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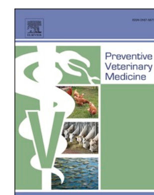

A Bayesian latent class estimation of the diagnostic accuracy of clinical examination and laboratory assays to identify bovine ephemeral fever virus infection in South African cattle

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ABSTRACT

Bovine ephemeral fever (BEF) is an economically important vector-borne viral disease of cattle and water buffalo in Africa, Australia and parts of Asia. The control of BEF is centred around vaccination, and therefore accurate, early identification of disease outbreaks are key to minimize its economic and welfare impact. In Africa, control programs are hampered by limited diagnostic capabilities and poor infrastructure for rapid transportation of diagnostic specimens. The primary objective of this study was to estimate the sensitivity (Se) and specificity (Sp) of four tests, namely clinical examination by a veterinarian, virus isolation and two different conventional PCR assays, to identify an acute bovine ephemeral fever virus (BEFV) infection in diseased, naturally infected South African cattle, without the assumption of a reference standard. Samples and data were collected from cattle with clinical signs suggestive of BEF rather than a random sample of cattle. A case was categorised as clinical examination positive if the examining veterinarian considered acute BEFV-infection as the most likely aetiology. Virus isolation was performed using the buffy coat of heparin blood samples on baby hamster kidney cell cultures, evaluating cytopathic effect and confirming virus morphology by transmission electron microscopy. PCR was performed using two previously published protocols: The *Ephemerovirus* L-gene PCR (targeting the RNA-dependent RNA polymerase gene) and a BEFV G-gene PCR (targeting the neutralising G1 epitope of the glycoprotein). A single population, four test Bayesian latent class model with conditional dependence between the two PCR assays was implemented. The prevalence of BEFV-infection was high in this study population of clinical suspects at 67 %, (95 % Probability Interval (PI) 52 %; 81 %). Clinical examination provided a reasonable indication of acute BEFV infection (Se of 86 % (PI 77 %; 93 %) and Sp of 67 % (PI 52 %; 82 %)). Virus isolation was the most specific (99 % (PI 97 %; 100 %)), but least sensitive assay (30 % (PI 20 %; 44 %)). Of the two conventional PCRs, the L-gene PCR outperformed the G-gene PCR: The L-gene Se was 64 % (PI 51 %; 76 %) and Sp 96 % (PI 84 %; 100 %) compared to Se of 50 % (PI 38 %; 61 %) and Sp of 89 % (PI 75 %; 98 %) for the G-gene. While the laboratory assays presented excellent positive predictive values within this high disease prevalence population, the poor negative predictive values limit their usefulness to field veterinarians attempting to exclude BEF as diagnosis. Novel pen-side diagnostics should be developed due to the limitations of currently available assays and infrastructure constraints prevalent in Africa.

1. Introduction

Bovine ephemeral fever (BEF) has been recognised as an economically important vector-borne viral disease of cattle and water buffalo in Africa, Australia and parts of Asia since the 19th century (Schweinfurth, 1874; Walker and Klement, 2015). It is caused by bovine ephemeral fever virus (BEFV), a member of the species *Ephemerovirus febris*, and characterised by acute onset fever, nasal and ocular discharge and muscle stiffness or shifting lameness, with affected animals usually recovering within three days (Bevan, 1907; Theiler, 1908; St. George, 2004). Although the mortality is typically low (less than 1 %) (Walker and Klement, 2015), affected herds can experience costly production losses, including up to an 85 % drop in milk production over the remainder of the affected lactation (Theodoridis et al., 1973; Davis et al.,

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1984; Yeruham et al., 2003) and condition losses in feedlot animals (Newton and Wheatley, 1970). Some animals can also develop acute pulmonary oedema and emphysema (Theodoridis and Coetzer, 1979), or suffer long-term paralysis (Barigye et al., 2016).

Vaccination remains the primary method to reduce the impact of the disease in BEF endemic regions (Walker and Klement, 2015). Unfortunately, vaccine uptake has been complicated by cost, irregular availability (production and distribution limitations) and the requirement for yearly boosters (Aziz-Boaron et al., 2014). As a consequence, many farmers rely on implementing strategic vaccination during high-risk periods. No official reports for vaccine use or BEF prevalence are available for South Africa, since BEF is not a controlled or notifiable disease. However, the data from the voluntary reporting network of the Ruminant Veterinary Association of South Africa (RuVASA) (https://www.ruvasa.co.za/) indicate that cases are seen throughout the country every year.

Often the first warning of the arrival of the virus in a region is when a presumptive diagnosis of BEF is made by a veterinarian. Although BEF is well known in Africa (Schweinfurth, 1874; St. George, 2004) and an experienced veterinarian can make a reasonably accurate clinical diagnosis of BEF based on the clinical signs and a quick recovery, none of the clinical signs are pathognomonic for BEF. Furthermore, predicting high-risk periods are almost impossible because of a poor understanding of the general epidemiology of BEFV (St. George, 2004; Walker, 2013; Walker and Klement, 2015). As a consequence, reliable, objective laboratory diagnostics are a crucial backup for the field veterinarian.

Limited availability of diagnostic laboratories is an important complicating factor for the diagnosis of infectious diseases for both human and veterinary medicine in Africa (Francis, 2022; Abdul-Karim et al., 2023). Samples often need to be transported substantial distances under suboptimal conditions with the risk of specimen degradation over time. Serology is therefore often preferred since antibodies are typically more stable in transport. Unfortunately, serology is of limited clinical value for the diagnosis of a BEFV outbreak because of the delay in seroconversion, the inability to date the exposure (chronic vs acute infection) and the inability to differentiate between natural infection and vaccine-induced immunity.

Assays aimed at detecting the pathogen are therefore more ideal for BEFV outbreak diagnostics. Virus isolation (VI) followed by confirmation of virus morphology by electron microscopy is currently the standard confirmatory test for BEFV in South Africa. VI relies on the presence of live, infectious virus, and is expected to have near perfect specificity. However, variations in isolation rates suggest that the sensitivity of the assay is likely not perfect (Spradbrow and Francis, 1969; Tanaka et al., 1969; Heuschele, 1970; Kemp et al., 1973; St. George et al., 1977; St. George, 1985; Zheng et al., 2011; Zaghawa et al., 2016; Hirashima et al., 2017; Karayel-Hacioglu et al., 2021).

