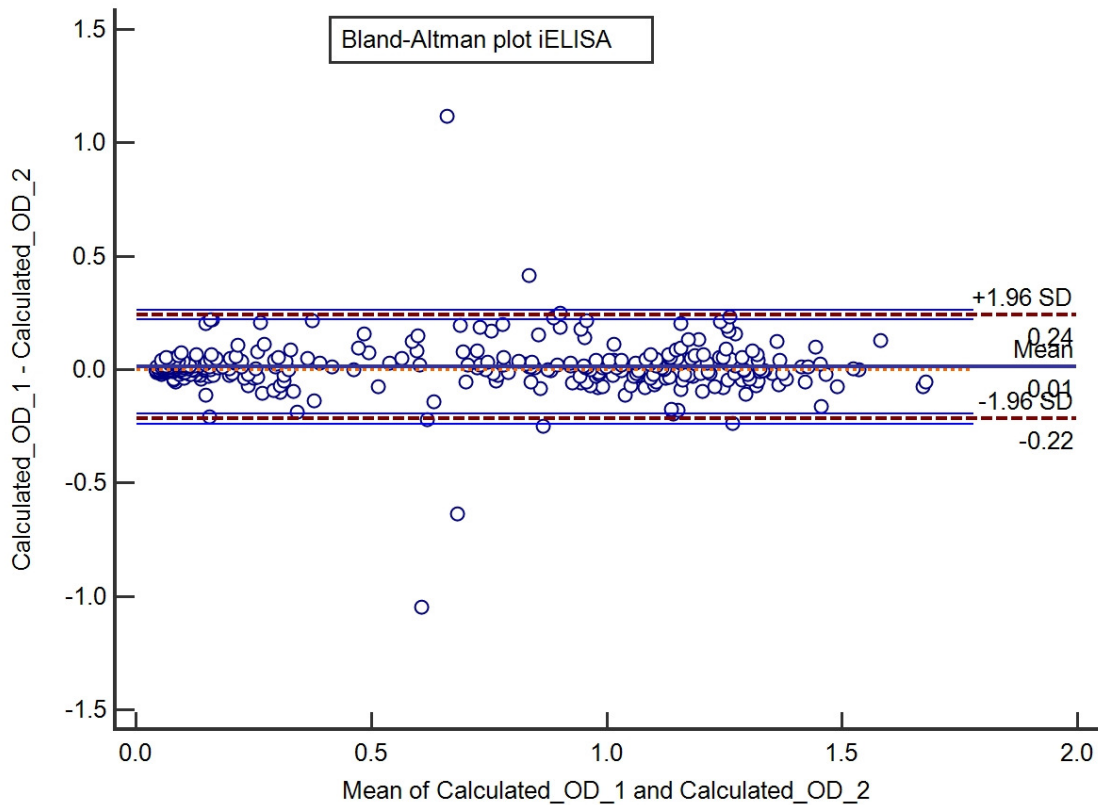
Figure 5-16 iELISA Bland-Altman plot

5.2.5.3 Complement fixation test (CFT)

The CFT titres for the uninfected reference panel were all 0 iU/ml. The CFT titres for the infected reference panel ranged from 0 to 784 IU/ml, with 65 with a titre of >20 iU/ml.

Diagnostic sensitivity (DSe) was calculated as 69.9%% (95% CI 69.4% - 87.2%) and diagnostic specificity as 100.0% (95% CI 100.0% - 100.0%) at the cut-off value of >20 iU/ml (Table 5-4).

Table 5-4 CFT 2x2 table for DSe and DSp with cut-off value of 20 iU/ml.

CFT (20iU/ml)	Known infected	Known uninfected	Total
Test positive	65	0	65
Test negative	28	107	135
Total	93	107	200
	Diagnostic sensitivity	Diagnostic specificity	
	69.9%	100.0%	
	Lower 95% CI	Lower 95% CI	
	69.4%	100.0%	
	Upper 95% CI	Upper 95% CI	
	87.2%	100.0%	

The area under the ROC curve (AUC) for the CFT was 0.871 (95% CI of 0.816 to 0.94) (Figure 5-17.).

DSe at cut-off value >0 iU/ml was 74.19% (95% CI 64.1% - 82.7%) and DSp 100% (95%CI 96.6% - 100%), and the performance index (sum of values of DSe plus DSp) (Gall & Nielsen 2004) was 174.19. This was the maximum value possible for performance index. The cut-off value of >0 iU/ml were recommended for the CFT.
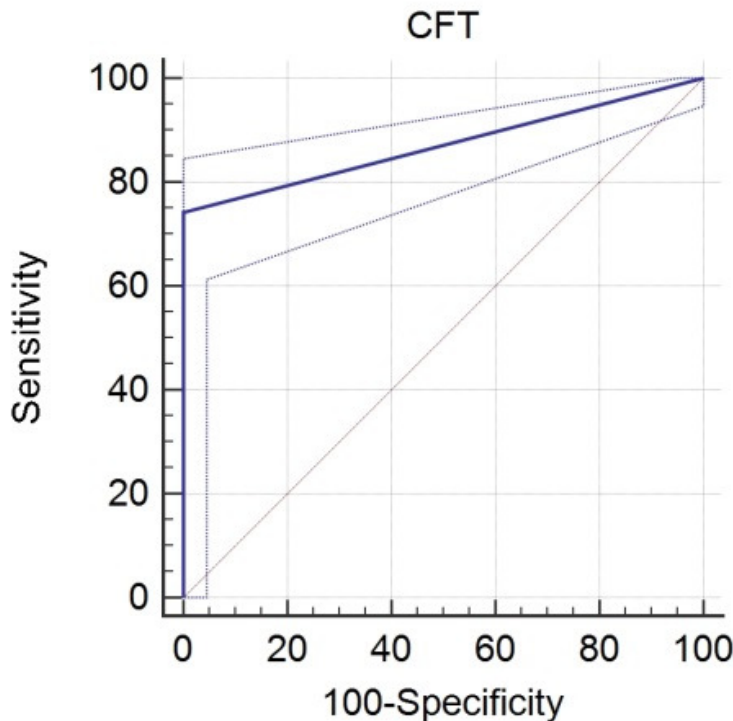
Figure 5-17 Receiver operator characteristics (ROC) curve CFT

Test agreement between categorised result (cut-off value >0 iU/ml) and infected and uninfected reference panel as evaluated by the unweighted Kappa coefficient was 0.7547 (95%CI 0.6654-0.844).

5.2.5.4 Fluorescence polarisation assay (FPA)

The final FPA results for the uninfected reference panel ranged from -101 to 16 ΔmP, with all the sera < 20 ΔmP, the manufacturer's recommended cut-off value (Diachemix). In the infected reference panel it ranged from -1 026 to 273 ΔmP, with 91 sera > 20 ΔmP (Table 5-5).

Table 5-5 FPA 2x2 table for DSe and DSp (cut-off value 20 ΔmP)

FPA (20ΔmP)	Known positive	Known negative	Total
Test result positive	91	0	91
Test result negative	2	107	109
Total	93	107	200
	Diagnostic sensitivity	Diagnostic specificity	
	97.8%	100.0%	
	Lower 95% CI	Lower 95% CI	
	94.9%	100.0%	
	Upper 95% CI	Upper 95% CI	
	100.8%	100.0%	

Diagnostic sensitivity (DSe) was calculated as 97.8% (95% CI 94.9% - 100.8%) and diagnostic specificity as 100.0% (95% CI 100.0% - 100.0%) at the cut-off value of 20 ΔmP.

The area under the ROC curve (AUC) was 0.989 (95% CI 0.962 to 0.998) (Figure 5-18)Figure 5-18 Receiver operator characteristic (ROC) curve FPA.

The ROC curve analyses data showed that DSe could be improved by reducing the cut-off value to >16 ΔmP without decrease in DSp. DSe at the cut-off value of > 16 ΔmP was 97.85% (95% CI 92.4% - 99.7%) and DSp 100% (95%CI 96.6% - 100%) and the performance index (sum of values of DSe plus DSp) (Gall & Nielsen 2004) was 197.85. This was the maximum value possible for performance index. The cut-off value of > 16 ΔmP was recommended for the FPA.
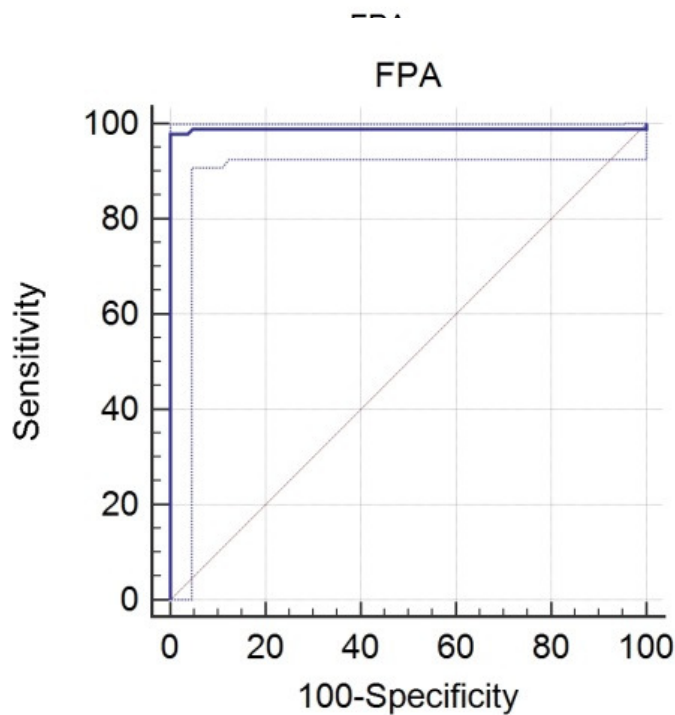
Figure 5-18 Receiver operator characteristic (ROC) curve FPA

Test agreement, between categorised result (cut-off value > 16 ΔmP) and infected and uninfected reference panel as evaluated by the unweighted Kappa coefficient was 0.9799 (95%CI 0.9521-1).

All sera were tested in duplicate by FPA, with duplicate analysis of 41 sera in a repeated run: 14 with wide variation between duplicate readouts, 8 with high readouts (≥173ΔmP), 6 with low readouts (≤5ΔmP), 6 with low positive readouts (23 to 83ΔmP) and 7 with suspect readouts (10 to 20ΔmP) according to interpretation recommended by manufacturer. There were 394 sets of duplicate ΔmP read outs for analyses of repeatability. The Pearson's correlation coefficient was 0.17. The points are roughly equally distributed both sides of the line of equality. Many points are close to the line of equality, while others diverge nearly at a right angle from the intersection of the X and Y axis at 0. The difference between duplicate read outs increased with increase in mean value of the two tests. There was weak correlation between duplicate tests, but no clear bias (Bland & Altman 2003) (Figure 5-19).

The mean of the difference between the duplicate tests was 25.53, which is far from zero. This indicated that the two tests were not true duplicate measurements (Bland & Altman 2003). The standard deviation was 314 and the repeatability coefficient 616. The frequency distribution curve of the differences had a bell shape, but skewed to the left, with an extended tail to the right. There were 94.4% of measurements within one standard deviation of the mean, 980% within two standard deviations and 98.5% within three standard deviations (Figure 5-20).

The Bland-Altman plot reveal differences that increase with increase in the mean value, as well as a number of measurements that are very high compared to the mean. At the same time, there are a large number of points that is close to the mean (Figure 5-21).

The distribution of the differences was not normal. The magnitude of the standard deviation was large compared to cut-off values.
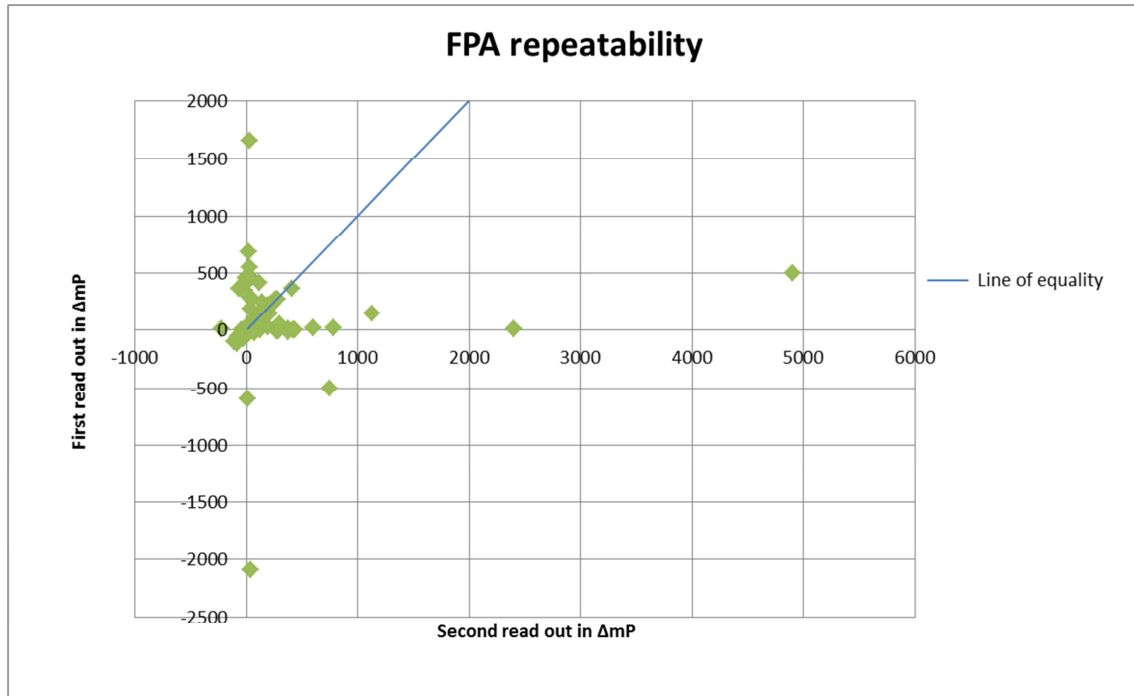
Figure 5-19 Scatter graph FPA duplicate read outs
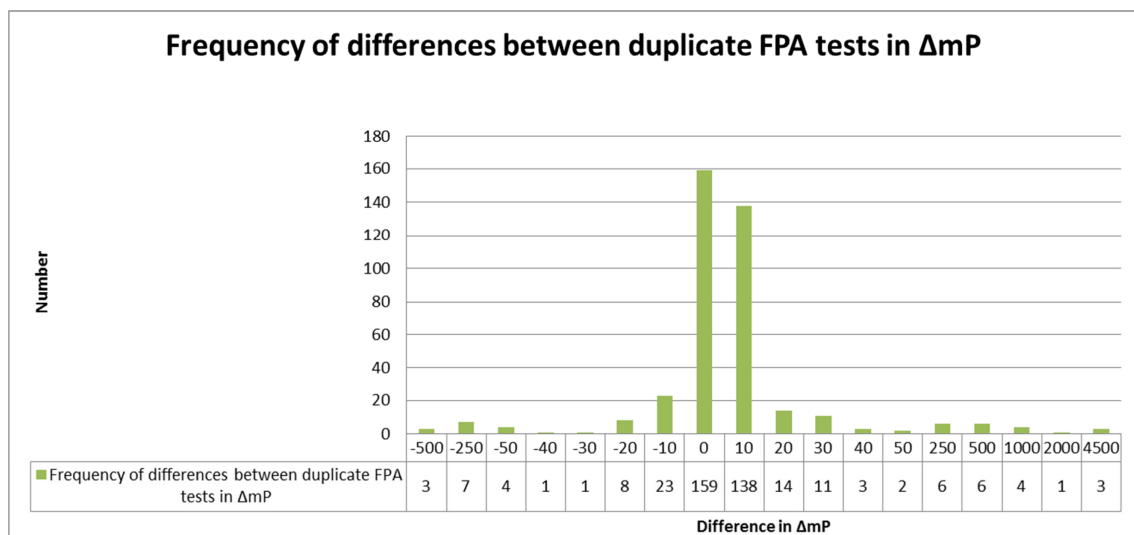
Figure 5-20 Histogram of distribution of differences in ΔmP measurements between duplicate FPA tests
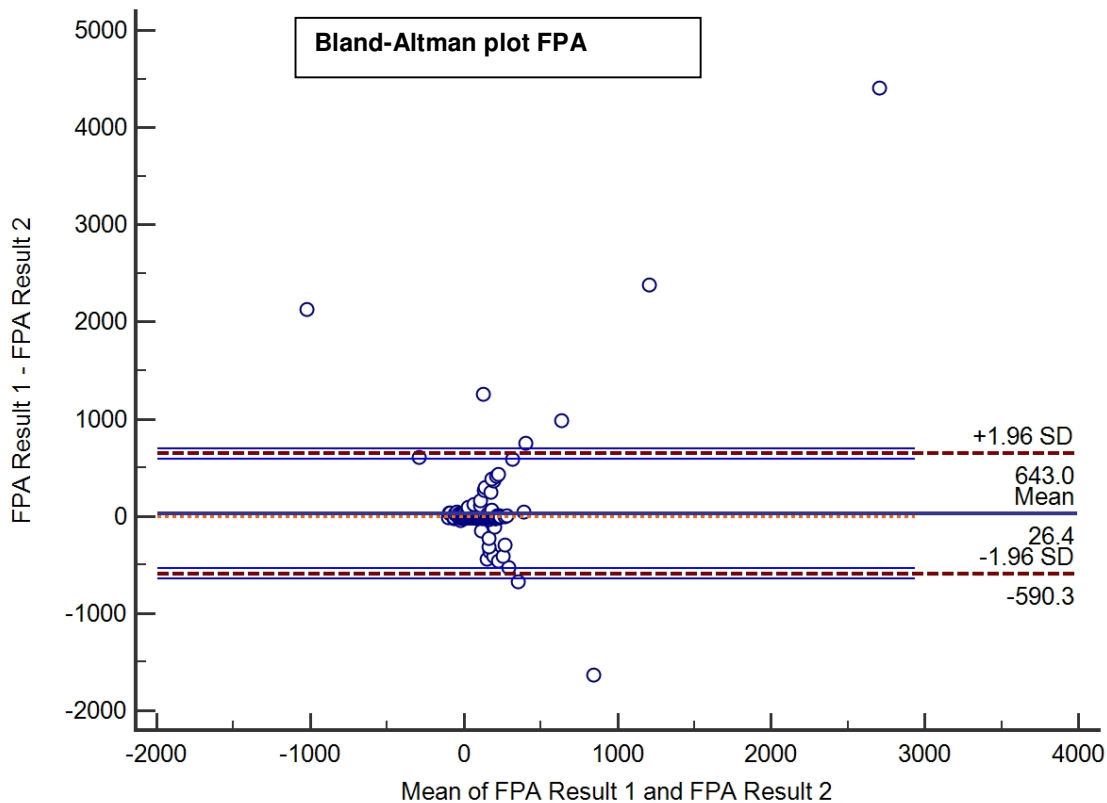
Figure 5-21 Bland Altman plot duplicate FPA results

Table 5-6 Diagnostic performance of index tests

Test	Cut-off	DSe	DSp	Performance index	Test agreement (Kappa)	AUC
RBT		98.9%	98.1%	197.0%	0.9699	
iELISA	>40.5%	98.9%	100.0%	198.9%	0.9899	1.000
CFT	>0iU/ml	74.2%	100.0%	174.2%	0.7547	0.871
FPA	>16ΔmP	97.9%	100.0%	197.9%	0.9799	0.989

(Note: test agreement refers to agreement with inclusion in infected/uninfected reference panels).

5.2.6 Test agreement between index tests

Unweighted Kappa values for test agreement between the index tests were the lowest for agreement between the CFT and RBT (0.26, 0.136 - 0.383 95%CI) followed closely by the iELISA and FPA versus the RBT with 0.263, 0.160 – 0.365 95% CI and 0.276, 0.173 – 0.379 95% CI respectively. Other values were: CFT vs iELISA: 0.414, 0.317 – 0.511 95% CI, FPA vs iELISA 0.685, 0.608 – 0.762 95% CI and FPA vs CFT 0.39, 0.291 – 0.49 95% CI (Table 5-7).

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The index for positive agreement (P$_{pos}$) was lowest for RBT vs CFT at 44.3% and RBT vs iELISA and CFT, both at 54%. FPA vs CFT was 56.8%, CFT vs iELISA 58.7% and FPA vs iELISA 83.8%.

The index for negative agreement (P$_{neg}$) was lowest for the RBT vs the IELISA at 69.4%, followed by the RBT vs FPA at 70.8%, FPA vs CFT at 77.3%, CFT vs iELISA at 77.5% and CFT vs RBT at 80.8%.

Table 5-7 Test agreement amongst index tests of categorised results using recommended cut-off values

	Unweighted Kappa	P$_{pos}$	P$_{neg}$
iELISA vs RBT	0.263	54%	69.40%
	0.160 - 0.365 95% CI	48.7% - 59.3% 95% CI	64.5% - 74.3% 95% CI
CFT vs RBT	0.26	44.30%	80.80%
	0.136 - 0.383 95% CI	39.1% - 49.6% 95% CI	76.6% - 85.0% 95% CI
FPA vs RBT	0.276	54%	70.80%
	0.173 - 0.379 95% CI	49.2% - 59.7% 95% CI	66.0% - 75.6% 95% CI
CFT vs iELISA	0.414	58.70%	77.50%
	0.317 - 0.511 95% CI	53.5% - 63.9% 95% CI	73.1% - 81.9% 95% CI
FPA vs iELISA	0.685	83.80%	84.70%
	0.608 - 0.762 95% CI	19.9% - 87.7% 95% CI	80.5% - 88.8 % 95% CI
FPA vs CFT	0.39	56.80%	77.30%
	0.291 - 0.490 95% CI	51.5% - 62.0% 95% CI	72.9% - 81.8% 95% CI

P$_{pos}$ is the index for positive agreement, and P$_{neg}$ the index for negative agreement.

