Indirect enzyme-linked immunosorbent assay for the detection of antibody against Rift Valley fever virus in domestic and wild ruminant sera

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ABSTRACT

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An indirect enzyme-linked immunosorbent assay (I-ELISA) for the detection of specific IgG immunoglobulins against Rift Valley fever virus (RVFV) was validated in-house. A total of 3055 sera from sheep ($n = 1\,159$), goats ($n = 636$), cattle ($n = 203$), African buffalo ($n = 928$), and other wild ruminants ($n = 129$), including eland, kudu, and black wildebeest, was used. Sera from domestic ruminants were collected in West ($n = 10$), South ($n = 1\,654$) and East Africa ($n = 334$), and sera from wild ruminants ($n = 1\,064$) were collected in South Africa. In addition, 136 sera from eight exper-imentally RVFV-infected sheep, taken during a period of 28 days post infection (dpi), were used to study the kinetics of RVFV antibody production. Field sera were tested by the serum neutralization (VN) test and experimental sera by VN and haemagglutination-inhibition (HI) test. Based on VN test results, negative sera were regarded as reference controls from RVFV-free, and positive sera were regarded as reference controls from RVFV-infected subpopulations of animals. ELISA data were expressed as the percentage positivity (PP) of an internal high positive control. The two-graph receiver operating characteristics approach was used for the selection and optimization of I-ELISA cut-offs including the misclassification costs term and Youden index (J). In addition, cut-off values were determined as the mean plus two-fold standard deviation of the result observed with the RVFV-free subpopulations. Established optimal cut-offs were different for each of the data sets analyzed, and ranged from 1.65 PP (buffalo) to 9.1 PP (goats). At the cut-off giving the highest estimate of combined measure of diagnostic accuracy (highest J value), the I-ELISA test parameters were deter-mined as follows:

(1) Diagnostic sensitivity (%): cattle—84.31, buffalo—94.44, sheep—98.91, goats—99.18

(2) Diagnostic specificity (%): cattle—99.34, buffalo—98.28, sheep—99.16, goats—99.23 and other game ruminants—99.26

In the group of RVFV-experimentally infected sheep, seroconversion in all individuals was detected by VN on 4–6 dpi, by HI on 5–7 dpi, and by I-ELISA on 6–7 dpi. All tests showed the same kinetic pattern of immunological response. Antibody levels were low for a very short period before increas-ing to high titres, after which it was easily detectable by all tests. Compared to traditional tests, the

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lower sensitivity of I-ELISA in the detection of the earliest stage of immunological response may be practically insignificant, particularly when this assay is used in population-based, disease-surveillance programmes. The high sensitivity and specificity of I-ELISA established in this study, especially for the statistically more representative subpopulations of animals tested, seem to support this prediction.

Test parameters determined in this study should, however, be regarded as in-house diagnostic decision limits, for which further updating is recommended, particularly for specimens from other countries, and preferably by applying a standardized method for sampling of new subpopulations of animals to be targeted by the assay.

INTRODUCTION

Rift Valley fever (RVF) is a mosquito-borne viral disease of ruminants and humans in Africa, mainly East and West Africa, southern Africa and Madagascar (Swanepoel & Coetzer 1994). The recent occurrence of the first confirmed outbreaks of RVF in humans and livestock outside the traditional endemic areas, namely in the Kingdom of Saudi Arabia and Yemen (CDC 2000), is of global medical and veterinary concern.