Given the difficulties of virus isolation for BEFV, various alternative diagnostic assays, especially molecular assays based on PCR have been reported. The availability of sequence data from Asia and Australia enabled these developments. However, molecular data from sub-Saharan Africa are scarce (Walker et al., 2015), and the available data suggest that the African strains are phylogenetically diverse and different to the Asian and Australian lineages (Dacheux et al., 2019; Omar et al., 2020; Mlingo et al., 2021). *In silico* analyses of available published assays have suggested misalignment between highly specific real-time PCR assays developed from Asian and Australian strains and African BEFV strains (Hsieh et al., 2005; Stram et al., 2005; Lew et al., 2006; Zheng et al., 2011; Zaghloul et al., 2012; Erster et al., 2017; Hou et al., 2018; Gao et al., 2020). Conventional PCRs are often considered to have poorer analytical sensitivity than real-time platforms but offer the advantage that PCR products can readily be sequenced. The L-gene PCR was developed to detect all *Ephemerovirus* species (Blasdell et al., 2013). It amplifies a short sequence (137 bp) of the relatively conserved *Ephemerovirus* L-gene, encoding the RNA-dependent RNA polymerase enzyme. Although some publications have indicated that this assay has poor sensitivity (Dacheux et al., 2019), its ability to identify multiple *Ephemerovirus* species makes it a favourable candidate for Africa given that the diversity of BEFV and *Ephemerovirus* genotypes is poorly described. The G-gene 420 base pair PCR amplifies the G1 neutralising epitope of the BEFV glycoprotein (Walker et al., 1992). This is one of the more commonly reported conventional PCRs (Zheng et al., 2007, 2009a, 2011; Hou et al., 2018) and has been used for outbreak descriptions (Chaisirirat et al., 2018; Amal et al., 2019; El-Habbaa and Radwan, 2019; Abo-Sakaya and Bazan, 2020). The glycoprotein is the main host-virus interaction and antibodies aimed at this protein are reported to provide virus neutralising immunity (Walker et al., 1991).

In this study, we considered the question of which of the mentioned assays (if any) would be the most appropriate for a South African field veterinarian. The primary objective was thus to estimate the diagnostic sensitivity (Se) and diagnostic specificity (Sp) of a clinical examination classification by the veterinarian (CS), virus isolation (VI), the L-gene PCR (LG) and the G-gene PCR (GG) to identify acute BEFV infection in diseased South African cattle with clinical signs suggestive of BEF. Since no gold standard test is available, a Bayesian latent class analysis (BLCA) was used. As a second objective, a preliminary analysis of the impact of transport time (i.e. the time from sample collection to processing) on VI and PCR was investigated.

2. Materials and methods

2.1. Study population

Prospective, convenience sampling was performed followed by retrospective data analysis to evaluate the diagnostic accuracy of the assays. Veterinarians affiliated with RuVASA were approached to contribute samples to a related project investigating the molecular characteristics of South African BEFV strains. Veterinarians were not requested to specifically search for suspected BEF cases nor were any incentives provided. However, veterinarians were requested to submit a heparin and an ethylenediaminetetraacetic acid (EDTA) anticoagulated blood sample, and to report the outcome of a clinical examination when submitting material for laboratory confirmation of BEFV.

The target population for this study consisted of the specific animals for which the selected BEFV-assays would typically be requested by a South African field veterinarian dealing with a potential BEF outbreak. This population was defined as 1) living within the borders of South Africa, 2) one year of age or older, 3) suffering from naturally acquired infection, 4) identified as “sick” by their primary caretakers, and 5) examined by a veterinarian affiliated with RuVASA with experience in African cattle diseases. No restrictions were placed on the sex, production stage, production system or vaccination status of the animal. The number of samples originating from a specific farm or region was also not restricted.

A case for inclusion was defined as a bovine that suffered from either fever and shifting lameness, fever and paresis/paralysis/stiffness, or acute onset respiratory distress associated with fever (van der Westhuizen, 1967). The animal had to display these signs at the time of examination by the veterinarian, or have a history of displaying these signs within the last five days. In addition to these clinical findings, the primary caretaker also had to either request or agree to laboratory confirmation of a presumptive diagnosis of BEF.

2.2. Clinical examination classification

In addition to the examination findings required for case enrolment, the examining veterinarian also assigned a clinical examination classification to each case. A case was considered clinical examination positive if the examining veterinarian considered acute BEF to be the most likely diagnosis, based on the clinical signs of the individual case and the supporting evidence (history, environment, management (Constable et al., 2017)) gathered during the clinical examination. Acute BEF was

considered the clinical expression of an active BEFV infection. A case was classified as a clinical examination negative if any other differential diagnosis was considered more likely than BEF, or if BEF was considered the most likely disease but the clinical signs had already been present for more than 48 hours.

2.3. Laboratory assays

All assays were performed in the laboratories of the Department of Veterinary Tropical Diseases (DVTD), Faculty of Veterinary Science, Onderstepoort, Pretoria. The heparin sample was for virus isolation while the EDTA sample was for PCR analysis. The personnel performing laboratory assays were aware that the samples were submitted for confirmation of suspected BEFV-infection, but were blinded to the classification of the individual cases as clinical examination positive or negative. Virus isolation and PCR assays were performed by different personnel, who were blinded to the reciprocal results. The two PCR assays were performed in parallel on the same day.

2.3.1. Virus Isolation

Virus isolation was performed according to van der Westhuizen (1967). Briefly, the buffy coat from heparinised blood samples was washed three times with phosphate-buffered saline (PBS) and inoculated unto prepared confluent monolayers of baby hamster kidney cells (BSR) in cell culture flasks (TPP® tissue culture flasks), with minimum essential media (MEM with Earle's salts, L-glutamina & non-essential amino acids, Biowest) supplemented with 5 % foetal calf serum (Gamma-irradiated Fetal bovine serum, Biowest) and 1 mg/ml gentamycin (Genta 50 Phenix®, Virbac), and incubated at 37°C with 5 % CO_2. Cell cultures were examined daily for signs of cytopathic effect (CPE). Samples were blindly passaged at least three times before being considered negative. Cultures with detectable CPE were sent for transmission electron microscopy (TEM). A sample was considered VI positive when TEM confirmed the presence of bullet- to cone-shaped rhabdovirus-like particles.

2.3.2. PCR

Two previously published conventional reverse transcription PCR were performed on whole blood samples collected in EDTA (Zheng et al., 2007; Blasdell et al., 2013). Upon arrival at the laboratory, whole blood was transferred to cryotubes and stored at −80°C until processing.