5.2.7 Serological diagnosis of brucellosis in infected buffalo herds

5.2.7.1 Diagnostic performance of tests used in combination

The DSe and DSp were calculated for all the possible combinations of two of the index tests in series and parallel at the cut-off points determined. Use of two tests in series improved the DSe to 100% in all combinations, while the use of two tests in parallel improved the DSp to 100% in all combinations. Where two of iELISA, CFT or FPA used in parallel the DSe and DSp was 100% (Table 5-8 and Table 5-9).

Table 5-8 Diagnostic performance of two tests used in series

	RBT pos AND iELISA > 40,5%	RBT pos AND FPA > 16 ΔmP	RBT pos AND CFT > 0 iU/ml	iELISA > 40,5% AND CFT > 0 iU/ml	iELISA > 40,5% AND FPA > 16 ΔmP	CFT > 0 iU/ml AND FPA > 16 ΔmP
DSe	97.8%	96.8%	73.1%	73.1%	96.8%	72.0%
No positive	91	90	68	68	90	67
DSp	100.0%	100.0%	100.0%	100.0%	100%	100.0%
No positive	0	0	0	0	0	0

Table 5-9 Diagnostic performance of two tests used in parallel

	RBT pos OR iELISA > 40,5%	RBT pos OR FPA > 16 ΔmP	RBT pos OR CFT > 0 iU/ml	iELISA > 40,5% OR CFT > 0 iU/ml	iELISA > 40,5% OR FPA > 16 ΔmP	CFT > 0 iU/ml OR FPA > 16 ΔmP
DSe	100.0%	100.0%	100.0%	100.0%	100%	100.0%
No positive	93	93	93	93	93	93
DSp	98.1%	98.1%	98.1%	100.0%	100%	100%
No positive	2	2	2	0	0	0

5.2.7.2 True prevalence and predictive values

True prevalence and predictive values was calculated for herds C and E where sampling was representative or the whole herd was sampled. PPV for the iELISA, CFT and FPA were 100% in both herds. The PPV of the RBT is 97.3% in herd C and 98.4 in herd E. The NPV was the highest for the RBT with 99.2% in herd C and 98.7% in herd E. The negative predictive value was the lowest for the CFT with 89% in herd C and 73.1% in herd E. Calculation of true prevalence with results from different tests gave different values (Table 5-4).

Table 5-10 True prevalence and positive and negative predictive values for herds C and E

Herd		RBT	iELISA >40.5%	CFT >0iU/ml	FPA >16ΔmP
	Number positive	71	125	41	116
	Apparent prevalence	41.5%	73.1%	24.0%	67.8%
	True prevalence	40.8%	73.9%	32.3%	69.3%
C	Sensitivity	98.9%	98.9%	74.2%	97.9%
	Specificity	98.1%	100.0%	100.0%	100.0%
	Positive predictive value	97.3%	100.0%	100.0%	100.0%
	Negative predictive value	99.2%	97.0%	89.0%	95.4%
	Number positive	30	38	24	37
	Apparent prevalence	54.5%	69.1%	43.6%	67.3%
	True prevalence	54.3%	69.9%	58.8%	68.8%
E	Sensitivity	98.9%	98.9%	74.2%	97.9%
	Specificity	98.1%	100.0%	100.0%	100.0%
	Positive predictive value	98.4%	100.0%	100.0%	100.0%
	Negative predictive value	98.7%	97.5%	73.1%	95.5%

6 DISCUSSION

Bovine brucellosis is endemic in the African buffalo population of the GKNPC and domestic cattle of South Africa. The risk of spread of the disease by translocation of African buffaloes is mitigated by repeated pre-movement serological testing. Conventional tests developed and validated for cattle, namely the RBT and CFT are used for this purpose (DAFF 2006). This study investigated the comparative performance of four serological tests, the RBT, iELISA, CFT and FPA for serological diagnosis of brucellosis in African buffaloes and compared their performance with that in cattle.

Two uninfected herds (A and B) and four infected herds (C, D, E and F) were recruited. Evidence of isolation of *B. abortus* biovar 1 from one or more animals per herd confirmed the infection of herds C, D, E and F with *B. abortus* biovar 1. Herd C, located in the GKNPC, was an endemically infected population known to be infected for more than 40 years (de Vos & van Niekerk 1969) and herds D, E and F were recently infected with abortions occurring at the time of sampling in herd E. The infected reference panel included sera from all four infected herds, presumably with sera from animals in all stages of infection, with and without clinical signs. Spectrum bias was therefore not considered to be a serious concern in this study (Greiner, Gardner 2000).

The infected herds were from five of the nine provinces. Herd C was from the GKNPC in Limpopo and Mpumalanga, and the others from commercial farms in North West, KwaZulu Natal and the Free State, which insured a good representation of the different environments and infectious agents potentially inducing cross-reactive immune responses in African buffaloes in South Africa. The infected reference panel included sera from both uninfected herds recruited. The uninfected herds were from two of the nine provinces in South Africa, one from the Hluhluwe-Imfolozi Park in KwaZulu Natal, and the other from a commercial farm in Mpumalanga, which only offered limited representation of the different environments and infectious agents potentially inducing cross-reactive immune responses African buffaloes in South Africa were exposed to. The low representation of profiles of exposure in the uninfected reference panel may have contributed to the very high DSp.

The infected reference panel consisted of 93 individual sera and the uninfected reference panel of 107 individual sera. This exceeded the OIE requirement for provisional validation of tests in wildlife species for which a test is available in a taxonomically related species (OIE 2014). It is also more than the 87 required for establishing a DSe or DSp of 94% to 99% allowing a 5% error with 95% confidence (OIE 2013). The DSe and DSp estimated were in this range for the RBT, iELISA, FPA but for the CFT the DSe was outside this range. Adequate numbers of specimens were included in the study for the findings to be accepted for provisional validation of the tests.

Overall the study was successful in estimating and comparing the DSe, DSp, performance index, and area under the ROC curve as indicators of the diagnostic performance characteristics of the tests in African buffaloes. Test agreement between tests was determined in terms of the unweighted Kappa

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and indices of positive and negative agreement. The study also provided data that may be used for provisional recognition of the tests by the OIE.

Comparing the index tests with each other, the iELISA had the highest performance index (198.9%) and area under the ROC curve (1.0), followed by the FPA (197.9% and 0.989). The RBT was third in terms of performance index (197%). The CFT had the lowest performance index (174.2%) and area under the ROC curve (0.871), though the area under the ROC curve could still be considered substantial. The lower performance of the CFT was due to the low diagnostic sensitivity. In terms of test agreement (index test categorised results compared with reference panels), the iELISA (0.9899), FPA (0.9799) and RBT (0.9699) were almost perfect and the CFT showed substantial test agreement (0.7547).

The RBT is a simple test that does not require expensive specialised equipment (Gall & Nielsen 2004) and performed very well in this study. Due to the lower specificity, the use of the RBT alone is not recommended where there is a very low level of infection, e.g. at the end of an eradication campaign or in brucellosis free populations where animals incorrectly identified as positive may be culled and/or extensive investigations done to establish the true status of the animal (Corbel 1985). Resources may be wasted this way and the declaration of a population as free from brucellosis be delayed (Corbel 1985). Screening tests are applied to healthy populations to detect presence of disease and the disease is usually confirmed by a second, confirmatory test (Cameron 2002). A test is required that is sensitive, relatively cheap and can be applied to large numbers of specimens (Cameron 2002). Based on the relatively high DSe the RBT and the fact that it is readily available, it should be retained as a screening test for brucellosis in African buffaloes.

The CFT is technically a complicated, labour intensive test that is not easily standardised and require high quality serum (Gall & Nielsen 2004). Other challenges with the CFT are anti-complementary reactions and the prozone phenomenon (Nielsen & Yu 2010, OIE 2009a). Three sera were excluded from selection into the reference panels because of anti-complementary reactions. The CFT was shown to have low sensitivity and should therefore not be used on its own where accurate identification of uninfected animals is essential, such as with international movements, where disease is spreading actively and early identification of infected animals is required or when the disease is nearly eradicated and infected animals incorrectly identified as uninfected bay cause a break down later (European Food Safety Authority 2006, Corbel 1985). The combination of CFT in series with the RBT as well as the FPA and iELISA had DSe <75% and should also be avoided under such circumstances, or tests must be interpreted in parallel. The CFT is suitable for confirmation of disease where correct identification of infected animals is critical based on the high, though one should be cautious since possible the low spectrum of exposure in this study may be biased towards a high DSp.

The iELISA is less complex than the CFT to standardise, and simpler to perform, it is rapid, reactivity is measured objectively and can be automated providing digital results (Nielsen & Yu 2010, Nielsen, Smith, Conde et al. 2004, Saegerman, de Waele, Gilson et al 2004). What also makes it attractive for use in wildlife species is its ability to analyse haemolysed sera and the possibility to adapt it to use in a field laboratory (Gorsich, Bengis, Ezenwa et al. 2015, Godfroid, Al Dahouk, Pappas et al. 2013). Both the DSp and DSe determined for the iELISA in this study were very high. The iELISA will therefore be suitable as both screening and confirmatory test of brucellosis in African buffaloes.

The FPA is a homogenous assay, simple to perform and can be adapted to field use, and applied to milk and blood as well as serum (Nielsen & Yu 2010, Nielsen, Smith, Gall et al. 2001, Nielsen, Lin, Gall et al. 2000). The DSe and DSp of the FPA were very high with perfect DSp and performance index and area under the FOC curve second to, and very close to the iELISA. Repeatability was, however, very weak. FPA requires samples of high quality, free of bacteria and particulate matter, though haemolysis does not affect the outcome (Nielsen, Lin, Gall et al. 2000). These characteristics make the FPA suitable for screening and confirmatory test of brucellosis in African buffaloes.

Test agreement between categorised results of index tests varied for different combinations, with all Kappa values above 0. It should be noted that test agreement indicates which tests can be used to replace each other without losing DSe or DSp, but does not indicate which of the two tests perform better (Cameron 2002). The highest agreement was between the FPA and the iELISA (unweighted Kappa = 0.685), which was considered substantial. Test agreement between the CFT and iELISA was moderate (unweighted Kappa = 0.414) and between other pairs of tests, fair (0.263 to 0.39). Agreement on positive (P_{pos}) and negative test results (P_{neg}) were also the highest between the FPA and iELISA (P_{pos} = 83.8% and P_{neg} = 84.7%). The lowest agreement on positive test results was between the CFT and the RBT (44.3%) and the lowest agreement on negative test results between the RBT and iELISA (69.5%).

P_{pos} is affected by the DSe and difference in detection of Ig classes by the index tests (Saegerman, Dohoo Wright et al 1986). The high P_{pos} between the FPA and iELISA could therefore be explained by the high DSe of both tests and detection of similar classes of IgG. The iELISA kit used in this study (IDEXX Brucellosis Serum X2 *Brucella abortus* antibody test kit) detects ruminant IgG (IDEXX n.d.), while no information is available on the specificity of the FPA for immunoglobulin classes. A strong correlation was seen between the results of the FPA and an iELISA in infected European cattle (McGiven, Tucker, Perret et al 2003). The lower P_{pos} between the iELISA and RBT could be explained by the detection of IgM and IgG by the RBT and IgG by the iELISA.

iELISAs which detects IgG1, IgG2 or both is more sensitive than the RBT and CFT in cattle with chronic infections (Saegerman, de Waele, Gilson et al 2004, Sanogo, Thys, Achi et al. 2013). The detection of different immunoglobulin classes by the iELISA and the CFT in African buffaloes, and possible differentiation of DSe in acute and chronic cases was supported by the comparison of

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infected reference panel titres between herds. iELISA titres of the infected reference panel showed a median of 99.1% for herd C with chronic endemic infection, and 83.4% in herd E which were known to be more recently infected and where the disease was actively spreading at the time of sampling. In comparison, the median of the infected reference sera from herd C's CFT titres (25.5 iU/ml) was lower than that of herd E (12 iU/ml). The higher sensitivity of the iELISA to the CFT found in this study may be related to the large proportion (60/93) of sera in the infected reference panel that came from the chronic endemic infected herd E. The study by Gorsich et al 2015 on the same herd, also found the CFT to be far less sensitive than the iELISA (Gorsich, Bengis, Ezenwa et al. 2015). The serum from one chronically infected animal of herd C, with *B. abortus* infected hygromas (B. Reininghaus pers. com. 25 July 2013) was positive on iELISA (S/P ratio of 113.2%), RBT and FPA (four readings between 131 and 185 ΔmP), but negative on CFT. The probability of prozone phenomenon was not excluded in this case.

P_{pos} between the CFT and RBT of 44.3% indicates that the sera that tested false negative with the CFT are not always the same sera that tested false negative with the RBT, which support parallel use of the two tests when accurate identification of negative results are essential.

The OIE require that diagnostic assay results must be interpretable with a defined meaning in terms of the diagnosis within the context of a specified purpose, before the assay will be recognised as 'fit for purpose' (OIE 2012). Using the values for DSe and DSp determined through this study, positive and negative predictive values for serological results of the four index tests were calculated. Classification of animals as infected or uninfected based on results of the four index tests proved successful in terms of the probability that the classification was correct. True prevalence of infection in herds was calculated based on the DSe and DSp of the four index tests. The four index tests were therefore considered fit for purpose for demonstration of freedom from infection when used in combination, e.g. for pre-movement testing, or used individually for screening for disease in populations, and for determination of prevalence of infection in herds.

The usefulness of a test in the field is not only determined by the DSe and DSp, but also the negative and positive predictive values (Larson 2008). Predictive values are affected by the prevalence of the disease, with the negative predictive value decreasing as prevalence increases, and the positive predictive value increasing as prevalence increase (Larson 2008). The positive predictive values of the iELISA, CFT and FPA were all 100%, and for the RBT 98.4% in herd C and 97.3% in herd D. For situations where confirmation of a positive RBT result is required, it was shown that testing RBT in series with any of the other tests will increase the DSp of the combination to 100%, allowing for serological confirmation of brucellosis. This is important where the disease is absent and where high DSp is important (Larson 2008), e.g. in herd A the positive RBT sera were classified as negative when interpreted in series with the iELISA, CFT or FPA.

The negative predictive values in herd C and E were very good at above 95% for the RBT, iELISA and FPA, but lower for the CFT at 89% in herd C and 73.1% in herd E. Where an accurate diagnosis of infected animals is of great importance, for example in pre-movement testing, or where the disease is actively spreading and abortion needs to be prevented, the tests can be used in parallel (Larson 2008). This will be especially applicable to situations of high prevalence of disease, since the negative predictive value will be lower with lower sensitivity as well as higher prevalence (Larson 2008). All combinations of two of the index tests used in parallel achieved a DSe of 100%. As example, one cow with *B. abortus* infected hygroma confirmed in herd C (B. Reininghaus pers. comm. 25 July 2013) tested negative with the CFT. When the CFT was applied in parallel with the RBT, iELISA of FPA the cow was correctly classified as infected.

Repeatability of tests within the same run for the iELISA was very strong. The repeatability of the RBT was almost perfect, and interoperator reproducibility of the RBT was substantial. The repeatability of the FPA was very weak. On the box and whiskers plots for all the FPA readings, many outliers were seen. The herds were most of the outliers occurred were herds A and C, with no outliers seen in herd E. Sera from herds A and C were stored for long periods compared to the sera from herd C. Solid material is known to interfere with the readings (Nielsen, Lin, Gall et al. 2000). Outliers were therefore considered artefacts due to low serum quality or solids related to prolonged storage in a freezer. After removal of readings identified as artefact, there were fewer and less extreme outliers. (Nielsen, Lin, Gall et al. 2000). Despite the very weak repeatability, categorised FPA results (cut-off value of > 16 ΔmP) showed substantial agreement with the reference panels. The centrifugation of serum with particulate matter is an optional preliminary step in the procedure (Diachemix n.d.) and should be considered with stored serum, irrespective of whether particulate matter is visible or not.

Gall and Nielsen (2004:989) reported the following mean DSe and DSp for serological tests for brucellosis in cattle: RBT: DSe 81.2% and DSp 86.3%, iELISA DSe 96% and DSp 93.8%, CFT DSe 89% and DSp 83.5% and FPA DSe 97.9% and DSp 98.9% (Gall & Nielsen 2004). Compared to these values, the difference between DSe and DSp of the tests in cattle and African buffalo were: for the RBT the DSe was found 18.7% higher and the DSp 11.8% higher, for the iELISA the DSe was found 3.9% higher and the DSp 6.2% higher, for the CFT the DSe was found 13.8% lower and the DSp 16.5% higher and for the FPA the DSe was found equal and the DSp 1.1% higher. The index tests performed better in the African buffaloes than the means reported for cattle, except for the DSe of the CFT that were lower.

Paweska, Potts and others 2002 reported on the validation of an iELISA and CFT in cattle using reference panels sourced from South Africa, North America and Europe. The performance characteristics reported were: iELISA DSe 100% and DSp 99.8%, CFT DSe 83.3% and DSp 100% (Paweska, Potts, Harris et al. 2002). Compared to this data, , the iELISA had a DSe of 1.1% less and

DSp of 0.2% more, and the CFT DSe of 9.1% less and equal DSp when applied on sera from African buffalo.

The diagnostic performance of the FPA and iELISA in African buffaloes were therefore comparable to the performance in cattle, with DSe and DSp differing 0% to 6.2% between species. The RBT performed better in the African buffaloes with DSe and DSp more than 10% higher than reported for cattle. The CFT performed poorer with regards to the DSe, by 9.2% to 13.8% but equal or better with DSp with 16.5%.

In agreement with this study, Gorsich, Bengis et al (2015) also found the RBT, CFT and iELISA useful diagnostic tool for brucellosis in African buffaloes, though the DSe for CFT was considerably lower than that of the RBT and iELISA. It is important to note that Gorsich, Bengis, Ezenwa et al (2015: 11) limited their study to African buffaloes in the KNP and used a different iELISA kit. The was intended to provide credibility to the serological test results for use in research studies in the KNP (Gorsich, Bengis, Ezenwa et al. 2015, Gorsich, Ezenwa, Cross et al. 2015) and it is therefore not necessarily applicable to the whole population of African buffaloes in South Africa.

Evaluation of diagnostic assays developed for cattle in American bison – also of the tribe Bovini- using a golden standard (*Brucella* isolation from various tissues after slaughter) indicated that the buffered antigen plate agglutination test (BPAT), CFT, iELISA and FPA all performed well, with the FPA and iELISA performing better than the BPAT and CFT and the CFT with the lowest sensitivity (Gall, Nielsen, Forbes et al. 2000). The performance and relative performance of the serological tests in American bison are comparable to the performance in African buffalo as determined in this study and what is known for cattle (Gall & Nielsen 2004).

It can be said that the comparable performance of the serological tests for brucellosis in three species of the tribe Bovini supports the OIE strategy of accepting data from smaller number of specimens for provisional validation of diagnostic tests in wildlife where there is a fully validated test in a related species (OIE 2014). The differences between the species, and between this study and the study by Gorsich, Bengis, Ezenwa et al 2015 supports the need for validation in every species with studies of sufficient size and representation of epidemiological settings (OIE 2010, Greiner, Gardner 2000).

Overestimation of diagnostic accuracy is a common problem (Rutjes, Reitsma, Di Nisio et al. 2006) especially for case-control studies and where there is no perfect reference standard (Whiting, Rutjes, Westwood et al. 2013, Whiting, Rutjes, Reitsma et al. 2004). In our study, the inclusion of the index tests in the criteria for the composite reference standard is a potential source of incorporation bias with overestimation of the performance, especially the diagnostic sensitivity (Greiner, Gardner 2000). The inclusion of false positive animals in the positive reference panel could not be entirely ruled out (Bisoffi, Buonfrate, Sequi et al. 2014) in our study, though the high specificity of 98.1% for the RBT and 100% for the CFT, FPA and iELISA at the cut-off values used in the inclusion criteria made it

highly unlikely. The exclusion of infected animals, presenting as false negatives when measured against the criteria for inclusion in the infected reference panel, from the infected reference panel was also possible. For example, one cow from herd E included in the infected reference panel had a vaginal discharge on the day of specimen collection and *B. abortus* was isolated, and was positive with the RBT and FPA (cut-off >20 ΔmP) and negative on the iELISA (cut-off >80%) and CFT (cut-off>20 iU/ml) tests. If the bacteriology results were not available, she would have been excluded from the infected reference panel. With the possibility of overestimation of the diagnostic accuracy, tests on individual animals should be interpreted in the context of the herd status where animals have to be certified as negative (Racloz, Schelling, Chitnis et al. 2013). We consider as a possible constraint in the determination of the DSp, which may have led to overestimation of the DSp, the low representation of the available uninfected reference panel of the African buffalo population of South Africa. This may be of particular concern in low prevalence situations, and multiple tests or further investigations, e.g. bacteriology, are recommended before an animal or herd that was previously considered free of the disease is declared infected.

Validation of diagnostic tests is a continuous process (OIE 2013, Jacobson 1998) and the evaluation of the diagnostic performance of the index tests should be continued with use in the general population. Increase in numbers and better representation of epidemiological settings will improve the confidence with which the tests can be applied to the all herds in South Africa, and the interpretation of the results.

Recommendations arising from this study for future research:

- Investigation into differential diagnoses for the clinical signs of brucellosis in African buffaloes.
- Investigations into the sensitivity of bacteriological culture and molecular techniques to identify infected animals, and their possible use as a golden reference standard for diagnostic accuracy studies in African buffaloes.
- Diagnostic accuracy studies that include a representation of epidemiological settings, especially in the uninfected reference panel, and animals affected with diseases causing the same clinical signs than brucellosis.