The increasing demand for high-quality veterinary certification worldwide aims to ensure the best protection of human and animal populations and to facilitate the free circulation of animals and animal products in international trade. Within national and international veterinary certification processes, diagnostic laboratories are suppliers of analytical test results that must be scientifically valid, quality controlled and based on internationally recognized methods and standards (Caporale, Nannini & Ricci 1998; Nannini, Giovannini, Fiore, Marabelli & Caporale 1999; Wiegers 2000). A validated serological assay consistently provides test results that identify animals as positive or negative for an antibody, and by inference, accurately predicts the infection status of animals with a predetermined degree of statistical certainty (Jacobson 2000). Numerous important reasons for the test validation are well known, including the need for reliable estimates of the diagnostic sensitivity and specificity that are of concern with respect to clinical diagnosis, risk assessment and risk-factors studies. The process of assay validation is, however, complex, time-consuming, expensive and vulnerable to many limitations, including availability of recommended standards and representatives of reference sera (Jacobson 1998a, b; Greiner & Gardner 2000a, b). These constraints are well evidenced by the fact that, for example, most enzyme-linked immunosorbent assays (ELISA) used in serological diagnosis of OIE List A and B diseases in wildlife, have not yet been validated (OIE 2000b).

The classical reference method for the detection of antibodies to RVF virus (RVFV) is based on various formats of the virus neutralization (VN) test. Although highly sensitive and specific (Swanepoel, Struthers, Erasmus, Shepherd, McGillivray, Erasmus & Barnard 1986a), they are expensive and time-consuming. A great disadvantage of these techniques is also the health risk to laboratory personnel (Smithburn, Mahaffy, Haddow, Kitchen & Smith 1949) and restrictions for their use outside RVF endemic areas (Barnard & Gerdes 2000).

A safe, cost-effective and alternative test for the serological diagnosis of RVF, based on an indirect enzyme-linked immunosorbent assay (I-ELISA) that employs an inactivated, cell-culture-produced antigen and protein G-peroxidase conjugate, has been developed (Paweska, Barnard & Williams 1995). This ELISA format is currently listed in the OIE Manual (Barnard & Gerdes 2000) as a diagnostic test which is suitable for the serological diagnosis of RVF within a local setting, and can also be used in the import/export of animals after bilateral agreement (OIE 2000a). A high diagnostic accuracy and particularly diagnostic sensitivity (97.3%) reported for the I-ELISA (Paweska et al. 1995), was, however, based only on results from 38 post-vaccination sera taken from seven sheep. In addition, methods used for the expression of the I-ELISA absorbance readings and the selection of the cut-off is now obsolete (Wright, Nilsson, Van Rooij, Lelenta & Jeggo 1993; Wright 1998; Jacobson 2000).

In response to an increasing local and international demand for the serological diagnosis of RVF, and to address the current international requirements for the validation of an ELISA, analytical data were generated to assess the diagnostic accuracy of the modified format of the previously developed I-ELISA (Paweska et al. 1995) for the detection of IgG antibody against RVFV in domestic and wild ruminants.

MATERIAL AND METHODS

Sera

Control sera

Control sera for the I-ELISA were obtained from Onderstepoort Biological Products, Onderstepoort, South Africa.

The source of positive control sera were sheep inoculated subcutaneously with a variant biologically cloned (clone 13) from the 74HB59 strain of RVFV (Muller, Saluzzo, Lopez, Dreier, Turell, Smith & Bouloy 1995) and subsequently challenged intravenously with 1 mℓ of tissue culture supernatant containing 10^6 MLD$_{50}$/mℓ of a RVFV strain recovered from an African buffalo (*Syncerus caffer*) in the Kruger National Park, South Africa (RVF isolate Buffalo/99). Sheep No. 3762 was selected as a donor for the high positive control (C++), and Sheep No. 3004 was used as a donor for the low positive control (C+) serum. Serum representing the negative control (C–) was obtained from Sheep No. 4085, which had no previous exposure to the virus and had tested serologically negative by VN and haemagglutination-inhibition (HI) tests. Aliquots of 1 mℓ of each control serum were freeze-dried in 5 mℓ glass vials, accordingly labelled (RVF C++ 3762, RVF C+ 3004, RVF C– 4085), and stored at 4 °C until use.