RNA extractions were performed with a commercial kit (QIAamp Viral RNA mini kit; Qiagen) according to the manufacturer's specifications following the spin protocol. In short, the kit makes use of a silica-based membrane that selectively binds RNA. A lysis buffer was added to the sample, 140 μL freeze-thawed whole-blood in EDTA, to denature proteins (including RNAses). This was followed by the addition of ethanol to optimise the binding of RNA to the membrane. Two washing steps were performed to remove contaminants. In the final step, purified RNA was eluted from the membrane with the elution/storage buffer. Extracted RNA was stored at −80°C until use. RNA extraction was performed once and both PCRs were run on the same RNA-extract.

Reverse transcription and amplification were performed in a single run using the OneStep *Ahead* RT-PCR commercial kit (Qiagen) according to the manufacturer's specifications. Briefly, the reverse transcription was performed first, followed by heat inactivation of the reverse transcriptase enzymes and heat activation of DNA-polymerase enzymes. This was followed by PCR cycling, according to the specifications of the specific primer set. Primers were supplied by Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, South Africa.

The L-gene PCR was performed as previously described (Blasdell et al., 2013), with minor changes to the reverse primer and annealing temperature. Both primers had degenerate nucleotides and were forward primer 5'-GGR TTD ACA ATG GCN GAT GA-3' and reverse primer 5'-CTT TGA TAR TTA ATT CCA TTT TTC ATA-3'. The cycling conditions were 10 minutes at 50°C, 5 minutes at 95°C, 40 cycles of 95°C for 10 seconds, 48°C for 30 seconds and 72°C for 10 seconds, followed by final extension of 2 minutes at 72°C.

The G420-gene PCR was performed as previously described (Zheng et al., 2009b), although the annealing temperature was increased to 54°C after temperature optimisation for local conditions. The primers used were forward primer 5'- TAA GAG AGC TTG GTG TGA GTA C-3' and reverse primer 5'- CCA ACC TAC AAC AGC AGA CA-3'. The cycling conditions were 10 minutes at 50°C, 5 minutes at 95°C, 40 cycles of 95°C for 10 seconds, 54°C for 30 seconds and 72°C for 10 seconds, followed by final extension of 2 minutes at 72°C.

Gel electrophoresis was performed to identify positive samples (Thorne, 1966). A field sample submitted in March 2020 for which the identity was confirmed by sequencing, was selected as positive control for PCR runs. RNAse-free water was used as negative control. Samples were considered PCR positive if an amplicon of the appropriate size (± 137 bp for the LG and ± 420 bp for GG) was present.

2.3.3. Effect of transport time

A preliminary assessment of the effect that the time from sampling to laboratory processing plays on laboratory test outcome was performed. After collection, blood samples were transported to the DVTD diagnostic laboratories in accordance with WOAH guidelines (WOAH, 2018) by either the veterinarian, the owner or a commercial courier service. Sample transport was performed according to the regular procedures of the submitting veterinarian and was not funded by this study. The time from sampling to processing was categorised into four categories: ≤ 24 hours; 25–48 hours; 49–72 hours and 73 hours or more. Cross tabulation was performed according to clinical examination classification and laboratory assay results. No attempt was made to correlate province of origin or shipping distance to transport time.

2.4. Analysis

Only animals with complete data for all four assays were included in the analyses.

Descriptive data were collected and processed in Microsoft® Excel. Agreement between pairs of diagnostic assays, kappa (K) (Cohen, 1960), was calculated in commercially available software (IBM SPSS Statistics Version 23, International Business Machines Corp., Amnonk, New York, USA). Kappa values were classified as poor (<0.20), fair (0.21–0.4), moderate (0.41–0.6), good (0.61–0.8) or very good (0.81–1.0) (Everitt, 1989).

The diagnostic sensitivity (Se) and specificity (Sp) of the assay (CS, VI, LG and GG) and the prevalence (prev) of disease within the selected population were estimated with BLCA, given that the true infection status of the subjects was unknown and that a gold standard test was not available. The latent condition was defined as the presence of BEFV-infection in the diseased bovine. For CS, this definition was interpreted as the clinical expression of acute BEFV infection. For VI, it was interpreted as the presence of characteristic virus particles while for PCRs, this was interpreted as the presence of the appropriate size fluorescent band on gel electrophoresis. Two BLCA models were analysed, based on the Hui-Walter paradigm adapted for Bayesian analysis (Hui and Walter, 1980; Enoe et al., 2000; Fosgate et al., 2002). The first model considered one population and all four assays conditionally independent. The second model considered one population with the CS and VI conditionally independent, the PCR assays (LG and GG) independent of CS and VI, but adjusted for conditional dependence between the two PCRs (Vacek, 1985). CS, VI and the PCR assays were considered independent since these make use of different biological principles (Gardner et al., 2000). However, the presence of conditional dependence could not be excluded for the PCR assays since these make use of the same biological samples (EDTA blood sample and RNA extraction sample) even though viral genes with dissimilar functions and viral expression profiles were targeted (Gardner et al., 2000). Both models were identifiable given that the available degrees of freedom (df = 15)

was greater than the number of parameters estimated (nine for the first and 11 for the second model) (Cheng et al., 2021). In addition to Se and Sp, the positive predictive value (PPV) and negative predictive value (NPV) for each test were calculated (Thrusfield, 2007). (The WinBUGS code for the analyses can be found in the Supplementary material: Addendum A. Results are reported according to the STARD-checklist (Table S2) (Bossuyt et al., 2015))

Prior probabilities were modelled as beta distributions (beta (a, b)). Where possible, quantitative or qualitative data from literature were used for prior elicitation (Table 1). Qualitative data from diagnostic assay development and disease outbreak reports were included in the elicitation of priors for Se of VI and Se of GG. For both these estimates, a combined mean and histogram of percentage positive cases was calculated and the beta distribution was modelled using open access software (Bognar, 2021) to follow these trends, resulting in beta (4.5, 11) for Se of VI and beta (13.5, 17) for Se of GG (Spradbrow and Francis, 1969; Kemp et al., 1973; St. George et al., 1977; St. George, 1985; Zheng et al., 2011; Zaghawa et al., 2016; Hirashima et al., 2017; Karayel-Hacioglu et al., 2021)). Insufficient quantitative data were available for the Se of LG and therefore a weakly informative prior guided by qualitative data was used (beta (22, 8) (Blasdell et al., 2013; Dacheux et al., 2019)). No data were available for the modelling of Sp, and therefore beta distributions were based on expert opinion. For VI, a highly specific test result was anticipated (beta (99,1)). Likewise, most PCR assays are expected to be relatively specific, although it was anticipated that LG (beta (11, 1.5)), developed for detecting all Ephemeroviruses and using degenerate primers, would be less specific than GG (beta (9, 1)). A non-specific (uniform) prior distribution was selected for the prevalence (beta (1, 1)), since no previous studies on the prevalence of BEFV in South African cattle with clinical signs suggestive of BEF could be identified.