7   CONCLUSION

The study provided diagnostic performance data for four bovine brucellosis serological tests (RBT, CFT, iELISA and FPA) for use in African buffaloes in South Africa. The data met the requirements towards provisional recognition of the RBT, CFT, iELISA and FPA by the OIE for diagnostic tests to be used for international movement of animals as set out in the OIE Validation Guidelines (OIE 2014) and found fit for purpose when used in combination for demonstration of freedom from infection in individual animals and populations for pre-movement testing, screening for disease in populations, and for determination of prevalence of infection in herds. Considering that the iELISA and FPA were found superior to the CFT in sensitivity and highly comparable in specificity, but are technically less challenging, easier to standardise (Nielsen & Yu 2010, Gall & Nielsen 2004) and can be adapted for field use (Gorsich, Bengis, Ezenwa et al. 2015, Nielsen, Gall, Smith et al. 2001), it is recommended that the iELISA and/or FPA is implemented in combination with the RBT for the routine diagnosis of brucellosis in African buffaloes. Interpretation of the tests in series or in parallel improved DSp and DSe and will depend on the epidemiological context and purpose of the testing (Abernethy, Menzies, Pfeifer et al 2006).   The diagnostic performance of the tests was found comparable to their performance in cattle. The values for DSe and DSp that were estimated will be of use in the interpretation of serological results and determination of diagnostic strategies in different circumstances.

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9   APPENDICES

9.1   Appendix 1: RBT and CFT results

Herd	Specimen	Box	Tube	RBT (student)	RBT (OVI staff)	CFT titre iU/ml	Exclusion
A	C16	I	A10	neg	neg	0	
A	C27	I	A11	neg	neg	0	
A	C32	I	A12	neg	neg	0	
A	B46	I	A5	neg	neg	0	
A	B65	I	A6	neg	neg	0	
A	25	I	A8	neg	neg	0	
A	C8	I	A9	neg	neg	0	
A	C17	I	B10	neg	neg	0	
A	C21	I	B11	neg	neg	0	
A	C33	I	B12	neg	pos	0	
A	B63	I	B5	neg	neg	0	
A	B55	I	B6	neg	neg	0	
A	C1	I	B8	neg	neg	0	
A	C9	I	B9	neg	neg	0	
A	C18	I	C10	neg	neg	0	
A	C23	I	C11	neg	neg	0	
A	C34	I	C12	neg	neg	0	
A	B46	I	C4	neg	neg	0	
A	B42	I	C5	neg	neg	0	
A	B54	I	C6	neg	neg	0	
A	C2	I	C8	neg	neg	0	
A	C10	I	C9	neg	neg	0	
A	C19	I	D10	neg	neg	0	
A	C26	I	D11	neg	neg	0	
A	C35	I	D12	neg	neg	0	
A	B61	I	D4	neg	neg	0	
A	B57	I	D5	neg	neg	0	
A	B52	I	D6	neg	neg	0	
A	C3	I	D8	neg	neg	0	
A	C11	I	D9	pos	neg	0	
A	C20	I	E10	neg	neg	0	
A	C28	I	E11	neg	neg	0	
A	C36	I	E12	neg	neg	0	
A	B47+	I	E4	neg	neg	0	
A	B59+	I	E5	neg	neg	0	
A	B51+	I	E6	neg	neg	0	
A	B64	I	E7	neg	neg	0	
A	C4	I	E8	neg	neg	0	
A	C12	I	E9	neg	neg	0	
A	C22	I	F10	neg	neg	0	
A	C29	I	F11	neg	neg	0	
A	C37	I	F12	neg	neg	0	

Herd	Specimen	Box	Tube	RBT (student)	RBT (OVI staff)	CFT titre iU/ml	Exclusion
A	A	I	F4	neg	neg	0	
A	B53	I	F5	neg	neg	0	
A	B50	I	F6	neg	neg	0	
A	B68	I	F7	neg	neg	0	
A	C5	I	F8	neg	neg	0	
A	C13	I	F9	neg	neg	0	
A	C24	I	G10	neg	neg	0	
A	C30	I	G11	neg	neg	0	
A	C38	I	G12	neg	neg	0	
A	40	I	G4	neg	neg	0	
A	B56	I	G5	neg	neg	0	
A	B49	I	G6	neg	neg	A/C 43	excluded
A	27	I	G7	neg	neg	0	
A	C6	I	G8	neg	neg	0	
A	C14	I	G9	neg	neg	0	
A	C25	I	H10	neg	neg	0	
A	C31	I	H11	neg	neg	0	
A	C39	I	H12	neg	neg	0	
A	B62	I	H4	neg	neg	0	
A	B45-	I	H5	neg	neg	0	
A	B44	I	H6	neg	neg	0	
A	28	I	H7	neg	neg	0	
A	C7	I	H8	neg	neg	0	
A	C15	I	H9	neg	neg	0	
A	no number	II	A2	neg	neg	0	
A	B4	II	A3	neg	neg	0	
A	B15	II	A4	neg	neg	0	
A	B32	II	A5	neg	neg	0	
A	B43	II	B2	neg	neg	0	
A	B7	II	B3	neg	neg	0	
A	B17	II	B4	neg	neg	0	
A	B34	II	B5	neg	neg	0	
A	B60	II	C2	neg	neg	0	
A	B9	II	C3	neg	no serum	No serum	excluded
A	B22	II	C4	neg	neg	0	
A	B35	II	C5	neg	neg	0	
A	B65	II	D2	neg	neg	0	
A	B10	II	D3	neg	neg	0	
A	B24	II	D4	neg	neg	0	
A	B38	II	D5	neg	neg	0	
A	C40	II	E1	neg	neg	0	
A	B67	II	E2	neg	neg	0	
A	B11	II	E3	neg	neg	0	
A	B26	II	E4	neg	neg	0	
A	B39	II	E5	neg	neg	0	
A	C41	II	F1	neg	neg	0	
A	B1	II	F2	neg	neg	0	
A	B12	II	F3	neg	neg	0	
A	B27	II	F4	neg	neg	0	

Herd	Specimen	Box	Tube	RBT (student)	RBT (OVI staff)	CFT titre iU/ml	Exclusion
A	B40	II	F5	neg	neg	0	
A	C42	II	G1	neg	neg	0	
A	B2	II	G2	neg	neg	0	
A	B13	II	G3	neg	neg	0	
A	B28	II	G4	neg	neg	0	
A	B41	II	G5	neg	neg	0	
A	C43	II	H1	neg	neg	0	
A	B3	II	H2	neg	neg	a/c 844	excluded
A	B14	II	H3	no serum	no serum	no serum	excluded
A	B31	II	H4	neg	neg	0	
B	RM4	I	A3	neg	neg	0	
B	F6B	I	B4	neg	neg	0	
B	252	I	A4	neg	neg	0	
B	329	I	H3	neg	neg	0	
B	560	I	F3	neg	neg	0	
B	34F	I	G3	neg	neg	0	
B	48	I	D3	neg	neg	0	
B	870	I	B3	neg	neg	0	
B	937	I	E3	neg	neg	0	
B	101	I	C3	neg	neg	0	
C1A	A1	IV	C8	not done	neg	0	
C1A	A2	IV	D8	not done	neg	0	
C1A	A3	IV	E8	not done	neg	0	
C1A	A4	IV	F8	not done	neg	0	
C1A	A5	IV	G8	not done	pos	18	
C1A	A6	IV	H8	not done	pos	172	
C1A	A7	IV	A9	not done	neg	0	
C1A	A8	IV	B9	not done	pos	0	
C1A	A9	IV	C9	not done	neg	0	
C1A	A10	IV	D9	not done	neg	0	
C1A	A11	IV	E9	not done	pos	392	
C1B	B1	IV	F9	not done	neg	0	
C1B	B2	IV	G9	not done	pos	0	
C1B	B3	IV	H9	not done	pos	0	
C1B	B4	IV	A10	not done	pos	98	
C1B	B5	IV	B10	not done	neg	0	
C1B	B6	IV	C10	not done	neg	0	
C1B	B7	IV	D10	not done	pos	43	
C1B	B8	IV	E10	not done	neg	0	
C1B	B9	IV	F10	not done	neg	0	
C1B	B10	IV	G10	not done	pos	43	
C1B	B11	IV	H10	not done	neg	0	
C1B	B12	IV	A11	not done	neg	0	
C1C	C1	IV	B11	not done	neg	21	
C1C	C2	IV	C11	not done	neg	0	
C1C	C3	IV	D11	not done	neg	0	
C1C	C4	IV	E11	not done	neg	0	
C1C	C5	IV	F11	not done	pos	0	
C1C	C6	IV	G11	not done	neg	0	

Herd	Specimen	Box	Tube	RBT (student)	RBT (OVI staff)	CFT titre iU/ml	Exclusion
C1C	C7	IV	H11	not done	neg	0	
C1C	C8	IV	A12	not done	neg	0	
C1C	C9	IV	B12	not done	pos	0	
C1C	C10	IV	C12	not done	pos	0	
C1C	C11 (BB1)	IV	D12	not done	neg	0	
C1C	C12 (BB2)	IV	E12	not done	neg	0	
C1C	C13 (BB3)	IV	F12	not done	neg	0	
C1C	C14	IV	G12	not done	neg	0	
C1C	C15	IV	H12	not done	pos	60	
C1C	C16 (BB4)	V	E1	not done	pos	344	
C1C	C17	V	F1	not done	pos	172	
C2	R2	II	B12	pos	pos	0	
C2	R3	II	C12	neg	neg	0	
C2	R10	III	F9	not done	pos	120	
C2	R11	III	H9	not done	neg	0	
C2	R12	III	B2	not done	pos	0	
C2	R13	III	C4	not done	pos	120	
C2	R16	II	E8	neg	neg	0	
C2	R17	II	F8	pos	pos	21	
C2	R18	II	A8	pos	neg	0	
C2	R19	III	D4	not done	neg	0	
C2	R20	III	C3	not done	pos	120	
C2	R28	II	G10	neg	neg	0	
C2	R30	III	A10	not done	pos	145	
C2	R33	III	F2	not done	no serum	no serum	excluded
C2	R36	III	F4	not done	neg	0	
C2	R41	II	A11	pos	pos	21	
C2	R51	III	B10	not done	neg	0	
C2	R52	III	C10	not done	neg	0	
C2	B11	II	A6	neg	neg	No serum	excluded
C2	B12	III	C8	not done	neg	0	
C2	B13b	II	D11	pos	pos	60	
C2	B14b	III	G1	not done	neg	0	
C2	B15	III	D8	not done	pos	0	
C2	B16	III	D11	not done	neg	0	
C2	B17	III	G6	not done	neg	0	
C2	B19	III	F5	not done	neg	0	
C2	B20	III	E6	not done	pos	0	
C2	B24	II	B6	pos	pos	86	
C2	B26b	III	G5	not done	neg	0	
C2	B27	III	E11	not done	neg	0	
C2	B29	II	E11	pos	pos	196	
C2	B2b	II	E7	neg	neg	0	
C2	B3	III	C11	not done	neg	0	
C2	B32	II	F11	neg	neg	0	
C2	B33	II	G11	pos	pos	0	
C2	B35	III	H5	not done	neg	0	
C2	B38	II	C6	neg	neg	0	
C2	B39c	III	E8	not done	neg	0	

Herd	Specimen	Box	Tube	RBT (student)	RBT (OVI staff)	CFT titre iU/ml	Exclusion
C2	B4	II	H5	neg	pos	0	
C2	B41	III	F8	not done	neg	0	
C2	B43	II	D6	pos	pos	43	
C2	B45	III	A6	not done	neg	0	
C2	B47b	III	H6	not done	pos	0	
C2	B50b	III	G8	not done	neg	0	
C2	B57	III	H8	not done	pos	0	
C2	B7	II	F9	pos	neg	0	
C2	B8b	III	F6	not done	neg	0	
C2	C1	III	G9	not done	neg	0	
C2	C2	III	H2	not done	neg	0	
C2	O1	III	B9	not done	neg	0	
C2	O12	III	F7	not done	pos	0	
C2	O13	III	F11	not done	neg	0	
C2	O14	III	A3	not done	neg	0	
C2	O15	II	F6	neg	neg	0	
C2	O16	III	G11	not done	neg	0	
C2	O18	III	C6	not done	neg	0	
C2	O19b	III	G7	not done	pos	43	
C2	O20	II	H11	neg	neg	0	
C2	O22	II	G9	neg	pos	480	
C2	O23b	II	F7	neg	neg	0	
C2	O24	II	G6	pos	pos	21	
C2	O25	II	C11	neg	neg	0	
C2	O26b	III	H7	not done	neg	0	
C2	O28b	III	H1	not done	pos	784	
C2	O29b	II	E9	neg	neg	0	
C2	O32c	III	A8	not done	neg	0	
C2	O33	III	H11	not done	pos	240	
C2	O36	III	B8	not done	neg	0	
C2	O37	II	G7	pos	neg	21	
C2	O4	II	E6	neg	pos	0	
C2	O40	III	A12	not done	pos	21	
C2	O41	III	C9	not done	pos	18	
C2	O46	II	H7	neg	neg	0	
C2	O48	II	H6	neg	neg	0	
C2	O50	III	D9	not done	neg	0	
C2	O6	III	B6	not done	pos	43	
C2	O7	III	E7	not done	neg	0	
C2	O86 O8b)	III	E9	not done	neg	0	
C2	O9	III	A9	not done	neg	0	
C2	R17(OR R47)	III	B3	not done	neg	0	
C2	R21c	III	C2	not done	pos	43	
C2	R23b	II	G8	neg	neg	0	
C2	R24b	III	A4	not done	pos	196	
C2	R26b	III	E4	not done	neg	0	
C2	R27b	II	F10	neg	neg	0	
C2	R29b	III	D3	not done	neg	0	

Herd	Specimen	Box	Tube	RBT (student)	RBT (OVI staff)	CFT titre iU/ml	Exclusion
C2	R31b	III	D2	not done	pos	18	
C2	R32b	II	D12	neg	neg	0	
C2	R37b	II	H10	pos	pos	0	
C2	R39b	II	E12	pos	pos	86	
C2	R42b	III	E2	not done	neg	0	
C2	R44b	II	H8	neg	neg	0	
C2	R46b	III	H4	not done	neg	0	
C2	R4c	III	G4	not done	pos	18	
C2	R50b	II	B11	pos	pos	21	
C2	R8b	II	E10	neg	neg	0	
C2	unknown	III	H3	not done	pos	240	
C2	W1b	II	B8	neg	neg	0	
C2	W2	III	E3	not done	neg	0	
C2	W4	II	A9	neg	neg	0	
C2	W5b	II	A12	neg	neg	0	
C2	W6	III	F3	not done	neg	0	
C2	Y10b	II	A10	pos	neg	A/C 120	excluded
C2	Y11	III	B5	not done	neg	30	
C2	Y12	II	G12	pos	pos	0	
C2	Y13	III	E10	not done	neg	0	
C2	Y14	III	C5	not done	neg	0	
C2	Y15c	II	H12	neg	neg	0	
C2	Y16b	II	C8	pos	pos	0	
C2	Y17	II	B10	neg	neg	0	
C2	Y18	III	F10	not done	neg	0	
C2	Y20i (Y20b)	III	E5	not done	neg	0	
C2	Y24b	II	D8	neg	neg	0	
C2	Y27	III	G10	not done	neg	0	
C2	Y28	III	H10	not done	neg	0	
C2	Y29	II	C9	neg	neg	0	
C2	Y2b	II	F12	pos	neg	172	
C2	Y3	II	B9	neg	neg	0	
C2	Y31d	III	F1	not done	pos	344	
C2	Y32b	II	C10	neg	pos	0	
C2	Y33b	III	A11	not done	neg	0	
C2	Y35d	III	D6	not done	neg	0	
C2	Y37b	III	G2	not done	pos	196	
C2	Y38b	III	B11	not done	pos	0	
C2	Y39b	III	B4	not done	neg	0	
C2	Y4	III	D10	not done	neg	0	
C2	Y42	III	D5	not done	neg	0	
C2	Y43b	II	D10	pos	pos	0	
C2	Y44c	II	D9	neg	neg	0	
C2	Y46b	III	E1	not done	neg	0	
C2	Y47	III	G3	not done	neg	no serum	excluded
C2	Y5c	II	H9	neg	neg	0	
C2	Y6	III	A5	not done	pos	784	
C2	Y9	III	A2	not done	neg	0	
C3	OC130529b	I	F1	pos	pos	0	excluded

Herd	Specimen	Box	Tube	RBT (student)	RBT (OVI staff)	CFT titre iU/ml	Exclusion
	5						
D	Buffalo *4	I	B2	pos	No serum	No serum (Allerton: 784)	
D	Buffalo *10	I	H1	pos	No serum	No serum (Allerton 344)	
D	Buffalo 6	I	G1	neg	neg	No serum (Allerton 0)	
D	Buffalo 13	I	A2	neg	No serum	No serum (Allerton 0)	
E	10	III	B12	not done	pos	784	
E	39	III	C12	not done	neg	0	
E	48	III	D12	not done	neg	0	
E	19	III	E12	not done	neg	0	
E	43	III	F12	not done	neg	0	
E	26	III	H12	not done	pos	784	
E	22	III	G12	not done	pos	30	
E	30	IV	E1	not done	pos	344	
E	40	IV	F1	not done	neg	0	
E	8	IV	G1	not done	neg	0	
E	32	IV	H1	not done	neg	0	
E	55	IV	A2	not done	pos	43	
E	41	IV	B2	not done	neg	0	
E	16	IV	C2	not done	pos	21	
E	18	IV	D2	not done	pos	196	
E	1	IV	E2	not done	neg	0	
E	46	IV	F2	not done	pos	120	
E	47	IV	G2	not done	neg	0	
E	27	IV	H2	not done	pos	784	
E	23	IV	A3	not done	neg	0	
E	17	IV	B3	not done	neg	0	
E	12	IV	C3	not done	pos	196	
E	6	IV	D3	not done	pos	60	
E	49	IV	E3	not done	pos	0	
E	34	IV	F3	not done	pos	120	
E	9	IV	G3	not done	pos	784	
E	3	IV	H3	not done	pos	0	
E	51	IV	A4	not done	neg	0	
E	4	IV	B4	not done	pos	480	
E	53	IV	C4	not done	pos	49	
E	54	IV	D4	not done	neg	0	
E	38	IV	E4	not done	neg	0	
E	28	IV	F4	not done	pos	0	
E	50	IV	G4	not done	neg	0	
E	44	IV	A5	not done	pos	0	
E	57	IV	B5	not done	pos	0	
E	45	IV	C5	not done	neg	0	
E	33	IV	D5	not done	pos	36	
E	36	IV	E5	not done	neg	0	

Herd	Specimen	Box	Tube	RBT (student)	RBT (OVI staff)	CFT titre iU/ml	Exclusion
E	52	IV	F5	not done	pos	98	
E	5	IV	G5	not done	pos	240	
E	56	IV	H5	not done	no serum	no serum	excluded
E	7	IV	A6	not done	pos	0	
E	11	IV	B6	not done	pos	196	
E	13	IV	C6	not done	neg	0	
E	14	IV	D6	not done	neg	60	
E	15	IV	E6	not done	pos	0	
E	20	IV	F6	not done	neg	0	
E	21	IV	G6	not done	neg	0	
E	24	IV	H6	not done	neg	784	
E	25	IV	E7	not done	neg	0	
E	29	IV	F7	not done	neg	0	
E	37	IV	G7	not done	pos	172	
E	2	IV	H7	not done	pos	784	
E	31	IV	A8	not done	pos	0	
E	35	IV	B8	not done	pos	60	
F	White 8	I	D2	pos	pos	120	
F	Yellow 28	I	E2	pos	pos	784	
F	Z.11	I	F2	pos	pos	0	
F	Yellow 6	I	H2	pos	pos	43	
F	Z.14	I	C2	pos	pos	145	
F	Z14	I	G2	pos	pos	145	excluded
F	B478/14, S5421/14	I	E1	pos	pos	688	
E	42	IV	H4	not done	neg	no serum	excluded

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9.2 Appendix 2: iELISA results