Bayesian latent class analysis was conducted using Markov chain Monte Carlo techniques in available statistical software (WinBUGS version 1.4, MRC Biostatistics Unit). For each model, three chains were run for 100 000 iterations, with only every 3rd iteration retained to reduce autocorrelation. The first 50 000 iterations were discarded as burn in and the next 50 000 were included in study inferences. Convergence was assessed by the Gelman-Rubin diagnostic (Gelman and Rubin, 1992). The median values and 95 % probability intervals as the 2.5th and 97.5th percentiles were calculated for each parameter.

To evaluate the sensitivity of the estimates to specified prior probability distributions, a sensitivity analysis was performed with an informative prior on prevalence (beta (26.23, 12.84)), uninformative priors on all four sensitivities (beta (1, 1)) and weakly informative priors on all four specificities (beta (2, 1)) in effort to guide the analysis into the correct parameter space.

3. Results

3.1. Descriptive statistics

Over the study period of March 2020 to March 2022, 124 samples from suspected BEF cases were received, 88 of which had data available for all four assays. For 14 cases no PCR or VI data were available because only clinical examination findings and serum samples were received; for 21 cases no VI data were available because only clinical examination findings and blood in EDTA were received and for one case without PCR data, only clinical examination findings and blood in heparin were received.

The majority of the samples originated from cattle classified as clinical examination positive (78 %) meaning that acute BEF was considered the most likely diagnosis by the examining veterinarian (Table S1). Overall, 57 % of the samples were BEFV positive on one or more laboratory assay. Independently, the laboratory assays had a low positive percentage, ranging from 20 % for VI to 43 % for LG (Table S1). There was moderate agreement between the two PCR assays ($K = 0.60$; 95 % confidence interval (0.43; 0.76)). Agreement between all other assay pairs were poor to fair ($K < 0.4$) (Cohen, 1960) (Table 2).

3.2. Latent class analysis

Nine cases (10 %) were positive on all four tests, while 18 (20 %) were negative on all four tests (Table 3). Only one case (1 %) classified as clinical examination negative tested positive on a laboratory assay and only on one assay, GG. Twenty clinical examination positive cases (23 %) tested negative on all laboratory assays.

Table 2
Outcome of kappa pairwise agreement assessment of clinical examination (CS), virus isolation (VI), L-gene PCR (LG) and G-gene (GG) PCR to identify the presence of BEFV in clinically diseased South African cattle.

Assay pair	Kappa	95 % Confidence interval
CS vs VI	0.13	0.05; 0.21
CS vs LG	0.35	0.21; 0.49
CS vs GG	0.18	0.07; 0.30
VI vs LG	0.06	−0.12; 0.24
VI vs GG	0.23	0.02; 0.44
LG vs GG	0.60	0.43; 0.76

Table 1
Prior distributions (Beta (a, b), Median (95 % probability interval (PI)), based on the 2.5th and 97.5th percentiles) used for BLCA estimation of the diagnostic sensitivity and specificity of clinical examination, virus isolation and two conventional PCRs for identifying BEFV-infection in South African cattle with clinical signs of BEF.

Assay	Parameter	Beta (a, b)	Median (95 % PI)	References
Clinical examination	Sensitivity	20, 1	0.67 (0.49, 0.82)	Clinical experience of the first author
	Specificity	21, 8.5	0.72 (0.54, 0.86)	
Virus isolation	Sensitivity	4.5, 11	0.28 (0.10, 0.53)	(Spradbrow and Francis, 1969; Kemp et al., 1973; St. George et al., 1977; St. George, 1985; Zheng et al., 2011; Zaghawa et al., 2016; Hirashima et al., 2017; Karayel-Hacioglu et al., 2021)
	Specificity	99, 1	0.99 (0.96, 0.99)	Authors' opinion
L-gene PCR	Sensitivity	22, 8	0.74 (0.56, 0.87)	(Blasdell et al., 2013; Dacheux et al., 2019)
	Specificity	11, 1.5	0.90 (0.66, 0.99)	(Blasdell et al., 2013)
G-gene PCR	Sensitivity	13.5, 17	0.44 (0.27, 0.62)	(Zheng et al., 2011; Chaisirirat et al., 2018; Hou et al., 2018; Amal et al., 2019; El-Habbaa and Radwan, 2019; Abo-Sakaya and Bazan, 2020)
	Specificity	9, 1	0.93 (0.66, 0.99)	Authors' opinion
Prevalence		1, 1	0.50 (0.03, 0.98)	Not applicable

Table 3
Cross tabulation of the test results of clinical examination classification vs three laboratory assays (virus isolation and two conventional PCRs) to identify the presence of BEFV in clinically diseased South African cattle.

	Laboratory assay outcome (Virus isolation/ L-gene PCR/ G-gene PCR)								
	+/+/+	+/+/-	+/-/+	+/-/-	-/+/+	-/+/-	-/-/+	-/-/-	Total
Clinical examination positive	9	0	1	8	16	13	2	20	69
Clinical examination negative	0	0	0	0	0	0	1	18	19
Total	9	0	1	8	16	13	3	38	88

For the majority of the estimated parameters, the posterior distributions deviated meaningfully from the priors (Fig. 1). The conditional dependence and independence models provided numerically similar estimates for most parameters, with the exception of Sp for GG and Se for LG (Table 5). The estimates for both these parameters were lower in the conditional dependent model than the independent model, 8 % lower in the case of Sp for GG and 5 % lower in the case of Se for LG. The 95 % PI of the conditional dependence variables for Se and Sp included zero, but displayed kernel density distributions towards the positive side.

Clinical examination classification by a veterinarian was the most sensitive test with a Se of 86 % (95 % Probability Interval (PI) 77 %; 93 %) and a reasonable specificity of 67 % (PI 52 %; 82 %) (Table 4). As expected, the laboratory assays had higher specificity than CS, with virus isolation being the best (99 %, PI 97 %; 100 %). Of the two PCR assays, the LG performed better than the GG in both sensitivity and specificity: the estimated Se for LG was 64 % (PI 51 %; 76) and the Sp 96 % (PI 84 %; 100 %) while the Se for GG was 50 % (PI 38 %; 61 %) and Sp was 89 % (PI 75 %; 98 %). The prevalence of BEFV was relatively high (67 %), which was anticipated for this specific population because it was not a random sample but rather a sample of sick cattle suspected of suffering from BEF. The high prevalence contributed to estimated PPV being higher than NPV for all evaluated assays.