Herd	ID	Box	Tube	Plate	Pos A1	Pos B1	Pos Mean	Neg C1	Neg D1	Neg Mean	CalcOD 1	CalcOD 2	CalcOD Mean	Pos corr OD	Sample corr OD	S/P ratio	Exclusion
A	C16	I	A10	2	0.931	0.918	0.925	0.420	0.420	0.420	0.159	0.134	0.147	0.505	-0.274	-54.2%	
A	C27	I	A11	2	0.931	0.918	0.925	0.420	0.420	0.420	0.062	0.068	0.065	0.505	-0.355	-70.4%	
A	C32	I	A12	2	0.931	0.918	0.925	0.420	0.420	0.420	0.300	0.327	0.314	0.505	-0.107	-21.1%	
A	B46	I	A5	1	1.047	0.984	1.016	0.041	0.390	0.216	0.150	0.143	0.147	0.800	-0.069	-8.6%	
A	B65	I	A6	1	1.047	0.984	1.016	0.041	0.390	0.216	0.555	0.524	0.540	0.800	0.324	40.5%	
A	25	I	A8	2	0.931	0.918	0.925	0.420	0.420	0.420	0.043	0.041	0.042	0.505	-0.378	-74.9%	
A	C8	I	A9	2	0.931	0.918	0.925	0.420	0.420	0.420	0.107	0.098	0.103	0.505	-0.318	-62.9%	
A	C17	I	B10	2	0.931	0.918	0.925	0.420	0.420	0.420	0.048	0.047	0.048	0.505	-0.373	-73.8%	
A	C21	I	B11	2	0.931	0.918	0.925	0.420	0.420	0.420	0.099	0.075	0.087	0.505	-0.333	-66.0%	
A	C33	I	B12	2	0.931	0.918	0.925	0.420	0.420	0.420	0.066	0.050	0.058	0.505	-0.362	-71.8%	
A	B63	I	B5	1	1.047	0.984	1.016	0.041	0.390	0.216	0.196	0.145	0.171	0.800	-0.045	-5.6%	
A	B55	I	B6	1	1.047	0.984	1.016	0.041	0.390	0.216	0.329	0.217	0.273	0.800	0.058	7.2%	
A	C1	I	B8	2	0.931	0.918	0.925	0.420	0.420	0.420	0.074	0.033	0.054	0.505	-0.367	-72.6%	
A	C9	I	B9	2	0.931	0.918	0.925	0.420	0.420	0.420	0.118	0.160	0.139	0.505	-0.281	-55.7%	
A	C18	I	C10	2	0.931	0.918	0.925	0.420	0.420	0.420	0.162	0.149	0.156	0.505	-0.265	-52.4%	
A	C23	I	C11	2	0.931	0.918	0.925	0.420	0.420	0.420	0.050	0.053	0.052	0.505	-0.369	-73.0%	
A	C34	I	C12	2	0.931	0.918	0.925	0.420	0.420	0.420	0.061	0.047	0.054	0.505	-0.366	-72.5%	
A	B46	I	C4	1	1.047	0.984	1.016	0.041	0.390	0.216	0.180	0.170	0.175	0.800	-0.041	-5.1%	
A	B42	I	C5	1	1.047	0.984	1.016	0.041	0.390	0.216	0.065	0.065	0.065	0.800	-0.151	-18.8%	
A	B54	I	C6	1	1.047	0.984	1.016	0.041	0.390	0.216	0.142	0.125	0.134	0.800	-0.082	-10.3%	
A	C2	I	C8	2	0.931	0.918	0.925	0.420	0.420	0.420	0.533	0.457	0.495	0.505	0.075	14.9%	
A	C10	I	C9	2	0.931	0.918	0.925	0.420	0.420	0.420	0.061	0.055	0.058	0.505	-0.362	-71.8%	
A	C19	I	D10	2	0.931	0.918	0.925	0.420	0.420	0.420	0.269	0.162	0.216	0.505	-0.205	-40.5%	
A	C26	I	D11	2	0.931	0.918	0.925	0.420	0.420	0.420	0.073	0.055	0.064	0.505	-0.356	-70.6%	
A	C35	I	D12	2	0.931	0.918	0.925	0.420	0.420	0.420	0.103	0.082	0.093	0.505	-0.328	-64.9%	
A	B61	I	D4	1	1.047	0.984	1.016	0.041	0.390	0.216	0.187	0.211	0.199	0.800	-0.017	-2.1%	

Herd	ID	Box	Tube	Plate	Pos A1	Pos B1	Pos Mean	Neg C1	Neg D1	Neg Mean	CalcOD 1	CalcOD 2	CalcOD Mean	Pos corr OD	Sample corr OD	S/P ratio	Exclusion
A	B57	I	D5	1	1.047	0.984	1.016	0.041	0.390	0.216	0.145	0.146	0.146	0.800	-0.070	-8.8%	
A	B52	I	D6	1	1.047	0.984	1.016	0.041	0.390	0.216	0.072	0.076	0.074	0.800	-0.142	-17.7%	
A	C3	I	D8	2	0.931	0.918	0.925	0.420	0.420	0.420	0.210	0.249	0.230	0.505	-0.191	-37.8%	
A	C11	I	D9	2	0.931	0.918	0.925	0.420	0.420	0.420	0.303	0.291	0.297	0.505	-0.123	-24.4%	
A	C20	I	E10	2	0.931	0.918	0.925	0.420	0.420	0.420	0.090	0.094	0.092	0.505	-0.328	-65.0%	
A	C28	I	E11	2	0.931	0.918	0.925	0.420	0.420	0.420	0.242	0.206	0.224	0.505	-0.196	-38.9%	
A	C36	I	E12	2	0.931	0.918	0.925	0.420	0.420	0.420	0.061	0.051	0.056	0.505	-0.364	-72.2%	
A	B47+	I	E4	1	1.047	0.984	1.016	0.041	0.390	0.216	0.068	0.073	0.071	0.800	-0.145	-18.1%	
A	B59+	I	E5	1	1.047	0.984	1.016	0.041	0.390	0.216	0.064	0.064	0.064	0.800	-0.152	-18.9%	
A	B51+	I	E6	1	1.047	0.984	1.016	0.041	0.390	0.216	0.079	0.084	0.082	0.800	-0.134	-16.8%	
A	B64	I	E7	2	0.931	0.918	0.925	0.420	0.420	0.420	0.097	0.047	0.072	0.505	-0.348	-69.0%	
A	C4	I	E8	2	0.931	0.918	0.925	0.420	0.420	0.420	0.297	0.219	0.258	0.505	-0.162	-32.1%	
A	C12	I	E9	2	0.931	0.918	0.925	0.420	0.420	0.420	0.099	0.097	0.098	0.505	-0.322	-63.8%	
A	C22	I	F10	2	0.931	0.918	0.925	0.420	0.420	0.420	0.049	0.052	0.051	0.505	-0.370	-73.2%	
A	C29	I	F11	2	0.931	0.918	0.925	0.420	0.420	0.420	0.122	0.058	0.090	0.505	-0.330	-65.4%	
A	C37	I	F12	2	0.931	0.918	0.925	0.420	0.420	0.420	0.103	0.063	0.083	0.505	-0.337	-66.8%	
A	A	I	F4	1	1.047	0.984	1.016	0.041	0.390	0.216	0.083	0.086	0.085	0.800	-0.131	-16.4%	
A	B53	I	F5	1	1.047	0.984	1.016	0.041	0.390	0.216	0.145	0.149	0.147	0.800	-0.069	-8.6%	
A	B50	I	F6	1	1.047	0.984	1.016	0.041	0.390	0.216	0.100	0.066	0.083	0.800	-0.133	-16.6%	
A	B68	I	F7	2	0.931	0.918	0.925	0.420	0.420	0.420	0.134	0.058	0.096	0.505	-0.324	-64.2%	
A	C5	I	F8	2	0.931	0.918	0.925	0.420	0.420	0.420	0.048	0.054	0.051	0.505	-0.369	-73.1%	
A	C13	I	F9	2	0.931	0.918	0.925	0.420	0.420	0.420	0.056	0.056	0.056	0.505	-0.364	-72.2%	
A	C24	I	G10	2	0.931	0.918	0.925	0.420	0.420	0.420	0.074	0.073	0.074	0.505	-0.347	-68.7%	
A	C30	I	G11	2	0.931	0.918	0.925	0.420	0.420	0.420	0.119	0.102	0.111	0.505	-0.310	-61.3%	
A	C38	I	G12	2	0.931	0.918	0.925	0.420	0.420	0.420	0.110	0.072	0.091	0.505	-0.329	-65.2%	
A	40	I	G4	1	1.047	0.984	1.016	0.041	0.390	0.216	0.053	0.062	0.058	0.800	-0.158	-19.8%	
A	B56	I	G5	1	1.047	0.984	1.016	0.041	0.390	0.216	0.075	0.079	0.077	0.800	-0.139	-17.3%	
A	B49	I	G6	1	1.047	0.984	1.016	0.041	0.390	0.216	0.088	0.077	0.083	0.800	-0.133	-16.6%	X

Herd	ID	Box	Tube	Plate	Pos A1	Pos B1	Pos Mean	Neg C1	Neg D1	Neg Mean	CalcOD 1	CalcOD 2	CalcOD Mean	Pos corr OD	Sample corr OD	S/P ratio	Exclusion
A	27	I	G7	2	0.931	0.918	0.925	0.420	0.420	0.420	0.044	0.064	0.054	0.505	-0.366	-72.5%	
A	C6	I	G8	2	0.931	0.918	0.925	0.420	0.420	0.420	0.249	0.047	0.148	0.505	-0.272	-53.9%	
A	C14	I	G9	2	0.931	0.918	0.925	0.420	0.420	0.420	0.055	0.056	0.056	0.505	-0.365	-72.2%	
A	C25	I	H10	2	0.931	0.918	0.925	0.420	0.420	0.420	0.101	0.085	0.093	0.505	-0.327	-64.8%	
A	C31	I	H11	2	0.931	0.918	0.925	0.420	0.420	0.420	0.091	0.039	0.065	0.505	-0.355	-70.4%	
A	C39	I	H12	2	0.931	0.918	0.925	0.420	0.420	0.420	0.094	0.065	0.080	0.505	-0.341	-67.5%	
A	B62	I	H4	1	1.047	0.984	1.016	0.041	0.390	0.216	0.051	0.062	0.057	0.800	-0.159	-19.9%	
A	B45-	I	H5	1	1.047	0.984	1.016	0.041	0.390	0.216	0.106	0.126	0.116	0.800	-0.100	-12.4%	
A	B44	I	H6	1	1.047	0.984	1.016	0.041	0.390	0.216	0.106	0.091	0.099	0.800	-0.117	-14.6%	
A	28	I	H7	2	0.931	0.918	0.925	0.420	0.420	0.420	0.050	0.055	0.053	0.505	-0.368	-72.8%	
A	C7	I	H8	2	0.931	0.918	0.925	0.420	0.420	0.420	0.052	0.038	0.045	0.505	-0.375	-74.3%	
A	C15	I	H9	2	0.931	0.918	0.925	0.420	0.420	0.420	0.226	0.194	0.210	0.505	-0.210	-41.6%	
A	no number	II	A2	3	1.107	0.929	1.018	0.430	0.410	0.420	0.045	0.049	0.047	0.598	-0.373	-62.4%	
A	B4	II	A3	3	1.107	0.929	1.018	0.430	0.410	0.420	0.390	0.340	0.365	0.598	-0.055	-9.2%	
A	B15	II	A4	3	1.107	0.929	1.018	0.430	0.410	0.420	0.055	0.058	0.057	0.598	-0.364	-60.8%	
A	B32	II	A5	3	1.107	0.929	1.018	0.430	0.410	0.420	0.082	0.075	0.079	0.598	-0.342	-57.1%	
A	B43	II	B2	3	1.107	0.929	1.018	0.430	0.410	0.420	0.065	0.055	0.060	0.598	-0.360	-60.2%	
A	B7	II	B3	3	1.107	0.929	1.018	0.430	0.410	0.420	0.051	0.052	0.052	0.598	-0.369	-61.6%	
A	B17	II	B4	3	1.107	0.929	1.018	0.430	0.410	0.420	0.117	0.138	0.128	0.598	-0.293	-48.9%	
A	B34	II	B5	3	1.107	0.929	1.018	0.430	0.410	0.420	0.050	0.055	0.053	0.598	-0.368	-61.5%	
A	B60	II	C2	3	1.107	0.929	1.018	0.430	0.410	0.420	0.225	0.177	0.201	0.598	-0.219	-36.6%	
A	B9	II	C3	3	1.107	0.929	1.018	0.430	0.410	0.420	0.067	0.054	0.061	0.598	-0.360	-60.1%	X
A	B22	II	C4	3	1.107	0.929	1.018	0.430	0.410	0.420	0.092	0.090	0.091	0.598	-0.329	-55.0%	
A	B35	II	C5	3	1.107	0.929	1.018	0.430	0.410	0.420	0.082	0.102	0.092	0.598	-0.328	-54.8%	
A	B65	II	D2	3	1.107	0.929	1.018	0.430	0.410	0.420	0.066	0.067	0.067	0.598	-0.354	-59.1%	
A	B10	II	D3	3	1.107	0.929	1.018	0.430	0.410	0.420	0.066	0.066	0.066	0.598	-0.354	-59.2%	
A	B24	II	D4	3	1.107	0.929	1.018	0.430	0.410	0.420	0.048	0.050	0.049	0.598	-0.371	-62.0%	

Herd	ID	Box	Tube	Plate	Pos A1	Pos B1	Pos Mean	Neg C1	Neg D1	Neg Mean	CalcOD 1	CalcOD 2	CalcOD Mean	Pos corr OD	Sample corr OD	S/P ratio	Exclusion
A	B38	II	D5	3	1.107	0.929	1.018	0.430	0.410	0.420	0.059	0.056	0.058	0.598	-0.363	-60.6%	
A	C40	II	E1	3	1.107	0.929	1.018	0.430	0.410	0.420	0.272	0.051	0.162	0.598	-0.259	-43.2%	
A	B67	II	E2	3	1.107	0.929	1.018	0.430	0.410	0.420	0.124	0.106	0.115	0.598	-0.305	-51.0%	
A	B11	II	E3	3	1.107	0.929	1.018	0.430	0.410	0.420	0.202	0.196	0.199	0.598	-0.221	-37.0%	
A	B26	II	E4	3	1.107	0.929	1.018	0.430	0.410	0.420	0.075	0.068	0.072	0.598	-0.349	-58.3%	
A	B39	II	E5	3	1.107	0.929	1.018	0.430	0.410	0.420	0.071	0.055	0.063	0.598	-0.357	-59.7%	
A	C41	II	F1	3	1.107	0.929	1.018	0.430	0.410	0.420	0.053	0.055	0.054	0.598	-0.366	-61.2%	
A	B1	II	F2	3	1.107	0.929	1.018	0.430	0.410	0.420	0.059	0.102	0.081	0.598	-0.340	-56.8%	
A	B12	II	F3	3	1.107	0.929	1.018	0.430	0.410	0.420	0.119	0.082	0.101	0.598	-0.320	-53.4%	
A	B27	II	F4	3	1.107	0.929	1.018	0.430	0.410	0.420	0.073	0.055	0.064	0.598	-0.356	-59.5%	
A	B40	II	F5	3	1.107	0.929	1.018	0.430	0.410	0.420	0.046	0.047	0.047	0.598	-0.374	-62.5%	
A	C42	II	G1	3	1.107	0.929	1.018	0.430	0.410	0.420	0.045	0.049	0.047	0.598	-0.373	-62.4%	
A	B2	II	G2	3	1.107	0.929	1.018	0.430	0.410	0.420	0.053	0.261	0.157	0.598	-0.263	-44.0%	
A	B13	II	G3	3	1.107	0.929	1.018	0.430	0.410	0.420	0.225	0.197	0.211	0.598	-0.209	-34.9%	
A	B28	II	G4	3	1.107	0.929	1.018	0.430	0.410	0.420	0.084	0.060	0.072	0.598	-0.348	-58.2%	
A	B41	II	G5	3	1.107	0.929	1.018	0.430	0.410	0.420	0.122	0.126	0.124	0.598	-0.296	-49.5%	
A	C43	II	H1	3	1.107	0.929	1.018	0.430	0.410	0.420	0.058	0.104	0.081	0.598	-0.339	-56.7%	
A	B3	II	H2	3	1.107	0.929	1.018	0.430	0.410	0.420	0.203	0.273	0.238	0.598	-0.182	-30.4%	X
A	B14	II	H3	3	1.107	0.929	1.018	0.430	0.410	0.420	0.094	0.082	0.088	0.598	-0.332	-55.5%	X
A	B31	II	H4	3	1.107	0.929	1.018	0.430	0.410	0.420	0.055	0.057	0.056	0.598	-0.364	-60.9%	
B	RM4	I	A3	1	1.047	0.984	1.016	0.041	0.390	0.216	0.050	0.048	0.049	0.800	-0.167	-20.8%	
B	F6B	I	B4	1	1.047	0.984	1.016	0.041	0.390	0.216	0.057	0.062	0.060	0.800	-0.156	-19.5%	
B	252	I	A4	1	1.047	0.984	1.016	0.041	0.390	0.216	0.074	0.084	0.079	0.800	-0.137	-17.1%	
B	329	I	H3	1	1.047	0.984	1.016	0.041	0.390	0.216	0.041	0.051	0.046	0.800	-0.170	-21.2%	
B	560	I	F3	1	1.047	0.984	1.016	0.041	0.390	0.216	0.039	0.050	0.045	0.800	-0.171	-21.4%	
B	34F	I	G3	1	1.047	0.984	1.016	0.041	0.390	0.216	0.045	0.057	0.051	0.800	-0.165	-20.6%	
B	48	I	D3	1	1.047	0.984	1.016	0.041	0.390	0.216	0.070	0.063	0.067	0.800	-0.149	-18.6%	
B	870	I	B3	1	1.047	0.984	1.016	0.041	0.390	0.216	0.323	0.325	0.324	0.800	0.109	13.6%	

Herd	ID	Box	Tube	Plate	Pos A1	Pos B1	Pos Mean	Neg C1	Neg D1	Neg Mean	CalcOD 1	CalcOD 2	CalcOD Mean	Pos corr OD	Sample corr OD	S/P ratio	Exclusion
B	937	I	E3	1	1.047	0.984	1.016	0.041	0.390	0.216	0.040	0.050	0.045	0.800	-0.171	-21.3%	
B	101	I	C3	1	1.047	0.984	1.016	0.041	0.390	0.216	0.057	0.052	0.055	0.800	-0.161	-20.1%	
C1A	A1	IV	C8	8	1.443	1.391	1.417	0.050	0.049	0.050	0.104	0.126	0.115	1.368	0.066	4.8%	
C1A	A2	IV	D8	8	1.443	1.391	1.417	0.050	0.049	0.050	1.075	1.046	1.061	1.368	1.011	73.9%	
C1A	A3	IV	E8	8	1.443	1.391	1.417	0.050	0.049	0.050	0.250	0.436	0.343	1.368	0.294	21.5%	
C1A	A4	IV	F8	8	1.443	1.391	1.417	0.050	0.049	0.050	0.762	0.730	0.746	1.368	0.697	50.9%	
C1A	A5	IV	G8	8	1.443	1.391	1.417	0.050	0.049	0.050	1.239	1.171	1.205	1.368	1.156	84.5%	
C1A	A6	IV	H8	8	1.443	1.391	1.417	0.050	0.049	0.050	1.645	1.518	1.582	1.368	1.532	112.0%	
C1A	A7	IV	A9	8	1.443	1.391	1.417	0.050	0.049	0.050	1.268	1.245	1.257	1.368	1.207	88.3%	
C1A	A8	IV	B9	8	1.443	1.391	1.417	0.050	0.049	0.050	1.536	1.537	1.537	1.368	1.487	108.7%	
C1A	A9	IV	C9	8	1.443	1.391	1.417	0.050	0.049	0.050	0.974	0.993	0.984	1.368	0.934	68.3%	
C1A	A10	IV	D9	8	1.443	1.391	1.417	0.050	0.049	0.050	1.043	1.021	1.032	1.368	0.983	71.8%	
C1A	A11	IV	E9	8	1.443	1.391	1.417	0.050	0.049	0.050	1.345	1.173	1.259	1.368	1.210	88.4%	
C1B	B1	IV	F9	8	1.443	1.391	1.417	0.050	0.049	0.050	0.996	0.807	0.902	1.368	0.852	62.3%	
C1B	B2	IV	G9	8	1.443	1.391	1.417	0.050	0.049	0.050	1.376	1.145	1.261	1.368	1.211	88.6%	
C1B	B3	IV	H9	8	1.443	1.391	1.417	0.050	0.049	0.050	1.351	1.194	1.273	1.368	1.223	89.4%	
C1B	B4	IV	A10	8	1.443	1.391	1.417	0.050	0.049	0.050	1.526	1.523	1.525	1.368	1.475	107.9%	
C1B	B5	IV	B10	8	1.443	1.391	1.417	0.050	0.049	0.050	1.290	1.294	1.292	1.368	1.243	90.9%	
C1B	B6	IV	C10	8	1.443	1.391	1.417	0.050	0.049	0.050	1.420	1.408	1.414	1.368	1.365	99.8%	
C1B	B7	IV	D10	8	1.443	1.391	1.417	0.050	0.049	0.050	1.186	1.139	1.163	1.368	1.113	81.4%	
C1B	B8	IV	E10	8	1.443	1.391	1.417	0.050	0.049	0.050	0.125	0.148	0.137	1.368	0.087	6.4%	
C1B	B9	IV	F10	8	1.443	1.391	1.417	0.050	0.049	0.050	1.204	1.110	1.157	1.368	1.108	81.0%	
C1B	B10	IV	G10	8	1.443	1.391	1.417	0.050	0.049	0.050	1.208	1.286	1.247	1.368	1.198	87.6%	
C1B	B11	IV	H10	8	1.443	1.391	1.417	0.050	0.049	0.050	1.192	1.104	1.148	1.368	1.099	80.3%	
C1B	B12	IV	A11	8	1.443	1.391	1.417	0.050	0.049	0.050	1.299	1.208	1.254	1.368	1.204	88.0%	
C1C	C1	IV	B11	8	1.443	1.391	1.417	0.050	0.049	0.050	0.875	0.873	0.874	1.368	0.825	60.3%	
C1C	C2	IV	C11	8	1.443	1.391	1.417	0.050	0.049	0.050	0.246	0.337	0.292	1.368	0.242	17.7%	
C1C	C3	IV	D11	8	1.443	1.391	1.417	0.050	0.049	0.050	0.056	0.059	0.058	1.368	0.008	0.6%	