3.2.1. *Sensitivity analysis*

The model with informative priors on the prevalence, uniform priors on all sensitivity estimates (beta (1,1)) and semi-informative priors on all specificity estimates (beta (2,1)) converged (Fig. 2). Most median estimates from this model differed less than 10 % from the estimates using informative priors, with the exceptions of Se of the CS and Sp of the GG.

3.3. *Effect of transport time*

Detection percentages were not formally compared but descriptively, positive results for both virus isolation and the PCR assays were less frequent with increased time from sampling to processing (Table 5). The percentage positive samples for virus isolation declined from 67 %, for samples processed within 24 hours, to 15 % for samples taking one day longer to reach the laboratory. The decrease for the PCR assays was less severe within the first 48 hours, but dropped markedly when samples spent more than 2 days in transit.

4. Discussion

Bovine ephemeral fever is endemic to Africa and its clinical signs are well-known to local farmers and veterinarians. However, verification of the disease (and specifically the presence of infectious virus) by laboratory assays are often required at the onset of a herd- or regional

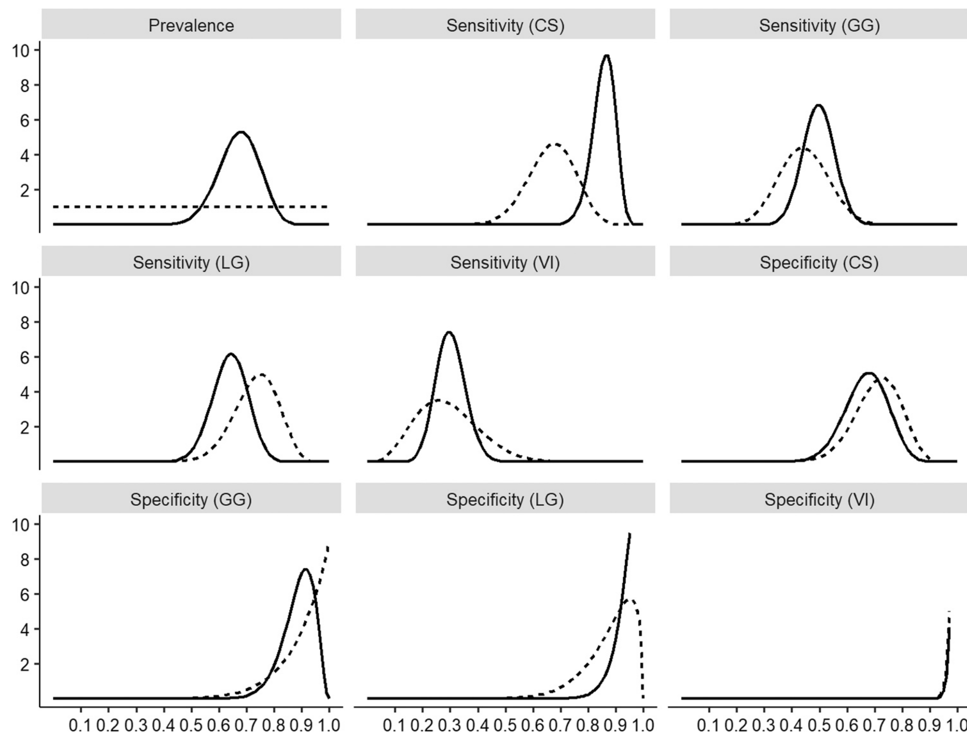

Fig. 1. Prior (dotted line) and posterior (solid line) probability distribution graphs for population prevalence, and diagnostic sensitivity and specificity of clinical examination (CS), G-gene conventional PCR (GG), L-gene conventional PCR (LG) and virus isolation (VI) to detect BEFV-infection in clinically diseases South African cattle as estimated by BLCA model for a single population and four assays and modelling conditional dependence between the two PCR assays.

Table 4
Estimated posterior probability distributions of diagnostic sensitivity and specificity of clinical examination, virus isolation and two conventional PCR's for detecting BEFV infection in clinically diseased South African cattle. Two BLCA models are represented, one considering conditional independence between all four assays and one considering conditional dependence between PCR assays.

		Independent assays	PCRs conditionally dependent
Assay	Parameter	Median (95 % PI)	Median (95 % PI)
Clinical examination	Sensitivity	0.87 (0.79; 0.93)	0.86 (0.77; 0.93)
	Specificity	0.63 (0.49; 0.77)	0.67 (0.52; 0.82)
	PPV	0.80 (0.66; 0.91)	0.84 (0.69; 0.94)
	NPV	0.73 (0.56; 0.87)	0.70 (0.46; 0.85)
Virus isolation	Sensitivity	0.31 (0.20; 0.43)	0.30 (0.20; 0.41)
	Specificity	0.99 (0.96; 1.00)	0.99 (0.97; 1.00)
	PPV	0.98 (0.91; 1.00)	0.99 (0.94; 1.00)
	NPV	0.45 (0.30; 0.61)	0.41 (0.24; 0.58)
L-gene PCR	Sensitivity	0.69 (0.57; 0.80)	0.64 (0.51; 0.76)
	Specificity	0.95 (0.87; 1.00)	0.96 (0.84; 1.00)
	PPV	0.96 (0.87; 1.00)	0.97 (0.86; 1.00)
	NPV	0.64 (0.44; 0.81)	0.56 (0.33; 0.75)
G-gene PCR	Sensitivity	0.49 (0.37; 0.60)	0.50 (0.38; 0.61)
	Specificity	0.96 (0.87; 1.00)	0.89 (0.75; 0.98)
	PPV	0.96 (0.85; 1.00)	0.91 (0.79; 0.98)
	NPV	0.52 (0.34; 0.68)	0.46 (0.26; 0.67)
Conditional dependence terms	Sensitivity	NA	0.03 (−0.01; 0.05)
	Specificity	NA	0.01 (−0.01; 0.07)
Prevalence		0.63 (0.50; 0.77)	0.67 (0.52; 0.81)

outbreak. Although several diagnostic assays have been reported in the last two decades (Hsieh et al., 2005; Stram et al., 2005; Lew et al., 2006; Zheng et al., 2011; Zaghloul et al., 2012; Erster et al., 2017; Hou et al., 2018; Gao et al., 2020), these have not been evaluated under local African conditions. In addition, many studies fail to report the diagnostic sensitivity and specificity of newly developed assays, or compare the performance to imperfect reference standards. This study aimed to estimate the sensitivity (Se) and specificity (Sp) of clinical examination by a veterinarians and three laboratory assays to identify BEFV-infection in South African cattle with BEF-like clinical signs without the assumption of a perfect reference assay.

In comparison to published results from similar study populations (i.e. cattle with clinical signs consistent with BEF), the positive percentage of the individual laboratory assays were towards the lower spectrum of the ranges reported in the literature (St. George et al., 1977; St. George, 1985; Hirashima et al., 2017; Chaisirirat et al., 2018; Amal et al., 2019; Abo-Sakaya and Bazan, 2020; Karayel-Hacioglu et al., 2021). This tendency towards a lower positive percentage might be due to the assays being implemented under African field conditions with natural disease exposure (Greiner and Gardner, 2000). There was poor pairwise agreement between the assays, except for the two PCRs. This could be a reflection of the differences in test principles: VI identifies live, infectious virus; PCR focuses on segments of viral RNA whereas the clinical examination is aimed at the clinical expression of BEFV infection in the bovine host.

According to our preliminary analysis of the impact of time from sample collection to processing, there appears to be a marked decrease in the ability of VI and PCR to detect BEFV when samples spend more time in transit. BEFV has a history of being difficult to isolate, attributable to its susceptibility to changes in pH, temperature and possibly a poor tropism for laboratory hosts (including cell cultures) (van der Westhuizen, 1967; Heuschele and Johnson, 1969; Tanaka et al., 1969; Heuschele, 1970; Theodoridis and Lecatsas, 1973). Our results suggest that attempts at isolation of infectious virus becomes almost futile unless the sample can reach the laboratory within 24 hours of collection. The effect on the PCR-assays was delayed by another 24 hours but the drop in the ability to detect viral RNA was worse than expected. Since PCR is aimed at detecting nucleic acid fragments without the need to preserve viral particle integrity, this large drop might indicate that the BEFV RNA genome is particularly fragile. While it is anticipated that heparin and EDTA provide limited to no ability to protect BEFV during sample transport, samples were requested in these tubes since these are the containers typically available to the South African veterinarian. Sample transfer to laboratories is a known impediment to laboratory diagnostics in Africa (Francis, 2022; Abdul-Karim et al., 2023) and our findings suggest that robust pen-side diagnostics would be more appropriate for this region.

The relatively high prevalence of BEFV in the selected population was expected given that the samples originated from bovines with BEF-like clinical signs rather than a random selection of bovines. No similar studies could be identified for comparison in the peer-reviewed, English literature although various studies evaluating the seroprevalence have been reported. Results from these studies vary greatly and positive percentages from 0 % to almost 100 % have been described in the Middle East and other areas of Africa (e.g. (Davies et al., 1975; Wang et al., 2001; Yeruham et al., 2010; Aziz-Boaron et al., 2012). A direct comparison between our results and these are not possible since positive serology indicates historic exposure to the virus, rather than the current study investigating the presence of the infectious pathogen.

While this is the first study to estimate the prevalence of BEFV within

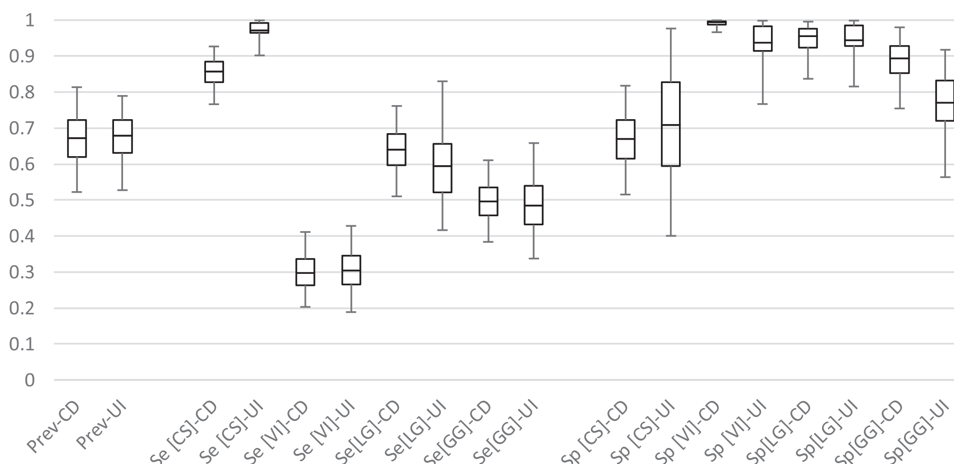

Fig. 2. Estimation of the sensitivity of the posterior probability distributions to the prior specifications of population prevalence (Prev) and diagnostic sensitivity (Se) and specificity (Sp) of four assays to detect BEFV-infection in clinically diseased South African cattle. CD – BLCA model incorporating conditional dependence of PCR assays. UI – the same model using uninformative priors on Se and weakly-informative priors on Sp. CS – Clinical examination classification. VI – Virus isolation. LG – L-gene PCR. GG – G-gene PCR.

Table 5
The impact of time from collection to laboratory processing on the number of positive samples when using virus isolation (VI) or PCR to detect BEFV-infection in clinical diseased South African cattle. Darker shading represents larger number of samples.

		Transport time			
Clinical examination classification	Assay status	≤24 hours	25-48 hours	49-72 hours	Unknown or >73 hours
Total number of samples that arrived within specific sample transport time		24	27	17	20
Virus isolation					
Clinical examination positive	VI positive	16	4	0	1
	VI negative	6	23	11	8
Clinical examination negative	VI positive	0	0	0	0
	VI negative	2	0	6	11
Any PCR					
Clinical examination positive	PCR positive	17	18	5	5
	PCR negative	5	9	6	4
Clinical examination negative	PCR positive	0	0	1	0
	PCR negative	2	0	5	11

diseased cattle, the results need to be interpreted with caution since it would not be applicable to the general bovine population (even in an endemic region). However, it is reported here because of its impact on the reported predictive values of the tests. Predictive values are not typically reported in diagnostic accuracy studies since they are dependent on both the diagnostic sensitivity and specificity of the assay and well as the disease prevalence within the sample population. In this study, the selected assays were applied within the specific clinical circumstances that the field veterinarian would require diagnostic testing. Predictive values are highly relevant to the clinical veterinarian since these are a better indication of the disease status of an individual patient but are of limited value when the same assays are used outside the specified test population.