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Herd	ID	Box	Tube	Plate	Pos A1	Pos B1	Pos Mean	Neg C1	Neg D1	Neg Mean	CalcOD 1	CalcOD 2	CalcOD Mean	Pos corr OD	Sample corr OD	S/P ratio	Exclusion
C1C	C4	IV	E11	8	1.443	1.391	1.417	0.050	0.049	0.050	0.112	0.108	0.110	1.368	0.061	4.4%	
C1C	C5	IV	F11	8	1.443	1.391	1.417	0.050	0.049	0.050	1.243	1.349	1.296	1.368	1.247	91.2%	
C1C	C6	IV	G11	8	1.443	1.391	1.417	0.050	0.049	0.050	0.257	0.252	0.255	1.368	0.205	15.0%	
C1C	C7	IV	H11	8	1.443	1.391	1.417	0.050	0.049	0.050	1.282	1.265	1.274	1.368	1.224	89.5%	
C1C	C8	IV	A12	8	1.443	1.391	1.417	0.050	0.049	0.050	0.060	0.061	0.061	1.368	0.011	0.8%	
C1C	C9	IV	B12	8	1.443	1.391	1.417	0.050	0.049	0.050	1.453	1.527	1.490	1.368	1.441	105.3%	
C1C	C10	IV	C12	8	1.443	1.391	1.417	0.050	0.049	0.050	1.651	1.705	1.678	1.368	1.629	119.1%	
C1C	C11(BB1)	IV	D12	8	1.443	1.391	1.417	0.050	0.049	0.050	0.906	0.885	0.896	1.368	0.846	61.9%	
C1C	C12(BB2)	IV	E12	8	1.443	1.391	1.417	0.050	0.049	0.050	1.150	1.386	1.268	1.368	1.219	89.1%	
C1C	C13(BB3)	IV	F12	8	1.443	1.391	1.417	0.050	0.049	0.050	0.331	0.275	0.303	1.368	0.254	18.5%	
C1C	C14	IV	G12	8	1.443	1.391	1.417	0.050	0.049	0.050	0.068	0.052	0.060	1.368	0.011	0.8%	
C1C	C15	IV	H12	8	1.443	1.391	1.417	0.050	0.049	0.050	1.233	1.280	1.257	1.368	1.207	88.3%	
C1C	C16(BB4)	V	E1	9	1.333	1.209	1.271	0.047	0.044	0.046	1.375	1.537	1.456	1.226	1.411	115.1%	
C1C	C17	V	F1	9	1.333	1.209	1.271	0.047	0.044	0.046	1.635	1.709	1.672	1.226	1.627	132.7%	
C2	R2	II	B12	4	1.123	1.043	1.083	0.046	0.042	0.044	0.898	0.956	0.927	1.039	0.883	85.0%	
C2	R3	II	C12	4	1.123	1.043	1.083	0.046	0.042	0.044	0.817	0.902	0.860	1.039	0.816	78.5%	
C2	R10	III	F9	6	1.305	1.286	1.296	0.050	0.042	0.046	1.351	1.286	1.319	1.250	1.273	101.8%	
C2	R11	III	H9	6	1.305	1.286	1.296	0.050	0.042	0.046	1.240	1.105	1.173	1.250	1.127	90.2%	
C2	R12	III	B2	5	1.321	1.350	1.336	0.046	0.044	0.045	1.332	1.337	1.335	1.291	1.290	99.9%	
C2	R13	III	C4	5	1.321	1.350	1.336	0.046	0.044	0.045	1.259	1.056	1.158	1.291	1.113	86.2%	
C2	R16	II	E8	4	1.123	1.043	1.083	0.046	0.042	0.044	0.716	0.694	0.705	1.039	0.661	63.6%	
C2	R17	II	F8	4	1.123	1.043	1.083	0.046	0.042	0.044	1.154	1.175	1.165	1.039	1.121	107.8%	
C2	R18	II	A8	4	1.123	1.043	1.083	0.046	0.042	0.044	0.850	0.843	0.847	1.039	0.803	77.2%	
C2	R19	III	D4	5	1.321	1.350	1.336	0.046	0.044	0.045	0.315	0.274	0.295	1.291	0.250	19.3%	
C2	R20	III	C3	5	1.321	1.350	1.336	0.046	0.044	0.045	1.343	1.301	1.322	1.291	1.277	99.0%	
C2	R28	II	G10	4	1.123	1.043	1.083	0.046	0.042	0.044	0.138	0.169	1.158	1.039	1.114	107.2%	
C2	R30	III	A10	6	1.305	1.286	1.296	0.050	0.042	0.046	1.433	1.420	1.427	1.250	1.381	110.5%	
C2	R33	III	F2	5	1.321	1.350	1.336	0.046	0.044	0.045	1.297	1.346	1.322	1.291	1.277	98.9%	X

Herd	ID	Box	Tube	Plate	Pos A1	Pos B1	Pos Mean	Neg C1	Neg D1	Neg Mean	CalcOD 1	CalcOD 2	CalcOD Mean	Pos corr OD	Sample corr OD	S/P ratio	Exclusion
C2	R36	III	F4	5	1.321	1.350	1.336	0.046	0.044	0.045	1.228	1.198	1.213	1.291	1.168	90.5%	
C2	R41	II	A11	4	1.123	1.043	1.083	0.046	0.042	0.044	1.217	1.156	1.187	1.039	1.143	110.0%	
C2	R51	III	B10	6	1.305	1.286	1.296	0.050	0.042	0.046	0.242	0.275	0.259	1.250	0.213	17.0%	
C2	R52	III	C10	6	1.305	1.286	1.296	0.050	0.042	0.046	0.462	0.464	0.463	1.250	0.417	33.4%	
C2	B11	II	A6	3	1.107	0.929	1.018	0.430	0.410	0.420	0.740	0.790	0.765	0.598	0.345	57.7%	X
C2	B12	III	C8	6	1.305	1.286	1.296	0.050	0.042	0.046	0.562	0.702	0.632	1.250	0.586	46.9%	
C2	B13b	II	D11	4	1.123	1.043	1.083	0.046	0.042	0.044	1.025	1.017	1.021	1.039	0.977	94.0%	
C2	B14b	III	G1	5	1.321	1.350	1.336	0.046	0.044	0.045	0.056	0.112	0.084	1.291	0.039	3.0%	
C2	B15	III	D8	6	1.305	1.286	1.296	0.050	0.042	0.046	1.275	1.220	1.248	1.250	1.202	96.2%	
C2	B16	III	D11	6	1.305	1.286	1.296	0.050	0.042	0.046	0.310	0.446	0.378	1.250	0.332	26.6%	
C2	B17	III	G6	5	1.321	1.350	1.336	0.046	0.044	0.045	1.137	1.184	1.161	1.291	1.116	86.4%	
C2	B19	III	F5	5	1.321	1.350	1.336	0.046	0.044	0.045	0.673	0.525	0.599	1.291	0.554	42.9%	
C2	B20	III	E6	5	1.321	1.350	1.336	0.046	0.044	0.045	0.167	0.132	0.150	1.291	0.105	8.1%	
C2	B24	II	B6	3	1.107	0.929	1.018	0.430	0.410	0.420	1.107	1.144	1.126	0.598	0.706	118.0%	
C2	B26b	III	G5	5	1.321	1.350	1.336	0.046	0.044	0.045	0.230	0.219	0.225	1.291	0.180	13.9%	
C2	B27	III	E11	6	1.305	1.286	1.296	0.050	0.042	0.046	0.928	0.958	0.943	1.250	0.897	71.8%	
C2	B29	II	E11	4	1.123	1.043	1.083	0.046	0.042	0.044	1.066	1.070	1.068	1.039	1.024	98.6%	
C2	B2b	II	E7	4	1.123	1.043	1.083	0.046	0.042	0.044	0.058	0.072	0.065	1.039	0.021	2.0%	
C2	B3	III	C11	6	1.305	1.286	1.296	0.050	0.042	0.046	0.613	0.591	0.602	1.250	0.556	44.5%	
C2	B32	II	F11	4	1.123	1.043	1.083	0.046	0.042	0.044	0.093	0.106	0.100	1.039	0.056	5.3%	
C2	B33	II	G11	4	1.123	1.043	1.083	0.046	0.042	0.044	0.995	0.988	0.225	1.039	0.181	17.4%	
C2	B35	III	H5	5	1.321	1.350	1.336	0.046	0.044	0.045	1.024	0.988	1.006	1.291	0.961	74.5%	
C2	B38	II	C6	3	1.107	0.929	1.018	0.430	0.410	0.420	0.639	0.554	0.597	0.598	0.177	29.5%	
C2	B39c	III	E8	6	1.305	1.286	1.296	0.050	0.042	0.046	1.023	0.882	0.953	1.250	0.907	72.5%	
C2	B4	II	H5	3	1.107	0.929	1.018	0.430	0.410	0.420	1.001	1.026	1.014	0.598	0.594	99.2%	
C2	B41	III	F8	6	1.305	1.286	1.296	0.050	0.042	0.046	0.845	0.833	0.839	1.250	0.793	63.5%	
C2	B43	II	D6	3	1.107	0.929	1.018	0.430	0.410	0.420	1.016	1.087	1.052	0.598	0.632	105.6%	
C2	B45	III	A6	5	1.321	1.350	1.336	0.046	0.044	0.045	0.405	0.374	0.390	1.291	0.345	26.7%	

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Herd	ID	Box	Tube	Plate	Pos A1	Pos B1	Pos Mean	Neg C1	Neg D1	Neg Mean	CalcOD 1	CalcOD 2	CalcOD Mean	Pos corr OD	Sample corr OD	S/P ratio	Exclusion
C2	B47b	III	H6	5	1.321	1.350	1.336	0.046	0.044	0.045	1.291	1.292	1.292	1.291	1.247	96.6%	
C2	B50b	III	G8	6	1.305	1.286	1.296	0.050	0.042	0.046	1.043	1.071	1.057	1.250	1.011	80.9%	
C2	B57	III	H8	6	1.305	1.286	1.296	0.050	0.042	0.046	1.052	1.010	1.031	1.250	0.985	78.8%	
C2	B7	II	F9	4	1.123	1.043	1.083	0.046	0.042	0.044	0.966	0.952	0.959	1.039	0.915	88.1%	
C2	B8b	III	F6	5	1.321	1.350	1.336	0.046	0.044	0.045	0.116	0.104	0.110	1.291	0.065	5.0%	
C2	C1	III	G9	6	1.305	1.286	1.296	0.050	0.042	0.046	1.065	0.850	0.958	1.250	0.912	72.9%	
C2	C2	III	H2	5	1.321	1.350	1.336	0.046	0.044	0.045	0.229	0.248	0.239	1.291	0.194	15.0%	
C2	O1	III	B9	6	1.305	1.286	1.296	0.050	0.042	0.046	0.812	0.867	0.840	1.250	0.794	63.5%	
C2	O12	III	F7	6	1.305	1.286	1.296	0.050	0.042	0.046	1.171	1.143	1.157	1.250	1.111	88.9%	
C2	O13	III	F11	6	1.305	1.286	1.296	0.050	0.042	0.046	1.155	1.252	1.204	1.250	1.158	92.6%	
C2	O14	III	A3	5	1.321	1.350	1.336	0.046	0.044	0.045	0.787	0.592	0.690	1.291	0.645	49.9%	
C2	O15	II	F6	3	1.107	0.929	1.018	0.430	0.410	0.420	1.006	0.993	1.000	0.598	0.580	96.9%	
C2	O16	III	G11	6	1.305	1.286	1.296	0.050	0.042	0.046	1.049	1.079	1.064	1.250	1.018	81.5%	
C2	O18	III	C6	5	1.321	1.350	1.336	0.046	0.044	0.045	0.197	0.213	0.205	1.291	0.160	12.4%	
C2	O19b	III	G7	6	1.305	1.286	1.296	0.050	0.042	0.046	1.329	1.332	1.331	1.250	1.285	102.8%	
C2	O20	II	H11	4	1.123	1.043	1.083	0.046	0.042	0.044	0.310	0.285	0.298	1.039	0.254	24.4%	
C2	O22	II	G9	4	1.123	1.043	1.083	0.046	0.042	0.044	1.128	1.084	1.106	1.039	1.062	102.2%	
C2	O23b	II	F7	4	1.123	1.043	1.083	0.046	0.042	0.044	0.271	0.341	0.306	1.039	0.262	25.2%	
C2	O24	II	G6	3	1.107	0.929	1.018	0.430	0.410	0.420	1.090	1.109	1.100	0.598	0.680	113.6%	
C2	O25	II	C11	4	1.123	1.043	1.083	0.046	0.042	0.044	0.840	0.817	0.829	1.039	0.785	75.5%	
C2	O26b	III	H7	6	1.305	1.286	1.296	0.050	0.042	0.046	1.210	1.228	1.219	1.250	1.173	93.9%	
C2	O28b	III	H1	5	1.321	1.350	1.336	0.046	0.044	0.045	1.465	1.442	1.454	1.291	1.409	109.1%	
C2	O29b	II	E9	4	1.123	1.043	1.083	0.046	0.042	0.044	0.076	0.082	0.079	1.039	0.035	3.4%	
C2	O32c	III	A8	6	1.305	1.286	1.296	0.050	0.042	0.046	1.264	1.279	1.272	1.250	1.226	98.1%	
C2	O33	III	H11	6	1.305	1.286	1.296	0.050	0.042	0.046	1.028	1.034	1.031	1.250	0.985	78.8%	
C2	O36	III	B8	6	1.305	1.286	1.296	0.050	0.042	0.046	0.589	0.541	0.565	1.250	0.519	41.5%	
C2	O37	II	G7	4	1.123	1.043	1.083	0.046	0.042	0.044	1.079	1.131	1.105	1.039	1.061	102.1%	
C2	O4	II	E6	3	1.107	0.929	1.018	0.430	0.410	0.420	0.954	1.028	0.991	0.598	0.571	95.5%	

Herd	ID	Box	Tube	Plate	Pos A1	Pos B1	Pos Mean	Neg C1	Neg D1	Neg Mean	CalcOD 1	CalcOD 2	CalcOD Mean	Pos corr OD	Sample corr OD	S/P ratio	Exclusion
C2	O40	III	A12	6	1.305	1.286	1.296	0.050	0.042	0.046	1.289	1.309	1.299	1.250	1.253	100.3%	
C2	O41	III	C9	6	1.305	1.286	1.296	0.050	0.042	0.046	1.321	1.330	1.326	1.250	1.280	102.4%	
C2	O46	II	H7	4	1.123	1.043	1.083	0.046	0.042	0.044	0.840	0.668	0.754	1.039	0.710	68.3%	
C2	O48	II	H6	3	1.107	0.929	1.018	0.430	0.410	0.420	0.808	0.753	0.781	0.598	0.361	60.3%	
C2	O50	III	D9	6	1.305	1.286	1.296	0.050	0.042	0.046	1.366	1.381	1.374	1.250	1.328	106.2%	
C2	O6	III	B6	5	1.321	1.350	1.336	0.046	0.044	0.045	1.393	1.449	1.421	1.291	1.376	106.6%	
C2	O7	III	E7	6	1.305	1.286	1.296	0.050	0.042	0.046	0.675	0.728	0.702	1.250	0.656	52.5%	
C2	O86/O8b	III	E9	6	1.305	1.286	1.296	0.050	0.042	0.046	0.077	0.063	0.070	1.250	0.024	1.9%	
C2	O9	III	A9	6	1.305	1.286	1.296	0.050	0.042	0.046	1.051	1.224	1.138	1.250	1.092	87.4%	
C2	R17/R47	III	B3	5	1.321	1.350	1.336	0.046	0.044	0.045	0.045	0.054	0.050	1.291	0.005	0.3%	
C2	R21c	III	C2	5	1.321	1.350	1.336	0.046	0.044	0.045	1.211	1.219	1.215	1.291	1.170	90.7%	
C2	R23b	II	G8	4	1.123	1.043	1.083	0.046	0.042	0.044	0.093	0.205	0.149	1.039	0.105	10.1%	
C2	R24b	III	A4	5	1.321	1.350	1.336	0.046	0.044	0.045	1.388	1.348	1.368	1.291	1.323	102.5%	
C2	R26b	III	E4	5	1.321	1.350	1.336	0.046	0.044	0.045	0.421	0.410	0.416	1.291	0.371	28.7%	
C2	R27b	II	F10	4	1.123	1.043	1.083	0.046	0.042	0.044	0.159	0.159	0.159	1.039	0.115	11.1%	
C2	R29b	III	D3	5	1.321	1.350	1.336	0.046	0.044	0.045	0.482	0.267	0.375	1.291	0.330	25.5%	
C2	R31b	III	D2	5	1.321	1.350	1.336	0.046	0.044	0.045	1.191	1.208	1.200	1.291	1.155	89.5%	
C2	R32b	II	D12	4	1.123	1.043	1.083	0.046	0.042	0.044	0.233	0.269	0.251	1.039	0.207	19.9%	
C2	R37b	II	H10	4	1.123	1.043	1.083	0.046	0.042	0.044	1.002	0.772	0.887	1.039	0.843	81.1%	
C2	R39b	II	E12	4	1.123	1.043	1.083	0.046	0.042	0.044	0.930	0.999	0.965	1.039	0.921	88.6%	
C2	R42b	III	E2	5	1.321	1.350	1.336	0.046	0.044	0.045	0.916	0.975	0.946	1.291	0.901	69.8%	
C2	R44b	II	H8	4	1.123	1.043	1.083	0.046	0.042	0.044	0.509	0.730	0.620	1.039	0.576	55.4%	
C2	R46b	III	H4	5	1.321	1.350	1.336	0.046	0.044	0.045	0.931	0.913	0.922	1.291	0.877	68.0%	
C2	R4c	III	G4	5	1.321	1.350	1.336	0.046	0.044	0.045	1.329	1.323	1.326	1.291	1.281	99.3%	
C2	R50b	II	B11	4	1.123	1.043	1.083	0.046	0.042	0.044	1.103	1.080	1.092	1.039	1.048	100.8%	
C2	R8b	II	E10	4	1.123	1.043	1.083	0.046	0.042	0.044	1.055	1.073	1.064	1.039	1.020	98.2%	
C2	unknown	III	H3	5	1.321	1.350	1.336	0.046	0.044	0.045	1.351	1.267	1.309	1.291	1.264	97.9%	
C2	W1b	II	B8	4	1.123	1.043	1.083	0.046	0.042	0.044	0.649	0.524	0.587	1.039	0.543	52.2%	

Herd	ID	Box	Tube	Plate	Pos A1	Pos B1	Pos Mean	Neg C1	Neg D1	Neg Mean	CalcOD 1	CalcOD 2	CalcOD Mean	Pos corr OD	Sample corr OD	S/P ratio	Exclusion
C2	W2	III	E3	5	1.321	1.350	1.336	0.046	0.044	0.045	0.373	0.284	0.329	1.291	0.284	22.0%	
C2	W4	II	A9	4	1.123	1.043	1.083	0.046	0.042	0.044	0.365	1.000	0.683	1.039	0.639	61.5%	
C2	W5b	II	A12	4	1.123	1.043	1.083	0.046	0.042	0.044	0.289	0.339	0.314	1.039	0.270	26.0%	
C2	W6	III	F3	5	1.321	1.350	1.336	0.046	0.044	0.045	0.824	0.636	0.730	1.291	0.685	53.1%	
C2	Y10b	II	A10	4	1.123	1.043	1.083	0.046	0.042	0.044	1.266	1.214	1.240	1.039	1.196	115.1%	X
C2	Y11	III	B5	5	1.321	1.350	1.336	0.046	0.044	0.045	1.361	1.401	1.381	1.291	1.336	103.5%	
C2	Y12	II	G12	4	1.123	1.043	1.083	0.046	0.042	0.044	1.042	0.626	0.329	1.039	0.285	27.4%	
C2	Y13	III	E10	6	1.305	1.286	1.296	0.050	0.042	0.046	0.959	0.941	0.950	1.250	0.904	72.3%	
C2	Y14	III	C5	5	1.321	1.350	1.336	0.046	0.044	0.045	1.164	1.187	1.176	1.291	1.131	87.6%	
C2	Y15c	II	H12	4	1.123	1.043	1.083	0.046	0.042	0.044	0.478	0.552	0.515	1.039	0.471	45.3%	
C2	Y16b	II	C8	4	1.123	1.043	1.083	0.046	0.042	0.044	0.042	0.046	0.044	1.039	0.000	0.0%	
C2	Y17	II	B10	4	1.123	1.043	1.083	0.046	0.042	0.044	0.976	0.988	0.982	1.039	0.938	90.3%	
C2	Y18	III	F10	6	1.305	1.286	1.296	0.050	0.042	0.046	0.521	0.424	0.473	1.250	0.427	34.1%	
C2	Y20 (Y20b)	III	E5	5	1.321	1.350	1.336	0.046	0.044	0.045	1.072	0.960	1.016	1.291	0.971	75.2%	
C2	Y24b	II	D8	4	1.123	1.043	1.083	0.046	0.042	0.044	0.042	0.046	0.044	1.039	0.000	0.0%	
C2	Y27	III	G10	6	1.305	1.286	1.296	0.050	0.042	0.046	0.767	0.684	0.726	1.250	0.680	54.4%	
C2	Y28	III	H10	6	1.305	1.286	1.296	0.050	0.042	0.046	1.456	1.475	1.466	1.250	1.420	113.6%	
C2	Y29	II	C9	4	1.123	1.043	1.083	0.046	0.042	0.044	0.066	0.054	0.060	1.039	0.016	1.5%	
C2	Y2b	II	F12	4	1.123	1.043	1.083	0.046	0.042	0.044	1.026	0.776	0.901	1.039	0.857	82.5%	
C2	Y3	II	B9	4	1.123	1.043	1.083	0.046	0.042	0.044	0.744	0.743	0.744	1.039	0.700	67.3%	
C2	Y31d	III	F1	5	1.321	1.350	1.336	0.046	0.044	0.045	1.315	1.260	1.288	1.291	1.243	96.3%	
C2	Y32b	II	C10	4	1.123	1.043	1.083	0.046	0.042	0.044	0.962	0.990	0.976	1.039	0.932	89.7%	
C2	Y33b	III	A11	6	1.305	1.286	1.296	0.050	0.042	0.046	0.057	0.053	0.055	1.250	0.009	0.7%	
C2	Y35d	III	D6	5	1.321	1.350	1.336	0.046	0.044	0.045	0.878	0.884	0.881	1.291	0.836	64.8%	
C2	Y37b	III	G2	5	1.321	1.350	1.336	0.046	0.044	0.045	1.344	1.359	1.352	1.291	1.307	101.2%	
C2	Y38b	III	B11	6	1.305	1.286	1.296	0.050	0.042	0.046	1.283	1.323	1.303	1.250	1.257	100.6%	
C2	Y39b	III	B4	5	1.321	1.350	1.336	0.046	0.044	0.045	1.347	1.136	1.242	1.291	1.197	92.7%	