The relatively good parameter estimates for clinical examination were unexpected given the ambiguous nature of BEF clinical signs. The diagnostic efficacy of clinical findings varies among diseases, but good Se and Sp has been reported for other infectious diseases including wildebeest associated malignant catarrhal fever in Kenya (Se of 99.1 % and Sp of 71.5 % (Orono et al., 2019) and bluetongue in the Netherlands (Elbers et al., 2008). Clinical diagnosis, including the interpretation of clinical signs within the context of the case history, environment, herd management (Constable et al., 2017), is currently the only cow-side test available for field veterinarians in South Africa. Experienced livestock veterinarians are familiar with the clinical signs of BEF and descriptions have been available for more than a century (Bevan, 1907; Theiler, 1908; van der Westhuizen, 1967), but none of the clinical signs are diagnostic in and of themselves. The familiarity with the disease was likely a major factor contributing to the relatively good Se estimate derived in this study. From an epidemiological perspective, these findings suggest that an experienced veterinarian could serve as a reasonable screening tool for detecting the disease within the general bovine population. Unfortunately, the widespread use of this "tool" is severely constraint by practical limitations: There is a deficiency of experienced veterinarians in Sub-Saharan Africa (Cheneau et al., 2004; Ilukor, 2017) and livestock veterinarians in areas where the disease is uncommon or recently introduced are less likely to have sufficient practical experience to achieve the diagnostic accuracy reported here. Despite being used in a population with a relative high disease prevalence, the diagnostic performance is unlikely to be good enough for the field veterinarian or farmer: roughly one in five animals diagnosed with BEFV infection would not be infected (PPV of 84 %) while three in every ten animals that are identified as not having the disease, would indeed be infected (NPV of 70 %). This would therefore not be a suitable assay to mitigate the economic impact of the disease during a potential outbreak scenario,

and emphasises why accurate laboratory assays are still required.

Virus isolation is currently the only accepted laboratory assay available for detecting BEFV-infection in South Africa. Very few studies investigating the diagnostic accuracy of virus isolation are presented in the literature (none for BEFV) probably because this presented the first laboratory means of detecting an infection with no comparator other than clinical signs of disease. A direct comparison of our estimates to published literature could therefore not be performed. However, the Se estimate (median 30 %) correlates well with the percentage of virus isolation positive cases detected from clinically sick cattle reported in some previous studies, e.g. 82 of 329 (25 %) samples (St. George et al., 1977) and 6 of 22 (27 %) samples (Karayel-Hacioglu et al., 2021). The excellent Sp estimate was expected given that virus isolation requires infectious viral particles and suggests that VI could be used as a confirmatory assay. However, the requirement for infectious particles is an important practical limitation because it requires viral titre and infectivity to be maximised (Leland and Ginocchio, 2007). BEFV is known to be difficult to isolate likely due to its instability outside the host and the relatively short duration of viraemia (typically 3 days), which does not necessarily overlap with the expression of clinical disease (van der Westhuizen, 1967; Heuschele and Johnson, 1969; Tanaka et al., 1969; Heuschele, 1970; Theodoridis and Lecatsas, 1973; Burgess and Spradbrow, 1977; Uren et al., 1989, 1992). Therefore, VI could be a useful assay for confirming the presence or absence of infectious BEFV in animals and animal products (such as semen) moving from endemic to epidemic regions. However, similar to CS, its value to the field veterinarian as a diagnostic tool is severely limited by the low Se.

The two conventional PCR assays were evaluated as potential candidates to replace VI. Similar to VI, a direct comparison of our results to published literature could not be performed because these estimates have not previously been reported. In general, the diagnostic accuracy of BEFV direct-detection assays is poorly reported in literature with reports instead focused on analytical Se and Sp. However, studies of the diagnostic accuracy of rabies virus and vesicular stomatitis virus assays (also members of the Rhabdoviridae) report excellent diagnostic Se and Sp for direct-detection assays including conventional PCRs (WOAH, 2023a, 2023b), suggesting that this should be possible for BEFV as well. Unfortunately, the diagnostic accuracy estimates indicate that neither of the PCRs evaluated here would be suitable as a diagnostic assay by themselves, due to the poor Se of these assays (64 % and 50 % for LG and GG respectively). Within this sample population with high disease prevalence, both assays had unacceptably low NPV (56 % for LG and 40 % for GG).

The reasons for the poor performance of the BEFV PCRs used in this study require further investigations, but we theorize that it might be caused by a combination of the (relatively) poor analytical sensitivity of conventional PCRs or low copy numbers of virus within clinical samples. Conventional PCR is generally considered to require a higher concentration of the target genome than real-time PCR, which has been reported in BEFV studies (Hou et al., 2018). For BEFV, with a relatively short-term viraemia, the host viral load can be low at the time of sample collection. These low viral quantities might be further degraded by the relative instability of the virus outside the host (Tanaka et al., 1969; Heuschele, 1970) and of its RNA genome (Lindahl, 1993; Relova et al., 2018). The drop in virus detection with longer transport times is in line with this theory. It is also possible that the breakdown of the viral genome can contribute to the difference in Se between the relatively small LG amplicon (± 140 bp) and the larger GG amplicon (420 bp) (Relova et al., 2018). These results support the argument for the development of point-of-care tests, preferably real-time, isothermal assays.

Similar to the VI estimates, the PCRs tested in this study performed better on Sp, which might make them useful as confirmatory assays. However, it was surprising that LG, designed to detect all ephemeroviruses, outperformed the BEFV-specific GG (Sp of 95 % vs 88 %). Further analysis of the one sample identified as positive on only GG (data not included), found that this was a specific false-positive result: *Anaplasma marginale* was identified upon sequencing of the amplicon. This can be considered a fatal flaw for further use of this assay. Bovine anaplasmosis is an economically important tick-borne disease with a worldwide distribution. It is considered endemic in many regions (including Africa) where most bovines have silent chronic infections (Kocan et al., 2010). Clinical disease is typically only seen after the bovine has gone through some form of stress, such as presence of other diseases, or immunosuppression (Klement and Smith, 2020). The possible misidentification of an *A. marginale* infection as BEFV could have serious consequences for the affected bovine and the control of both diseases within a region, especially since a case fatality of up to 30 % has been reported for untreated cases of Anaplasmosis (St. George, 2004). At present, it is unknown whether this positive animal was suffering from clinical anaplasmosis following a BEFV outbreak (a complication of BEFV that has been suggested by farmers and field veterinarians) or if the assay simply identified a chronic carrier. Given the quality of the PCR product, the former is considered the more likely scenario. From a clinical perspective, this suggests that further research into the longer term effects of BEFV-infection, especially for animals suffering from chronic debilities after the episode, is warranted. For the development of BEFV assays, this suggests that future studies should include a wider array of bovine pathogens in studies evaluating the analytical specificity of a new assay.

Two BLCA models were included in this study. The model including conditional dependence between the two PCR assays was selected since the conditional dependence terms were positive. Also, although the estimates from the two models were mostly quantitatively similar, there were noticeable differences (4–8 %) in the estimates of Se and NPV for LG and Sp, PPV and NPV for GG between the two models. The sensitivity analysis with an informative prior on prevalence, uninformative priors on all four sensitivities and weakly informative priors on all four specificities converged. Most of the estimates were found to be robust with the weakly informative priors. The exceptions were Se of CS, which increased more than 10 %, and the Sp of GG which decreased with more than 10 % when applying weakly informative priors. The discrepancies in the GG estimates align with the specific false positive identified, although the impact of this false positive seems disproportionate. Given the lack of data to guide prior specifications for this parameter, and the relatively small number of samples available for this study, further analyses are warranted.

Several study limitations have been identified. Firstly, there is the lack of pre-study sample size calculation. Since sample collection was completed before this analysis was considered, the sample size was fixed. However, calculations would be complicated by the lack of prevalence and diagnostic accuracy estimates for this specific population and assays (Greiner and Gardner, 2000). The resulting sample size was relatively small leading to imprecision in estimates as well as a high prevalence compared to what would be expected from a random selection of cattle. Also, presented results specifically relate to populations of BEF clinical suspects and Se estimates in particular might be lower if the assays were applied to cattle without clinical signs (i.e used for screening). As an extreme example, the sensitivity of clinical examination would be zero in a population of healthy cattle.

The interpretation of predictive values reported in this study require consideration. As mentioned, the predictive values are dependent on the disease prevalence within the tested population. The impact of changes in disease prevalence was evaluated (Figure S1). For example, if these assays were implemented in the 1999 focal BEF outbreak in Taiwan where a 7.4 % morbidity percentage was reported (Hsieh et al., 2005b), the NPV would increase for all assays (98 %, 95 %, 97 % and 96 % for CS, VI, LG and GG respectively), while the PPV would decrease (17 %, 71 %, 56 % and 27 % for CS, VI, LG and GG respectively). Conversely, if these assays were implemented in a high prevalence outbreak, such as the 2013 outbreak in Turkey, with a morbidity percentage of 83 % (Mirzaie et al., 2017), the PPV for all assays would be more than 90 %

(93 %, 99 %, 99 % and 96 % for CS, VI, LG and GG respectively) while the NPV would range from 22 % (VI) to 50 % (CS). It is therefore critical that the composition of the test population is scrutinised before applying the reported predictive values.

Further analyses of the effect of transport time on assay outcome are also required. No statistical analyses were performed because of the limited amount of data. Although the study did not provide incentives to veterinarians or subsidise transport costs, RuVASA veterinarians were aware of the related project and specifically the importance of virus recovery. It is therefore possible that the collection and transport conditions were better than would be the typical situation for diagnostic field samples resulting in better performance of the laboratory assays than what would be anticipated for standard field application. On the other hand, because this was a field study and therefore made use of the sample collection tubes that would be available to the field veterinarian, it is possible that the diagnostic assays could perform better if more specific sample preservation (for example RNA stabilisation) was performed. Nonetheless, we believe that the calculated estimates can provide useful information for further studies.

Another drawback of the study is the definition of the latent condition. For this study, the latent condition was defined as the presence of BEFV in a patient with BEF-like disease signs. From the perspective of the end-user (farmer/veterinarian) this definition does not rule out BEF as the prevailing disease in an outbreak. However, the estimates from this study provide valuable insights for the interpretation of current laboratory results and give some basic guidelines for maximising the probability of successfully identifying the presence of the virus, especially for virus isolation.

This study identified a need for the development of new diagnostic assays for the detection of BEFV suitable for implementation under African conditions (Vudriko et al., 2021) and for African genotypes (Dacheux et al., 2019; Omar et al., 2020; Mlingo et al., 2021). These assays must have excellent analytical sensitivity to deal with the expected low levels of virus encountered in natural infections and should preferably be available as a point-of-care assay to minimise transport-associated false negatives. Within an endemic disease area, the consequences of false negatives can be serious, since BEFV is known to readily spread over vast distances (Aziz-Boaron et al., 2012), and therefore the outcome of a screening test in a single animal can have a profound impact on disease control for animals within an entire region. Various new assays for the detection of BEFV have been developed in the last decade, including an isothermal lateral-flow dipstick assay (Hou et al., 2018). Unfortunately, *in silico* comparison of the proposed primers and probes to the African genotypes identified several nucleotide mismatches, but further investigation particularly under field conditions is warranted.

To our knowledge, this was the first analysis that estimated the diagnostic accuracy of available BEFV assays without the assumption of a perfect reference assay. Our findings suggest that neither the presence of clinical signs, virus isolation or PCR are perfect reference standards and we recommend that this should be considered for future assay development.

5. Conclusion

Clinical examination by a skilled veterinarian is currently the most sensitive screening assay available for detecting BEFV infection in clinically diseased South African cattle. Although the laboratory assays, including virus isolation showed excellent specificity, the poor sensitivity indicate that negative results should be interpreted with caution. Given the multitude of practical limitations still present in Africa, there is a palpable need for the development of more accurate and robust diagnostic assays.

Ethics approval

Sample collection and analyses were approved by the Research Ethics Committee of the University of Pretoria, Faculty of Veterinary Science (Reference number: REC046–19) and the Department of Agriculture, Land Reform and Rural development (SDAH-Epi-19071106491).

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Authorship

All those designated as authors should meet all four criteria for authorship, and all who meet the four criteria should be identified as authors.

CRediT authorship contribution statement

Grobler Miemie: Writing – review & editing, Writing – original draft, Visualization, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Crafford Jan Ernst:** Writing – review & editing, Supervision. **Fosgate Geoffrey:** Writing – review & editing, Supervision, Software, Methodology, Formal analysis, Conceptualization. **Swanepoel Robert:** Supervision, Methodology.

Declaration of Competing Interest

We wish to confirm that there are no known conflicts of interest associated with this publication.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.prevetmed.2025.106475.

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