Herd	ID	Box	Tube	Plate	Pos A1	Pos B1	Pos Mean	Neg C1	Neg D1	Neg Mean	CalcOD 1	CalcOD 2	CalcOD Mean	Pos corr OD	Sample corr OD	S/P ratio	Exclusion
C2	Y4	III	D10	6	1.305	1.286	1.296	0.050	0.042	0.046	0.258	0.356	0.307	1.250	0.261	20.9%	
C2	Y42	III	D5	5	1.321	1.350	1.336	0.046	0.044	0.045	0.240	0.183	0.212	1.291	0.167	12.9%	
C2	Y43b	II	D10	4	1.123	1.043	1.083	0.046	0.042	0.044	0.966	0.964	0.965	1.039	0.921	88.6%	
C2	Y44c	II	D9	4	1.123	1.043	1.083	0.046	0.042	0.044	0.066	0.054	0.060	1.039	0.016	1.5%	
C2	Y46b	III	E1	5	1.321	1.350	1.336	0.046	0.044	0.045	0.287	0.383	0.335	1.291	0.290	22.5%	
C2	Y47	III	G3	5	1.321	1.350	1.336	0.046	0.044	0.045	0.367	0.160	0.264	1.291	0.219	16.9%	X
C2	Y5c	II	H9	4	1.123	1.043	1.083	0.046	0.042	0.044	0.840	0.829	0.835	1.039	0.791	76.1%	
C2	Y6	III	A5	5	1.321	1.350	1.336	0.046	0.044	0.045	1.282	1.351	1.317	1.291	1.272	98.5%	
C2	Y9	III	A2	5	1.321	1.316	1.319	0.046	0.045	0.046	1.168	1.198	1.183	1.273	1.138	89.4%	
C3	OC1305 29b5	I	F1	1	1.047	0.984	1.016	0.041	0.390	0.216	1.121	1.121	1.121	0.800	0.906	113.2%	X
D	Buffalo *4	I	B2	1	1.047	0.984	1.016	0.041	0.390	0.216	1.188	1.155	1.172	0.800	0.956	119.5%	
D	Buffalo *10	I	H1	1	1.047	0.984	1.016	0.041	0.390	0.216	1.203	1.154	1.179	0.800	0.963	120.4%	
D	Buffalo 6	I	G1	1	1.047	0.984	1.016	0.041	0.390	0.216	0.120	0.122	0.121	0.800	-0.095	-11.8%	
D	Buffalo 13	I	A2	1	1.047	0.984	1.016	0.041	0.390	0.216	0.784	0.796	0.790	0.800	0.575	71.8%	
E	10	III	B12	6	1.305	1.286	1.296	0.050	0.042	0.046	1.286	1.239	1.263	1.250	1.217	97.4%	
E	39	III	C12	6	1.305	1.286	1.296	0.050	0.042	0.046	0.270	0.048	0.159	1.250	0.113	9.0%	
E	48	III	D12	6	1.305	1.286	1.296	0.050	0.042	0.046	0.756	0.781	0.769	1.250	0.723	57.8%	
E	19	III	E12	6	1.305	1.286	1.296	0.050	0.042	0.046	1.261	1.130	1.196	1.250	1.150	92.0%	
E	43	III	F12	6	1.305	1.286	1.296	0.050	0.042	0.046	1.220	0.103	0.662	1.250	0.616	49.3%	
E	26	III	H12	6	1.305	1.286	1.296	0.050	0.042	0.046	1.424	1.300	1.362	1.250	1.316	105.3%	
E	22	III	G12	6	1.305	1.286	1.296	0.050	0.042	0.046	1.351	1.159	1.255	1.250	1.209	96.8%	
E	30	IV	E1	7	1.286	1.317	1.302	0.049	0.047	0.048	0.741	0.720	0.731	1.254	0.683	54.4%	
E	40	IV	F1	7	1.286	1.317	1.302	0.049	0.047	0.048	0.071	0.063	0.067	1.254	0.019	1.5%	
E	8	IV	G1	7	1.286	1.317	1.302	0.049	0.047	0.048	0.563	0.405	0.484	1.254	0.436	34.8%	

Herd	ID	Box	Tube	Plate	Pos A1	Pos B1	Pos Mean	Neg C1	Neg D1	Neg Mean	CalcOD 1	CalcOD 2	CalcOD Mean	Pos corr OD	Sample corr OD	S/P ratio	Exclusion
E	32	IV	H1	7	1.286	1.317	1.302	0.049	0.047	0.048	0.791	0.770	0.781	1.254	0.733	58.4%	
E	55	IV	A2	7	1.286	1.317	1.302	0.049	0.047	0.048	0.736	0.655	0.696	1.254	0.648	51.7%	
E	41	IV	B2	7	1.286	1.317	1.302	0.049	0.047	0.048	0.133	0.116	0.125	1.254	0.077	6.1%	
E	16	IV	C2	7	1.286	1.317	1.302	0.049	0.047	0.048	1.069	1.134	0.125	1.254	0.077	6.1%	
E	18	IV	D2	7	1.286	1.317	1.302	0.049	0.047	0.048	1.044	1.238	1.141	1.254	1.093	87.2%	
E	1	IV	E2	7	1.286	1.317	1.302	0.049	0.047	0.048	0.217	0.321	0.269	1.254	0.221	17.6%	
E	46	IV	F2	7	1.286	1.317	1.302	0.049	0.047	0.048	0.084	1.131	0.608	1.254	0.560	44.6%	
E	47	IV	G2	7	1.286	1.317	1.302	0.049	0.047	0.048	0.181	0.135	0.158	1.254	0.110	8.8%	
E	27	IV	H2	7	1.286	1.317	1.302	0.049	0.047	0.048	1.116	1.151	1.134	1.254	1.086	86.6%	
E	23	IV	A3	7	1.286	1.317	1.302	0.049	0.047	0.048	0.939	0.908	0.924	1.254	0.876	69.8%	
E	17	IV	B3	7	1.286	1.317	1.302	0.049	0.047	0.048	1.113	1.202	1.158	1.254	1.110	88.5%	
E	12	IV	C3	7	1.286	1.317	1.302	0.049	0.047	0.048	1.026	0.984	1.005	1.254	0.957	76.3%	
E	6	IV	D3	7	1.286	1.317	1.302	0.049	0.047	0.048	1.035	0.856	0.946	1.254	0.898	71.6%	
E	49	IV	E3	7	1.286	1.317	1.302	0.049	0.047	0.048	0.729	0.721	0.725	1.254	0.677	54.0%	
E	34	IV	F3	7	1.286	1.317	1.302	0.049	0.047	0.048	1.063	1.241	1.152	1.254	1.104	88.1%	
E	9	IV	G3	7	1.286	1.317	1.302	0.049	0.047	0.048	1.164	1.097	1.131	1.254	1.083	86.4%	
E	3	IV	H3	7	1.286	1.317	1.302	0.049	0.047	0.048	0.959	0.950	0.955	1.254	0.907	72.3%	
E	51	IV	A4	7	1.286	1.317	1.302	0.049	0.047	0.048	0.069	0.068	0.069	1.254	0.021	1.6%	
E	4	IV	B4	7	1.286	1.317	1.302	0.049	0.047	0.048	1.264	1.311	1.288	1.254	1.240	98.9%	
E	53	IV	C4	7	1.286	1.317	1.302	0.049	0.047	0.048	1.126	1.122	1.124	1.254	1.076	85.8%	
E	54	IV	D4	7	1.286	1.317	1.302	0.049	0.047	0.048	0.751	0.768	0.760	1.254	0.712	56.8%	
E	38	IV	E4	7	1.286	1.317	1.302	0.049	0.047	0.048	0.060	0.052	0.056	1.254	0.008	0.6%	
E	28	IV	F4	7	1.286	1.317	1.302	0.049	0.047	0.048	0.190	0.177	0.184	1.254	0.136	10.8%	
E	50	IV	G4	7	1.286	1.317	1.302	0.049	0.047	0.048	0.162	0.096	0.129	1.254	0.081	6.5%	
E	44	IV	A5	7	1.286	1.317	1.302	0.049	0.047	0.048	1.042	1.119	1.081	1.254	1.033	82.4%	
E	57	IV	B5	7	1.286	1.317	1.302	0.049	0.047	0.048	1.076	1.112	1.094	1.254	1.046	83.4%	
E	45	IV	C5	7	1.286	1.317	1.302	0.049	0.047	0.048	0.087	0.082	0.085	1.254	0.037	2.9%	
E	33	IV	D5	7	1.286	1.317	1.302	0.049	0.047	0.048	0.997	0.955	0.976	1.254	0.928	74.0%	

Herd	ID	Box	Tube	Plate	Pos A1	Pos B1	Pos Mean	Neg C1	Neg D1	Neg Mean	CalcOD 1	CalcOD 2	CalcOD Mean	Pos corr OD	Sample corr OD	S/P ratio	Exclusion
E	36	IV	E5	7	1.286	1.317	1.302	0.049	0.047	0.048	0.097	0.110	0.104	1.254	0.056	4.4%	
E	52	IV	F5	7	1.286	1.317	1.302	0.049	0.047	0.048	0.932	0.778	0.855	1.254	0.807	64.4%	
E	5	IV	G5	7	1.286	1.317	1.302	0.049	0.047	0.048	0.983	1.097	1.040	1.254	0.992	79.1%	
E	56	IV	H5	7	1.286	1.317	1.302	0.049	0.047	0.048	1.333	1.399	1.366	1.254	1.318	105.1%	X
E	7	IV	A6	7	1.286	1.317	1.302	0.049	0.047	0.048	0.859	0.824	0.842	1.254	0.794	63.3%	
E	11	IV	B6	7	1.286	1.317	1.302	0.049	0.047	0.048	1.161	1.108	1.135	1.254	1.087	86.7%	
E	13	IV	C6	7	1.286	1.317	1.302	0.049	0.047	0.048	0.083	0.120	0.102	1.254	0.054	4.3%	
E	14	IV	D6	7	1.286	1.317	1.302	0.049	0.047	0.048	0.741	0.990	0.866	1.254	0.818	65.2%	
E	15	IV	E6	7	1.286	1.317	1.302	0.049	0.047	0.048	0.930	0.982	0.956	1.254	0.908	72.4%	
E	20	IV	F6	7	1.286	1.317	1.302	0.049	0.047	0.048	0.152	0.176	0.164	1.254	0.116	9.3%	
E	21	IV	G6	7	1.286	1.317	1.302	0.049	0.047	0.048	0.194	0.128	0.161	1.254	0.113	9.0%	
E	24	IV	H6	7	1.286	1.317	1.302	0.049	0.047	0.048	0.942	1.021	0.982	1.254	0.934	74.5%	
E	25	IV	E7	8	1.443	1.391	1.417	0.050	0.049	0.050	0.066	0.070	0.068	1.368	0.019	1.4%	
E	29	IV	F7	8	1.443	1.391	1.417	0.050	0.049	0.050	0.337	0.299	0.318	1.368	0.269	19.6%	
E	37	IV	G7	8	1.443	1.391	1.417	0.050	0.049	0.050	0.831	0.793	0.812	1.368	0.763	55.8%	
E	2	IV	H7	8	1.443	1.391	1.417	0.050	0.049	0.050	1.191	1.266	1.229	1.368	1.179	86.2%	
E	31	IV	A8	8	1.443	1.391	1.417	0.050	0.049	0.050	1.493	1.393	1.443	1.368	1.394	101.9%	
E	35	IV	B8	8	1.443	1.391	1.417	0.050	0.049	0.050	1.127	1.061	1.094	1.368	1.045	76.4%	
F	White 8	I	D2	1	1.047	0.984	1.016	0.041	0.390	0.216	1.108	1.066	1.087	0.800	0.872	108.9%	
F	Yellow 28	I	E2	1	1.047	0.984	1.016	0.041	0.390	0.216	1.175	1.125	1.150	0.800	0.935	116.8%	
F	Z.11	I	F2	1	1.047	0.984	1.016	0.041	0.390	0.216	1.107	1.060	1.084	0.800	0.868	108.5%	
F	Yellow 6	I	H2	1	1.047	0.984	1.016	0.041	0.390	0.216	1.159	1.109	1.134	0.800	0.919	114.8%	
F	Z.14	I	C2	1	1.047	0.984	1.016	0.041	0.390	0.216	1.035	0.995	1.015	0.800	0.800	99.9%	
F	Z14	I	G2	1	1.047	0.984	1.016	0.041	0.390	0.216	1.112	1.097	1.105	0.800	0.889	111.1%	X
F	B478/14, S5421/14	I	E1	1	1.047	0.984	1.016	0.041	0.390	0.216	1.121	1.113	1.117	0.800	0.902	112.7%	
E	42	IV	H4	7	1.286	1.317	1.302	0.049	0.047	0.048	0.878	0.680	0.779	1.254	0.731	58.3%	X

9.3   Appendix 3: FPA results

Herd	Specimen label/ID	Box	Tube	FPA Result 1	FPA Result 2	FPA rerun 1	FPA rerun 2	Exclusion	Reason FPA repeat
A	C16	I	A10	-35	-22				
A	C27	I	A11	10	-23				
A	C32	I	A12	-32	-31				
A	B46	I	A5	-17	-21				
A	B65	I	A6	-9	-5				
A	25	I	A8	18	11	3	8		suspect
A	C8	I	A9	-6	-6				
A	C17	I	B10	-34	-29				
A	C21	I	B11	-26	-73				
A	C33	I	B12	3	3				
A	B63	I	B5	290	-10	-7	1		high variation
A	B55	I	B6	-8	-12				
A	C1	I	B8	7	-3				
A	C9	I	B9	-12	-27				
A	C18	I	C10	-8	-9				
A	C23	I	C11	17	-7				
A	C34	I	C12	9	5				
A	B46	I	C4	4	11				
A	B42	I	C5	269	-3	-8	-1		high variation
A	B54	I	C6	-9	-7				
A	C2	I	C8	-77	-109				
A	C10	I	C9	0	-1				
A	C19	I	D10	1	2				
A	C26	I	D11	-1	-18				
A	C35	I	D12	-24	-27				
A	B61	I	D4	418	5	-2	-3		high variation
A	B57	I	D5	375	-12	-20	-17		high variation
A	B52	I	D6	-10	398	0	0		high variation

Herd	Specimen label/ID	Box	Tube	FPA Result 1	FPA Result 2	FPA rerun 1	FPA rerun 2	Exclusion	Reason FPA repeat
A	C3	I	D8	-32	-45				
A	C11	I	D9	-20	-29				
A	C20	I	E10	-37	-32				
A	C28	I	E11	-8	-25				
A	C36	I	E12	-1	-2				
A	B47+	I	E4	-13	-10				
A	B59+	I	E5	-7	3				
A	B51+	I	E6	-8	-10				
A	B64	I	E7	-78	-54				
A	C4	I	E8	-22	-47				
A	C12	I	E9	14	16	-1	8		suspect
A	C22	I	F10	-2	-12				
A	C29	I	F11	-79	-105				
A	C37	I	F12	10	13				
A	A	I	F4	-18	-16				
A	B53	I	F5	127	2	-24	-10		high variation
A	B50	I	F6	-1	-10				
A	B68	I	F7	-3	4				
A	C5	I	F8	-48	-76				
A	C13	I	F9	-19	-18				
A	C24	I	G10	-15	-14				
A	C30	I	G11	-29	-41				
A	C38	I	G12	12	6				
A	40	I	G4	2398	13				
A	B56	I	G5	372	10	-4	3		high variation
A	B49	I	G6	2	5			excluded	
A	27	I	G7	7	13				
A	C6	I	G8	-11	-15				
A	C14	I	G9	0	-7				
A	C25	I	H10	-27	-14				

Herd	Specimen label/ID	Box	Tube	FPA Result 1	FPA Result 2	FPA rerun 1	FPA rerun 2	Exclusion	Reason FPA repeat
A	C31	I	H11	9	7				
A	C39	I	H12	-105	-96				
A	B62	I	H4	76	-21	-26	-27		high variation
A	B45-	I	H5	-14	0				
A	B44	I	H6	1	-2				
A	28	I	H7	17	17	16	15		suspect
A	C7	I	H8	-14	-16				
A	C15	I	H9	-17	-21				
A	no number	II	A2	10	10				
A	B4	II	A3	9	8				
A	B15	II	A4	-7	-9				
A	B32	II	A5	-4	-2				
A	B43	II	B2	7	3				
A	B7	II	B3	15	9	-5	2		suspect
A	B17	II	B4	-40	1				
A	B34	II	B5	-8	-7	-16	350		low
A	B60	II	C2	-12	-15	-27	-15		low
A	B9	II	C3	no serum	no serum			excluded	
A	B22	II	C4	6	9				
A	B35	II	C5	10	13				
A	B65	II	D2	0	-1	-9	-7		low
A	B10	II	D3	-221	10	4	12		high variation
A	B24	II	D4	13	7				
A	B38	II	D5	0	10				
A	C40	II	E1	-14	-34				
A	B67	II	E2	-6	-27				
A	B11	II	E3	3	-5				
A	B26	II	E4	3	4				
A	B39	II	E5	8	11				
A	C41	II	F1	-42	-45				

Herd	Specimen label/ID	Box	Tube	FPA Result 1	FPA Result 2	FPA rerun 1	FPA rerun 2	Exclusion	Reason FPA repeat
A	B1	II	F2	-2	6				
A	B12	II	F3	10	10				
A	B27	II	F4	5	6				
A	B40	II	F5	9	8				
A	C42	II	G1	5	6	436	6		low
A	B2	II	G2	-16	-11	3	-11		low
A	B13	II	G3	11	7				
A	B28	II	G4	-1	6				
A	B41	II	G5	4	6				
A	C43	II	H1	-68	368	411	366		high variation
A	B3	II	H2	2	5			excluded	
A	B14	II	H3	14	12			excluded	
A	B31	II	H4	16	12	5	10		suspect
B	RM4	I	A3	-8	-3				
B	F6B	I	B4	-80	-116				
B	252	I	A4	-23	-29				
B	329	I	H3	-2	-4				
B	560	I	F3	-26	-13				
B	34F	I	G3	-73	-56				
B	48	I	D3	-5	457	7	17		high variation
B	870	I	B3	-9	-2				
B	937	I	E3	-15	6				
B	101	I	C3	-11	-4				
C1A	A1	IV	C8	8	8				
C1A	A2	IV	D8	19	16				
C1A	A3	IV	E8	18	14				
C1A	A4	IV	F8	24	25				
C1A	A5	IV	G8	76	72				
C1A	A6	IV	H8	199	215				
C1A	A7	IV	A9	17	16				

Herd	Specimen label/ID	Box	Tube	FPA Result 1	FPA Result 2	FPA rerun 1	FPA rerun 2	Exclusion	Reason FPA repeat
C1A	A8	IV	B9	86	79				
C1A	A9	IV	C9	12	15				
C1A	A10	IV	D9	13	16				
C1A	A11	IV	E9	177	159				
C1B	B1	IV	F9	28	23				
C1B	B2	IV	G9	102	94				
C1B	B3	IV	H9	91	67				
C1B	B4	IV	A10	154	143				
C1B	B5	IV	B10	20	14				
C1B	B6	IV	C10	54	45				
C1B	B7	IV	D10	37	39				
C1B	B8	IV	E10	13	15				
C1B	B9	IV	F10	56	50				
C1B	B10	IV	G10	98	94				
C1B	B11	IV	H10	35	35				
C1B	B12	IV	A11	16	16				
C1C	C1	IV	B11	15	11				
C1C	C2	IV	C11	11	15				
C1C	C3	IV	D11	15	12				
C1C	C4	IV	E11	16	18				
C1C	C5	IV	F11	126	129				
C1C	C6	IV	G11	26	22				
C1C	C7	IV	H11	29	27				
C1C	C8	IV	A12	16	16				
C1C	C9	IV	B12	41	50				
C1C	C10	IV	C12	109	101				
C1C	C11 (BB1)	IV	D12	19	20				
C1C	C12 (BB2)	IV	E12	26	31				
C1C	C13 (BB3)	IV	F12	23	19				
C1C	C14	IV	G12	14	17				

Herd	Specimen label/ID	Box	Tube	FPA Result 1	FPA Result 2	FPA rerun 1	FPA rerun 2	Exclusion	Reason FPA repeat
C1C	C15	IV	H12	40	-2092				
C1C	C16 (BB4)	V	E1	68	74				
C1C	C17	V	F1	104	111				
C2	R2	II	B12	25	26				
C2	R3	II	C12	1	5				
C2	R10	III	F9	192	219				
C2	R11	III	H9	39	26				
C2	R12	III	B2	45	47				
C2	R13	III	C4	92	73				
C2	R16	II	E8	9	9				
C2	R17	II	F8	140	138				
C2	R18	II	A8	22	29				
C2	R19	III	D4	19	22				
C2	R20	III	C3	161	177				
C2	R28	II	G10	4	4				
C2	R30	III	A10	171	176				
C2	R33	III	F2	60	70			excluded	
C2	R36	III	F4	24	25				
C2	R41	II	A11	196	188				
C2	R51	III	B10	13	11				
C2	R52	III	C10	15	15				
C2	B11	II	A6	18	15	605	20	excluded	suspect
C2	B12	III	C8	19	13				
C2	B13b	II	D11	134	121				
C2	B14b	III	G1	20	20				
C2	B15	III	D8	54	49				
C2	B16	III	D11	15	15				
C2	B17	III	G6	23	18				
C2	B19	III	F5	17	688				
C2	B20	III	E6	25	19				

Herd	Specimen label/ID	Box	Tube	FPA Result 1	FPA Result 2	FPA rerun 1	FPA rerun 2	Exclusion	Reason FPA repeat
C2	B24	II	B6	166	142				
C2	B26b	III	G5	191	26				
C2	B27	III	E11	26	24				
C2	B29	II	E11	134	135				
C2	B2b	II	E7	2	0				
C2	B3	III	C11	16	16				
C2	B32	II	F11	2	1				
C2	B33	II	G11	32	34				
C2	B35	III	H5	2	317				
C2	B38	II	C6	-21	-22	-42	-33		low
C2	B39c	III	E8	17	15				
C2	B4	II	H5	61	71	52	42		low pos
C2	B41	III	F8	17	24				
C2	B43	II	D6	164	157				
C2	B45	III	A6	17	16				
C2	B47b	III	H6	44	42				
C2	B50b	III	G8	34	26				
C2	B57	III	H8	18	15				
C2	B7	II	F9	-22	-15				
C2	B8b	III	F6	21	18				
C2	C1	III	G9	36	23				
C2	C2	III	H2	18	18				
C2	O1	III	B9	11	7				
C2	O12	III	F7	31	32				
C2	O13	III	F11	28	22				
C2	O14	III	A3	18	20				
C2	O15	II	F6	30	30	299	54		low pos
C2	O16	III	G11	19	19				
C2	O18	III	C6	9	18				
C2	O19b	III	G7	137	139				

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Herd	Specimen label/ID	Box	Tube	FPA Result 1	FPA Result 2	FPA rerun 1	FPA rerun 2	Exclusion	Reason FPA repeat
C2	O20	II	H11	-4	2				
C2	O22	II	G9	230	225				
C2	O23b	II	F7	4	1				
C2	O24	II	G6	83	78	49	21		low pos
C2	O25	II	C11	12	7				
C2	O26b	III	H7	30	30				
C2	O28b	III	H1	238	237				
C2	O29b	II	E9	0	-2				
C2	O32c	III	A8	20	17				
C2	O33	III	H11	26	1660				
C2	O36	III	B8	11	9				
C2	O37	II	G7	101	95				
C2	O4	II	E6	54	47	4905	500		low pos
C2	O40	III	A12	56	73				
C2	O41	III	C9	47	45				
C2	O46	II	H7	18	13				
C2	O48	II	H6	25	23	15	-595		low pos
C2	O50	III	D9	23	29				
C2	O6	III	B6	77	101				
C2	O7	III	E7	18	16				
C2	O86 O8b)	III	E9	-9	9				
C2	O9	III	A9	15	16				
C2	R17(OR R47)	III	B3	15	18				
C2	R21c	III	C2	51	56				
C2	R23b	II	G8	-1	-7				
C2	R24b	III	A4	84	85				
C2	R26b	III	E4	18	19				
C2	R27b	II	F10	4	-4				
C2	R29b	III	D3	779	23	751	-503		high variation
C2	R31b	III	D2	72	51				

Herd	Specimen label/ID	Box	Tube	FPA Result 1	FPA Result 2	FPA rerun 1	FPA rerun 2	Exclusion	Reason FPA repeat
C2	R32b	II	D12	2	5				
C2	R37b	II	H10	115	125				
C2	R39b	II	E12	61	57				
C2	R42b	III	E2	24	24				
C2	R44b	II	H8	9	3				
C2	R46b	III	H4	23	21				
C2	R4c	III	G4	66	73				
C2	R50b	II	B11	12	10				
C2	R8b	II	E10	49	47				
C2	unknown	III	H3	193	213				
C2	W1b	II	B8	6	3				
C2	W2	III	E3	20	14				
C2	W4	II	A9	15	19				
C2	W5b	II	A12	2	2				
C2	W6	III	F3	22	22				
C2	Y10b	II	A10	180	170			excluded	
C2	Y11	III	B5	52	273	47	458		high variation
C2	Y12	II	G12	93	85				
C2	Y13	III	E10	22	15				
C2	Y14	III	C5	26	551	13	19		high variation
C2	Y15c	II	H12	0	-6				
C2	Y16b	II	C8	69	73				
C2	Y17	II	B10	-2	-1				
C2	Y18	III	F10	12	18				
C2	Y20i (Y20b)	III	E5	38	186				
C2	Y24b	II	D8	4	5				
C2	Y27	III	G10	19	19				
C2	Y28	III	H10	185	206				
C2	Y29	II	C9	3	5				
C2	Y2b	II	F12	194	192				

Herd	Specimen label/ID	Box	Tube	FPA Result 1	FPA Result 2	FPA rerun 1	FPA rerun 2	Exclusion	Reason FPA repeat
C2	Y3	II	B9	8	9				
C2	Y31d	III	F1	159	155				
C2	Y32b	II	C10	28	29				
C2	Y33b	III	A11	9	13				
C2	Y35d	III	D6	18	18				
C2	Y37b	III	G2	150	161				
C2	Y38b	III	B11	54	53				
C2	Y39b	III	B4	27	26	25	22		low pos
C2	Y4	III	D10	19	20				
C2	Y42	III	D5	18	24				
C2	Y43b	II	D10	60	65				
C2	Y44c	II	D9	0	3				
C2	Y46b	III	E1	18	14				
C2	Y47	III	G3	15	19			excluded	
C2	Y5c	II	H9	10	10				
C2	Y6	III	A5	142	249				
C2	Y9	III	A2	23	23				
C3	OC130529b5	I	F1	181	185	137	131	excluded	High
D	Buffalo *4	I	B2	219	216				
D	Buffalo *10	I	H1	173	183	1131	143		High
D	Buffalo 6	I	G1	17	26	15	17		suspect
D	Buffalo 13	I	A2	19	24				
E	10	III	B12	223	232				
E	39	III	C12	3	8				
E	48	III	D12	13	7				
E	19	III	E12	26	29				
E	43	III	F12	1	0				
E	26	III	H12	204	204				
E	22	III	G12	105	84				
E	30	IV	E1	78	77				

Herd	Specimen label/ID	Box	Tube	FPA Result 1	FPA Result 2	FPA rerun 1	FPA rerun 2	Exclusion	Reason FPA repeat
E	40	IV	F1	-1	3				
E	8	IV	G1	22	21				
E	32	IV	H1	18	20				
E	55	IV	A2	82	77				
E	41	IV	B2	5	0				
E	16	IV	C2	73	77				
E	18	IV	D2	101	102				
E	1	IV	E2	19	13				
E	46	IV	F2	74	73				
E	47	IV	G2	5	8				
E	27	IV	H2	231	231				
E	23	IV	A3	28	29				
E	17	IV	B3	32	28				
E	12	IV	C3	211	149				
E	6	IV	D3	121	102				
E	49	IV	E3	18	19				
E	34	IV	F3	153	149				
E	9	IV	G3	233	230				
E	3	IV	H3	142	115				
E	51	IV	A4	-10	-7				
E	4	IV	B4	145	176				
E	53	IV	C4	45	52				
E	54	IV	D4	15	15				
E	38	IV	E4	1	16				
E	28	IV	F4	29	20				
E	50	IV	G4	-18	-10				
E	44	IV	A5	19	21				
E	57	IV	B5	34	42				
E	45	IV	C5	0	6				
E	33	IV	D5	79	99				

Herd	Specimen label/ID	Box	Tube	FPA Result 1	FPA Result 2	FPA rerun 1	FPA rerun 2	Exclusion	Reason FPA repeat
E	36	IV	E5	10	17				
E	52	IV	F5	82	92				
E	5	IV	G5	167	168				
E	56	IV	H5	199	194			excluded	
E	7	IV	A6	19	18				
E	11	IV	B6	108	107				
E	13	IV	C6	4	5				
E	14	IV	D6	45	62				
E	15	IV	E6	163	50				
E	20	IV	F6	18	13				
E	21	IV	G6	23	17				
E	24	IV	H6	13	17				
E	25	IV	E7	4	9				
E	29	IV	F7	12	2				
E	37	IV	G7	46	39				
E	2	IV	H7	189	189				
E	31	IV	A8	27	35				
E	35	IV	B8	109	99				
F	White 8	I	D2	222	217	140	134		High
F	Yellow 28	I	E2	279	275	267	270		High
F	Z.11	I	F2	197	195	116	413		High
F	Yellow 6	I	H2	200	196	134	128		High
F	Z.14	I	C2	197	192	110	130		High
F	Z14	I	G2	192	192			excluded	
F	B478/14, S5421/14	I	E1	225	226	204	207		High
E	42	IV	H4	14	16			excluded	

9.4 Appendix 4: iELISA ROC data

Variable	iELISA
Classification variable	DIAGNOSIS
Sample size	200
Positive group [a]	93 (46.50%)
Negative group [b]	107 (53.50%)

[a] DIAGNOSIS = 1
[b] DIAGNOSIS = 0

Disease prevalence (%)	unknown

Area under the ROC curve (AUC)

Area under the ROC curve (AUC)	1.000
Standard Error [a]	0.000448
95% Confidence interval [b]	0.981 to 1.000
z statistic	1114.821
Significance level P (Area=0.5)	<0.0001

[a] DeLong et al., 1988
[b] Binomial exact

Youden index

Youden index J	0.9892
Associated criterion	>0.405
Sensitivity	98.92
Specificity	100.00

Criterion values and coordinates of the ROC curve

Criterion	Sensitivity	95% CI	Specificity	95% CI	+LR	-LR
≥-0.749	100.00	96.1 - 100.0	0.00	0.0 - 3.4	1.00	
>-0.749	100.00	96.1 - 100.0	0.93	0.02 - 5.1	1.01	0.00
>-0.743	100.00	96.1 - 100.0	1.87	0.2 - 6.6	1.02	0.00
>-0.738	100.00	96.1 - 100.0	2.80	0.6 - 8.0	1.03	0.00
>-0.732	100.00	96.1 - 100.0	3.74	1.0 - 9.3	1.04	0.00
>-0.731	100.00	96.1 - 100.0	4.67	1.5 - 10.6	1.05	0.00
>-0.73	100.00	96.1 - 100.0	5.61	2.1 - 11.8	1.06	0.00
>-0.728	100.00	96.1 - 100.0	6.54	2.7 - 13.0	1.07	0.00

Criterion	Sensitivity	95% CI	Specificity	95% CI	+LR	-LR
>-0.726	100.00	96.1 - 100.0	7.48	3.3 - 14.2	1.08	0.00
>-0.725	100.00	96.1 - 100.0	9.35	4.6 - 16.5	1.10	0.00
>-0.722	100.00	96.1 - 100.0	12.15	6.6 - 19.9	1.14	0.00
>-0.718	100.00	96.1 - 100.0	14.02	8.1 - 22.1	1.16	0.00
>-0.706	100.00	96.1 - 100.0	14.95	8.8 - 23.1	1.18	0.00
>-0.704	100.00	96.1 - 100.0	16.82	10.3 - 25.3	1.20	0.00
>-0.69	100.00	96.1 - 100.0	17.76	11.0 - 26.3	1.22	0.00
>-0.687	100.00	96.1 - 100.0	18.69	11.8 - 27.4	1.23	0.00
>-0.675	100.00	96.1 - 100.0	19.63	12.6 - 28.4	1.24	0.00
>-0.668	100.00	96.1 - 100.0	20.56	13.4 - 29.5	1.26	0.00
>-0.66	100.00	96.1 - 100.0	21.50	14.1 - 30.5	1.27	0.00
>-0.654	100.00	96.1 - 100.0	22.43	14.9 - 31.5	1.29	0.00
>-0.652	100.00	96.1 - 100.0	23.36	15.7 - 32.5	1.30	0.00
>-0.65	100.00	96.1 - 100.0	24.30	16.5 - 33.5	1.32	0.00
>-0.649	100.00	96.1 - 100.0	25.23	17.3 - 34.6	1.34	0.00
>-0.648	100.00	96.1 - 100.0	26.17	18.1 - 35.6	1.35	0.00
>-0.642	100.00	96.1 - 100.0	27.10	19.0 - 36.6	1.37	0.00
>-0.638	100.00	96.1 - 100.0	28.04	19.8 - 37.5	1.39	0.00
>-0.629	100.00	96.1 - 100.0	28.97	20.6 - 38.5	1.41	0.00
>-0.625	100.00	96.1 - 100.0	29.91	21.4 - 39.5	1.43	0.00
>-0.624	100.00	96.1 - 100.0	31.78	23.1 - 41.5	1.47	0.00
>-0.62	100.00	96.1 - 100.0	32.71	24.0 - 42.5	1.49	0.00
>-0.616	100.00	96.1 - 100.0	33.64	24.8 - 43.4	1.51	0.00
>-0.615	100.00	96.1 - 100.0	34.58	25.6 - 44.4	1.53	0.00
>-0.613	100.00	96.1 - 100.0	35.51	26.5 - 45.4	1.55	0.00
>-0.612	100.00	96.1 - 100.0	36.45	27.4 - 46.3	1.57	0.00
>-0.609	100.00	96.1 - 100.0	37.38	28.2 - 47.3	1.60	0.00
>-0.608	100.00	96.1 - 100.0	38.32	29.1 - 48.2	1.62	0.00
>-0.606	100.00	96.1 - 100.0	39.25	30.0 - 49.2	1.65	0.00
>-0.602	100.00	96.1 - 100.0	40.19	30.8 - 50.1	1.67	0.00
>-0.597	100.00	96.1 - 100.0	41.12	31.7 - 51.0	1.70	0.00
>-0.595	100.00	96.1 - 100.0	42.06	32.6 - 52.0	1.73	0.00
>-0.592	100.00	96.1 - 100.0	42.99	33.5 - 52.9	1.75	0.00
>-0.591	100.00	96.1 - 100.0	43.93	34.3 - 53.9	1.78	0.00

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Criterion	Sensitivity	95% CI	Specificity	95% CI	+LR	-LR
>-0.583	100.00	96.1 - 100.0	44.86	35.2 - 54.8	1.81	0.00
>-0.582	100.00	96.1 - 100.0	45.79	36.1 - 55.7	1.84	0.00
>-0.571	100.00	96.1 - 100.0	46.73	37.0 - 56.6	1.88	0.00
>-0.568	100.00	96.1 - 100.0	47.66	37.9 - 57.5	1.91	0.00
>-0.567	100.00	96.1 - 100.0	48.60	38.8 - 58.5	1.95	0.00
>-0.557	100.00	96.1 - 100.0	49.53	39.7 - 59.4	1.98	0.00
>-0.55	100.00	96.1 - 100.0	50.47	40.6 - 60.3	2.02	0.00
>-0.548	100.00	96.1 - 100.0	51.40	41.5 - 61.2	2.06	0.00
>-0.542	100.00	96.1 - 100.0	52.34	42.5 - 62.1	2.10	0.00
>-0.539	100.00	96.1 - 100.0	53.27	43.4 - 63.0	2.14	0.00
>-0.534	100.00	96.1 - 100.0	54.21	44.3 - 63.9	2.18	0.00
>-0.524	100.00	96.1 - 100.0	55.14	45.2 - 64.8	2.23	0.00
>-0.51	100.00	96.1 - 100.0	56.07	46.1 - 65.7	2.28	0.00
>-0.495	100.00	96.1 - 100.0	57.01	47.1 - 66.5	2.33	0.00
>-0.489	100.00	96.1 - 100.0	57.94	48.0 - 67.4	2.38	0.00
>-0.44	100.00	96.1 - 100.0	58.88	49.0 - 68.3	2.43	0.00
>-0.432	100.00	96.1 - 100.0	59.81	49.9 - 69.2	2.49	0.00
>-0.416	100.00	96.1 - 100.0	60.75	50.8 - 70.0	2.55	0.00
>-0.405	100.00	96.1 - 100.0	61.68	51.8 - 70.9	2.61	0.00
>-0.389	100.00	96.1 - 100.0	62.62	52.7 - 71.8	2.67	0.00
>-0.378	100.00	96.1 - 100.0	63.55	53.7 - 72.6	2.74	0.00
>-0.37	100.00	96.1 - 100.0	64.49	54.6 - 73.5	2.82	0.00
>-0.366	100.00	96.1 - 100.0	65.42	55.6 - 74.4	2.89	0.00
>-0.349	100.00	96.1 - 100.0	66.36	56.6 - 75.2	2.97	0.00
>-0.321	100.00	96.1 - 100.0	67.29	57.5 - 76.0	3.06	0.00
>-0.244	100.00	96.1 - 100.0	68.22	58.5 - 76.9	3.15	0.00
>-0.214	100.00	96.1 - 100.0	69.16	59.5 - 77.7	3.24	0.00
>-0.213	100.00	96.1 - 100.0	70.09	60.5 - 78.6	3.34	0.00
>-0.212	100.00	96.1 - 100.0	71.03	61.5 - 79.4	3.45	0.00
>-0.211	100.00	96.1 - 100.0	71.96	62.5 - 80.2	3.57	0.00
>-0.208	100.00	96.1 - 100.0	72.90	63.4 - 81.0	3.69	0.00
>-0.206	100.00	96.1 - 100.0	73.83	64.4 - 81.9	3.82	0.00
>-0.201	100.00	96.1 - 100.0	74.77	65.4 - 82.7	3.96	0.00
>-0.199	100.00	96.1 - 100.0	75.70	66.5 - 83.5	4.12	0.00

Criterion	Sensitivity	95% CI	Specificity	95% CI	+LR	-LR
>-0.198	100.00	96.1 - 100.0	76.64	67.5 - 84.3	4.28	0.00
>-0.195	100.00	96.1 - 100.0	77.57	68.5 - 85.1	4.46	0.00
>-0.189	100.00	96.1 - 100.0	78.50	69.5 - 85.9	4.65	0.00
>-0.188	100.00	96.1 - 100.0	79.44	70.5 - 86.6	4.86	0.00
>-0.186	100.00	96.1 - 100.0	80.37	71.6 - 87.4	5.10	0.00
>-0.181	100.00	96.1 - 100.0	81.31	72.6 - 88.2	5.35	0.00
>-0.177	100.00	96.1 - 100.0	82.24	73.7 - 89.0	5.63	0.00
>-0.173	100.00	96.1 - 100.0	83.18	74.7 - 89.7	5.94	0.00
>-0.171	100.00	96.1 - 100.0	84.11	75.8 - 90.5	6.29	0.00
>-0.168	100.00	96.1 - 100.0	85.05	76.9 - 91.2	6.69	0.00
>-0.166	100.00	96.1 - 100.0	85.98	77.9 - 91.9	7.13	0.00
>-0.164	100.00	96.1 - 100.0	86.92	79.0 - 92.7	7.64	0.00
>-0.146	100.00	96.1 - 100.0	87.85	80.1 - 93.4	8.23	0.00
>-0.124	100.00	96.1 - 100.0	88.79	81.2 - 94.1	8.92	0.00
>-0.103	100.00	96.1 - 100.0	89.72	82.3 - 94.8	9.73	0.00
>-0.092	100.00	96.1 - 100.0	90.65	83.5 - 95.4	10.70	0.00
>-0.088	100.00	96.1 - 100.0	91.59	84.6 - 96.1	11.89	0.00
>-0.086	100.00	96.1 - 100.0	93.46	87.0 - 97.3	15.29	0.00
>-0.056	100.00	96.1 - 100.0	94.39	88.2 - 97.9	17.83	0.00
>-0.051	100.00	96.1 - 100.0	95.33	89.4 - 98.5	21.40	0.00
>-0.021	100.00	96.1 - 100.0	96.26	90.7 - 99.0	26.75	0.00
>0.061	98.92	94.2 - 100.0	96.26	90.7 - 99.0	26.46	0.011
>0.072	98.92	94.2 - 100.0	97.20	92.0 - 99.4	35.28	0.011
>0.136	98.92	94.2 - 100.0	98.13	93.4 - 99.8	52.92	0.011
>0.149	98.92	94.2 - 100.0	99.07	94.9 - 100.0	105.85	0.011
>0.405	**98.92**	**94.2 - 100.0**	**100.00**	**96.6 - 100.0**		**0.011**
>0.446	97.85	92.4 - 99.7	100.00	96.6 - 100.0		0.022
>0.517	96.77	90.9 - 99.3	100.00	96.6 - 100.0		0.032
>0.544	95.70	89.4 - 98.8	100.00	96.6 - 100.0		0.043
>0.558	94.62	87.9 - 98.2	100.00	96.6 - 100.0		0.054
>0.644	93.55	86.5 - 97.6	100.00	96.6 - 100.0		0.065
>0.716	92.47	85.1 - 96.9	100.00	96.6 - 100.0		0.075
>0.723	91.40	83.8 - 96.2	100.00	96.6 - 100.0		0.086
>0.74	90.32	82.4 - 95.5	100.00	96.6 - 100.0		0.097

Criterion	Sensitivity	95% CI	Specificity	95% CI	+LR	-LR
>0.763	89.25	81.1 - 94.7	100.00	96.6 - 100.0		0.11
>0.764	88.17	79.8 - 93.9	100.00	96.6 - 100.0		0.12
>0.788	87.10	78.5 - 93.2	100.00	96.6 - 100.0		0.13
>0.791	86.02	77.3 - 92.3	100.00	96.6 - 100.0		0.14
>0.811	84.95	76.0 - 91.5	100.00	96.6 - 100.0		0.15
>0.814	83.87	74.8 - 90.7	100.00	96.6 - 100.0		0.16
>0.825	82.80	73.6 - 89.8	100.00	96.6 - 100.0		0.17
>0.834	81.72	72.4 - 89.0	100.00	96.6 - 100.0		0.18
>0.845	80.65	71.1 - 88.1	100.00	96.6 - 100.0		0.19
>0.85	79.57	69.9 - 87.2	100.00	96.6 - 100.0		0.20
>0.858	78.49	68.8 - 86.3	100.00	96.6 - 100.0		0.22
>0.862	76.34	66.4 - 84.5	100.00	96.6 - 100.0		0.24
>0.864	75.27	65.2 - 83.6	100.00	96.6 - 100.0		0.25
>0.866	74.19	64.1 - 82.7	100.00	96.6 - 100.0		0.26
>0.867	73.12	62.9 - 81.8	100.00	96.6 - 100.0		0.27
>0.868	72.04	61.8 - 80.9	100.00	96.6 - 100.0		0.28
>0.872	70.97	60.6 - 79.9	100.00	96.6 - 100.0		0.29
>0.876	69.89	59.5 - 79.0	100.00	96.6 - 100.0		0.30
>0.881	68.82	58.4 - 78.0	100.00	96.6 - 100.0		0.31
>0.883	67.74	57.3 - 77.1	100.00	96.6 - 100.0		0.32
>0.884	66.67	56.1 - 76.1	100.00	96.6 - 100.0		0.33
>0.886	63.44	52.8 - 73.2	100.00	96.6 - 100.0		0.37
>0.889	62.37	51.7 - 72.2	100.00	96.6 - 100.0		0.38
>0.894	61.29	50.6 - 71.2	100.00	96.6 - 100.0		0.39
>0.895	60.22	49.5 - 70.2	100.00	96.6 - 100.0		0.40
>0.897	59.14	48.5 - 69.2	100.00	96.6 - 100.0		0.41
>0.907	58.06	47.4 - 68.2	100.00	96.6 - 100.0		0.42
>0.912	56.99	46.3 - 67.2	100.00	96.6 - 100.0		0.43
>0.94	55.91	45.2 - 66.2	100.00	96.6 - 100.0		0.44
>0.955	54.84	44.2 - 65.2	100.00	96.6 - 100.0		0.45
>0.962	53.76	43.1 - 64.2	100.00	96.6 - 100.0		0.46
>0.963	52.69	42.1 - 63.1	100.00	96.6 - 100.0		0.47
>0.966	51.61	41.0 - 62.1	100.00	96.6 - 100.0		0.48
>0.968	50.54	40.0 - 61.1	100.00	96.6 - 100.0		0.49

Criterion	Sensitivity	95% CI	Specificity	95% CI	+LR	-LR
>0.974	49.46	38.9 - 60.0	100.00	96.6 - 100.0		0.51
>0.979	48.39	37.9 - 59.0	100.00	96.6 - 100.0		0.52
>0.985	47.31	36.9 - 57.9	100.00	96.6 - 100.0		0.53
>0.986	45.16	34.8 - 55.8	100.00	96.6 - 100.0		0.55
>0.989	44.09	33.8 - 54.8	100.00	96.6 - 100.0		0.56
>0.99	43.01	32.8 - 53.7	100.00	96.6 - 100.0		0.57
>0.992	41.94	31.8 - 52.6	100.00	96.6 - 100.0		0.58
>0.993	40.86	30.8 - 51.5	100.00	96.6 - 100.0		0.59
>0.999	38.71	28.8 - 49.4	100.00	96.6 - 100.0		0.61
>1.003	37.63	27.8 - 48.3	100.00	96.6 - 100.0		0.62
>1.006	36.56	26.8 - 47.2	100.00	96.6 - 100.0		0.63
>1.008	35.48	25.8 - 46.1	100.00	96.6 - 100.0		0.65
>1.012	34.41	24.9 - 45.0	100.00	96.6 - 100.0		0.66
>1.018	33.33	23.9 - 43.9	100.00	96.6 - 100.0		0.67
>1.019	32.26	22.9 - 42.7	100.00	96.6 - 100.0		0.68
>1.021	31.18	22.0 - 41.6	100.00	96.6 - 100.0		0.69
>1.022	30.11	21.0 - 40.5	100.00	96.6 - 100.0		0.70
>1.024	29.03	20.1 - 39.4	100.00	96.6 - 100.0		0.71
>1.025	27.96	19.1 - 38.2	100.00	96.6 - 100.0		0.72
>1.028	26.88	18.2 - 37.1	100.00	96.6 - 100.0		0.73
>1.035	25.81	17.3 - 35.9	100.00	96.6 - 100.0		0.74
>1.053	23.66	15.5 - 33.6	100.00	96.6 - 100.0		0.76
>1.056	22.58	14.6 - 32.4	100.00	96.6 - 100.0		0.77
>1.066	21.51	13.7 - 31.2	100.00	96.6 - 100.0		0.78
>1.078	20.43	12.8 - 30.1	100.00	96.6 - 100.0		0.80
>1.079	19.35	11.9 - 28.9	100.00	96.6 - 100.0		0.81
>1.085	18.28	11.0 - 27.6	100.00	96.6 - 100.0		0.82
>1.087	17.20	10.2 - 26.4	100.00	96.6 - 100.0		0.83
>1.089	16.13	9.3 - 25.2	100.00	96.6 - 100.0		0.84
>1.091	15.05	8.5 - 24.0	100.00	96.6 - 100.0		0.85
>1.1	13.98	7.7 - 22.7	100.00	96.6 - 100.0		0.86
>1.105	12.90	6.8 - 21.5	100.00	96.6 - 100.0		0.87
>1.12	11.83	6.1 - 20.2	100.00	96.6 - 100.0		0.88
>1.127	10.75	5.3 - 18.9	100.00	96.6 - 100.0		0.89

Criterion	Sensitivity	95% CI	Specificity	95% CI	+LR	-LR
>1.132	9.68	4.5 - 17.6	100.00	96.6 - 100.0		0.90
>1.136	8.60	3.8 - 16.2	100.00	96.6 - 100.0		0.91
>1.148	7.53	3.1 - 14.9	100.00	96.6 - 100.0		0.92
>1.151	6.45	2.4 - 13.5	100.00	96.6 - 100.0		0.94
>1.168	5.38	1.8 - 12.1	100.00	96.6 - 100.0		0.95
>1.18	4.30	1.2 - 10.6	100.00	96.6 - 100.0		0.96
>1.191	3.23	0.7 - 9.1	100.00	96.6 - 100.0		0.97
>1.195	2.15	0.3 - 7.6	100.00	96.6 - 100.0		0.98
>1.204	1.08	0.03 - 5.8	100.00	96.6 - 100.0		0.99
>1.327	0.00	0.0 - 3.9	100.00	96.6 - 100.0		1.00

9.5 Appendix 5: CFT ROC data

Variable	CFT
Classification variable	DIAGNOSIS
Sample size	200
Positive group [a]	93 (46.50%)
Negative group [b]	107 (53.50%)

[a] DIAGNOSIS = 1
[b] DIAGNOSIS = 0

Disease prevalence (%)	unknown

Area under the ROC curve (AUC)

Area under the ROC curve (AUC)	0.871
Standard Error [a]	0.0228
95% Confidence interval [b]	0.816 to 0.914
z statistic	16.263
Significance level P (Area=0.5)	<0.0001

[a] DeLong et al., 1988
[b] Binomial exact

Youden index

Youden index J	0.7419
Associated criterion	>0
Sensitivity	74.19
Specificity	100.00

Criterion values and coordinates of the ROC curve

Criterion	Sensitivity	95% CI	Specificity	95% CI	+LR	-LR
≥0	100.00	96.1 - 100.0	0.00	0.0 - 3.4	1.00	
>0	74.19	64.1 - 82.7	100.00	96.6 - 100.0		0.26
>18	69.89	59.5 - 79.0	100.00	96.6 - 100.0		0.30
>21	62.37	51.7 - 72.2	100.00	96.6 - 100.0		0.38
>30	60.22	49.5 - 70.2	100.00	96.6 - 100.0		0.40
>36	59.14	48.5 - 69.2	100.00	96.6 - 100.0		0.41
>43	50.54	40.0 - 61.1	100.00	96.6 - 100.0		0.49
>49	49.46	38.9 - 60.0	100.00	96.6 - 100.0		0.51

Criterion	Sensitivity	95% CI	Specificity	95% CI	+LR	-LR
>60	45.16	34.8 - 55.8	100.00	96.6 - 100.0		0.55
>86	43.01	32.8 - 53.7	100.00	96.6 - 100.0		0.57
>98	40.86	30.8 - 51.5	100.00	96.6 - 100.0		0.59
>120	34.41	24.9 - 45.0	100.00	96.6 - 100.0		0.66
>145	32.26	22.9 - 42.7	100.00	96.6 - 100.0		0.68
>172	27.96	19.1 - 38.2	100.00	96.6 - 100.0		0.72
>196	21.51	13.7 - 31.2	100.00	96.6 - 100.0		0.78
>240	18.28	11.0 - 27.6	100.00	96.6 - 100.0		0.82
>344	13.98	7.7 - 22.7	100.00	96.6 - 100.0		0.86
>392	12.90	6.8 - 21.5	100.00	96.6 - 100.0		0.87
>480	10.75	5.3 - 18.9	100.00	96.6 - 100.0		0.89
>688	9.68	4.5 - 17.6	100.00	96.6 - 100.0		0.90
>784	0.00	0.0 - 3.9	100.00	96.6 - 100.0		1.00

9.6   Appendix 6: FPA ROC data

Variable	FPA
Classification variable	DIAGNOSIS
Sample size	200
Positive group [a]	93 (46.50%)
Negative group [b]	107 (53.50%)

[a] DIAGNOSIS = 1

[b] DIAGNOSIS = 0

Disease prevalence (%)	unknown

Area under the ROC curve (AUC)

Area under the ROC curve (AUC)	0.989
Standard Error [a]	0.0108
95% Confidence interval [b]	0.962 to 0.998
z statistic	45.430
Significance level P (Area=0.5)	<0.0001

[a] DeLong et al., 1988

[b] Binomial exact

Youden index

Youden index J	0.9785
Associated criterion	>16
Sensitivity	97.85
Specificity	100.00

Criterion	Sensitivity	95% CI	Specificity	Perf index	95% CI	+LR	-LR
≥-1026	100.00	96.1 - 100.0	0.00		0.0 - 3.4	1.00	
>-1026	98.92	94.2 - 100.0	0.00		0.0 - 3.4	0.99	
>-101	98.92	94.2 - 100.0	0.93		0.02 - 5.1	1.00	1.15
>-98	98.92	94.2 - 100.0	1.87		0.2 - 6.6	1.01	0.58
>-93	98.92	94.2 - 100.0	2.80		0.6 - 8.0	1.02	0.38
>-92	98.92	94.2 - 100.0	3.74		1.0 - 9.3	1.03	0.29
>-68	98.92	94.2 - 100.0	4.67		1.5 - 10.6	1.04	0.23
>-66	98.92	94.2 - 100.0	5.61		2.1 - 11.8	1.05	0.19
>-65	98.92	94.2 - 100.0	6.54		2.7 - 13.0	1.06	0.16
>-62	98.92	94.2 - 100.0	7.48		3.3 - 14.2	1.07	0.14
>-50	98.92	94.2 - 100.0	8.41		3.9 - 15.4	1.08	0.13

Criterion	Sensitivity	95% CI	Specificity	Perf index	95% CI	+LR	-LR
>-49	98.92	94.2 - 100.0	9.35		4.6 - 16.5	1.09	0.12
>-44	98.92	94.2 - 100.0	10.28		5.2 - 17.7	1.10	0.10
>-39	98.92	94.2 - 100.0	11.21		5.9 - 18.8	1.11	0.096
>-35	98.92	94.2 - 100.0	14.02		8.1 - 22.1	1.15	0.077
>-32	98.92	94.2 - 100.0	15.89		9.5 - 24.2	1.18	0.068
>-29	98.92	94.2 - 100.0	16.82		10.3 - 25.3	1.19	0.064
>-26	98.92	94.2 - 100.0	18.69		11.8 - 27.4	1.22	0.058
>-25	98.92	94.2 - 100.0	20.56		13.4 - 29.5	1.25	0.052
>-24	98.92	94.2 - 100.0	21.50		14.1 - 30.5	1.26	0.050
>-21	98.92	94.2 - 100.0	22.43		14.9 - 31.5	1.28	0.048
>-20	98.92	94.2 - 100.0	25.23		17.3 - 34.6	1.32	0.043
>-19	98.92	94.2 - 100.0	28.04		19.8 - 37.5	1.37	0.038
>-17	98.92	94.2 - 100.0	31.78		23.1 - 41.5	1.45	0.034
>-16	98.92	94.2 - 100.0	32.71		24.0 - 42.5	1.47	0.033
>-15	98.92	94.2 - 100.0	34.58		25.6 - 44.4	1.51	0.031
>-13	98.92	94.2 - 100.0	35.51		26.5 - 45.4	1.53	0.030
>-12	98.92	94.2 - 100.0	36.45		27.4 - 46.3	1.56	0.030
>-11	98.92	94.2 - 100.0	37.38		28.2 - 47.3	1.58	0.029
>-10	98.92	94.2 - 100.0	40.19		30.8 - 50.1	1.65	0.027
>-9	98.92	94.2 - 100.0	42.99		33.5 - 52.9	1.74	0.025
>-8	98.92	94.2 - 100.0	45.79		36.1 - 55.7	1.82	0.023
>-7	98.92	94.2 - 100.0	50.47		40.6 - 60.3	2.00	0.021
>-6	98.92	94.2 - 100.0	54.21		44.3 - 63.9	2.16	0.020
>-5	98.92	94.2 - 100.0	56.07		46.1 - 65.7	2.25	0.019
>-4	98.92	94.2 - 100.0	58.88		49.0 - 68.3	2.41	0.018
>-3	98.92	94.2 - 100.0	63.55		53.7 - 72.6	2.71	0.017
>-2	98.92	94.2 - 100.0	67.29		57.5 - 76.0	3.02	0.016
>-1	98.92	94.2 - 100.0	71.96		62.5 - 80.2	3.53	0.015
>0	98.92	94.2 - 100.0	72.90		63.4 - 81.0	3.65	0.015
>1	98.92	94.2 - 100.0	73.83		64.4 - 81.9	3.78	0.015
>2	98.92	94.2 - 100.0	74.77		65.4 - 82.7	3.92	0.014
>3	98.92	94.2 - 100.0	75.70		66.5 - 83.5	4.07	0.014
>4	98.92	94.2 - 100.0	76.64		67.5 - 84.3	4.23	0.014
>5	98.92	94.2 - 100.0	78.50		69.5 - 85.9	4.60	0.014

Criterion	Sensitivity	95% CI	Specificity	Perf index	95% CI	+LR	-LR
>6	98.92	94.2 - 100.0	82.24		73.7 - 89.0	5.57	0.013
>7	98.92	94.2 - 100.0	83.18		74.7 - 89.7	5.88	0.013
>8	98.92	94.2 - 100.0	85.98		77.9 - 91.9	7.06	0.013
>9	98.92	94.2 - 100.0	90.65		83.5 - 95.4	10.58	0.012
>10	98.92	94.2 - 100.0	95.33		89.4 - 98.5	21.17	0.011
>11	97.85	92.4 - 99.7	96.26		90.7 - 99.0	26.17	0.022
>12	97.85	92.4 - 99.7	98.13		93.4 - 99.8	52.35	0.022
>13	97.85	92.4 - 99.7	99.07		94.9 - 100.0	104.70	0.022
>16	**97.85**	**92.4 - 99.7**	**100.00**	**197.85**	**96.6 - 100.0**		**0.022**
>26	95.70	89.4 - 98.8	100.00		96.6 - 100.0		0.043
>29	94.62	87.9 - 98.2	100.00		96.6 - 100.0		0.054
>31	93.55	86.5 - 97.6	100.00		96.6 - 100.0		0.065
>32	92.47	85.1 - 96.9	100.00		96.6 - 100.0		0.075
>33	91.40	83.8 - 96.2	100.00		96.6 - 100.0		0.086
>35	90.32	82.4 - 95.5	100.00		96.6 - 100.0		0.097
>38	88.17	79.8 - 93.9	100.00		96.6 - 100.0		0.12
>43	86.02	77.3 - 92.3	100.00		96.6 - 100.0		0.14
>46	82.80	73.6 - 89.8	100.00		96.6 - 100.0		0.17
>49	81.72	72.4 - 89.0	100.00		96.6 - 100.0		0.18
>50	80.65	71.1 - 88.1	100.00		96.6 - 100.0		0.19
>51	79.57	69.9 - 87.2	100.00		96.6 - 100.0		0.20
>52	78.49	68.8 - 86.3	100.00		96.6 - 100.0		0.22
>54	76.34	66.4 - 84.5	100.00		96.6 - 100.0		0.24
>57	75.27	65.2 - 83.6	100.00		96.6 - 100.0		0.25
>59	74.19	64.1 - 82.7	100.00		96.6 - 100.0		0.26
>62	73.12	62.9 - 81.8	100.00		96.6 - 100.0		0.27
>63	72.04	61.8 - 80.9	100.00		96.6 - 100.0		0.28
>65	70.97	60.6 - 79.9	100.00		96.6 - 100.0		0.29
>70	69.89	59.5 - 79.0	100.00		96.6 - 100.0		0.30
>71	68.82	58.4 - 78.0	100.00		96.6 - 100.0		0.31
>74	66.67	56.1 - 76.1	100.00		96.6 - 100.0		0.33
>75	65.59	55.0 - 75.1	100.00		96.6 - 100.0		0.34
>78	64.52	53.9 - 74.2	100.00		96.6 - 100.0		0.35
>79	63.44	52.8 - 73.2	100.00		96.6 - 100.0		0.37

Criterion	Sensitivity	95% CI	Specificity	Perf index	95% CI	+LR	-LR
>80	62.37	51.7 - 72.2	100.00		96.6 - 100.0		0.38
>83	60.22	49.5 - 70.2	100.00		96.6 - 100.0		0.40
>85	59.14	48.5 - 69.2	100.00		96.6 - 100.0		0.41
>87	58.06	47.4 - 68.2	100.00		96.6 - 100.0		0.42
>89	54.84	44.2 - 65.2	100.00		96.6 - 100.0		0.45
>95	53.76	43.1 - 64.2	100.00		96.6 - 100.0		0.46
>96	52.69	42.1 - 63.1	100.00		96.6 - 100.0		0.47
>98	50.54	40.0 - 61.1	100.00		96.6 - 100.0		0.49
>102	49.46	38.9 - 60.0	100.00		96.6 - 100.0		0.51
>104	48.39	37.9 - 59.0	100.00		96.6 - 100.0		0.52
>105	47.31	36.9 - 57.9	100.00		96.6 - 100.0		0.53
>108	45.16	34.8 - 55.8	100.00		96.6 - 100.0		0.55
>112	44.09	33.8 - 54.8	100.00		96.6 - 100.0		0.56
>120	43.01	32.8 - 53.7	100.00		96.6 - 100.0		0.57
>128	40.86	30.8 - 51.5	100.00		96.6 - 100.0		0.59
>129	39.78	29.8 - 50.5	100.00		96.6 - 100.0		0.60
>135	38.71	28.8 - 49.4	100.00		96.6 - 100.0		0.61
>138	37.63	27.8 - 48.3	100.00		96.6 - 100.0		0.62
>139	36.56	26.8 - 47.2	100.00		96.6 - 100.0		0.63
>149	35.48	25.8 - 46.1	100.00		96.6 - 100.0		0.65
>151	34.41	24.9 - 45.0	100.00		96.6 - 100.0		0.66
>154	33.33	23.9 - 43.9	100.00		96.6 - 100.0		0.67
>156	32.26	22.9 - 42.7	100.00		96.6 - 100.0		0.68
>157	30.11	21.0 - 40.5	100.00		96.6 - 100.0		0.70
>159	29.03	20.1 - 39.4	100.00		96.6 - 100.0		0.71
>161	26.88	18.2 - 37.1	100.00		96.6 - 100.0		0.73
>165	25.81	17.3 - 35.9	100.00		96.6 - 100.0		0.74
>166	24.73	16.4 - 34.8	100.00		96.6 - 100.0		0.75
>168	22.58	14.6 - 32.4	100.00		96.6 - 100.0		0.77
>169	20.43	12.8 - 30.1	100.00		96.6 - 100.0		0.80
>174	19.35	11.9 - 28.9	100.00		96.6 - 100.0		0.81
>178	18.28	11.0 - 27.6	100.00		96.6 - 100.0		0.82
>180	17.20	10.2 - 26.4	100.00		96.6 - 100.0		0.83
>189	16.13	9.3 - 25.2	100.00		96.6 - 100.0		0.84

Criterion	Sensitivity	95% CI	Specificity	Perf index	95% CI	+LR	-LR
>192	15.05	8.5 - 24.0	100.00		96.6 - 100.0		0.85
>193	13.98	7.7 - 22.7	100.00		96.6 - 100.0		0.86
>196	12.90	6.8 - 21.5	100.00		96.6 - 100.0		0.87
>203	11.83	6.1 - 20.2	100.00		96.6 - 100.0		0.88
>204	10.75	5.3 - 18.9	100.00		96.6 - 100.0		0.89
>206	9.68	4.5 - 17.6	100.00		96.6 - 100.0		0.90
>207	8.60	3.8 - 16.2	100.00		96.6 - 100.0		0.91
>216	7.53	3.1 - 14.9	100.00		96.6 - 100.0		0.92
>218	6.45	2.4 - 13.5	100.00		96.6 - 100.0		0.94
>228	4.30	1.2 - 10.6	100.00		96.6 - 100.0		0.96
>231	3.23	0.7 - 9.1	100.00		96.6 - 100.0		0.97
>232	2.15	0.3 - 7.6	100.00		96.6 - 100.0		0.98
>238	1.08	0.03 - 5.8	100.00		96.6 - 100.0		0.99
>273	0.00	0.0 - 3.9	100.00		96.6 - 100.0		1.00