Field sera

A total of 3 062 field sera from sheep (*n* = 1 159), goats (*n* = 636), cattle (*n* = 203), African buffalo (*n* = 928), eland (*Taurotragus oryx*) (*n* = 14), kudu (*Tragelaphus strepsiceros*) (*n* = 50), and black wildebeest (*Connochaetes taurinus*) (*n* = 65) was used. Sera from domestic ruminants were collected in Kenya (four cattle, 211 goats and 64 sheep), Senegal (ten sheep), Somalia (three cattle, 17 goats and eight sheep), South Africa (196 cattle, 391 goats and 1 067 sheep), and Tanzania (17 goats and ten sheep). Sera from wild ruminants (*n* = 1 064) were collected in South Africa.

The vaccination or infection status of sampled animals was unknown. However, East African sera were specifically taken to monitor the 1997–1998 outbreak of RVF and to determine its extent in this region (Woods, Karpati, Grein, McCarthy, Gaturuku, Muchiri, Dunster, Henderson, Khan, Swanepoel, Bonmarin, Martin, Mann, Smoak, Ryan, Ksiazek, Arthur, Ndikuyeze, Agata, Peters & WHO Hemorrhagic Fever Task Force 2002).

Experimental sera

Eight sheep were inoculated subcutaneously with 1 mℓ inoculum comprising the supernatant of a cell culture fluid containing $10^{6.5}$ TCID$_{50}$/mℓ of the AR 20368 strain of RVF virus isolated in 1981 from *Culex zombaensis* in South Africa. Blood samples from inoculated animals were taken daily for 2 weeks and then every 7 days for 2 more weeks (*n* = 136).

Field sera were tested by the VN test and experimental sera both by the VN and HI tests. The VN-negative sera were regarded in this study as a reference control panel from RVF-non-infected, and the VN-positive sera as a reference control panel from RVF-infected sub-populations of animals. ELISA data generated from testing the field VN-defined sera were used for the selection of cut-off values and determination of diagnostic accuracy of the I-ELISA. Data obtained from experimental sera were used to study the kinetics of RVFV antibody production by the ELISA, VN and HI tests. The following were analyzed in the reference panels shown:

Panel I   South African sheep VN-negative sera (*n* = 1 067)

Panel II   West (*n* = 10) and East (*n* = 82) African sheep VN-positive sera

Panel III   South African goat VN-negative sera (*n* = 391)

Panel IV   West African goat VN-positive sera (*n* = 245)

Panel V   South African cattle VN-negative sera (*n* = 152)

Panel VI   South (*n* = 44) and West (*n* = 7) African cattle VN-positive sera

Panel VII   South African buffalo VN-negative sera (*n* = 874)

Panel VIII   South African buffalo VN-positive sera (*n* = 54)

Panel IX   South African other wild ruminants VN-negative sera (*n* = 129)

Panel X   South African experimental sheep sera (*n* = 136)

Serological tests

Serum neutralization test

The SN test was conducted according to a previously described method (Swanepoel *et al.* 1986a),

except for using the AR 20368 isolate of RVFV. The titre was expressed as the reciprocal of the serum dilution that inhibited $\geq 75\%$ of viral cytopathic effect (CPE). A serum sample was considered seropositive when it had a SN titre of $\geq \log_{10}$ 0.6, equivalent to a serum dilution $\geq 1:4$.

Haemagglutination-inhibition test

The HI test was conducted according to the method by Clarke & Casals (1958) with modification described by Swanepoel et al. (1986a). A serum sample was considered seropositive when it had a HI titre of $\geq \log_{10} 1.3$, equivalent to a serum dilution $\geq 1:20$.

Indirect ELISA

PRODUCTION OF ANTIGEN

Production of ELISA RVFV antigen and control antigen was carried out according to Paweska et al. (1995) with some modifications. Monolayers of Madin-Darby-bovine-kidney (MDBK) cells prepared in 20 x 220 cm^2 Roux flasks ($\sim$ 7.5 x 10^6 cells/flask) were inoculated with 100 mℓ of Eagles modified essential medium (EMEM) containing 10$^{0.3}$ TCID$_{50}$/mℓ of the AR 20368 strain of RVFV and 2.5 % foetal calf serum. Inoculated flasks were incubated at 37 °C until full CPE was observed (usually after 4 days). Thereafter, the infectious cell suspension was harvested and stored at 4 °C for 2 days, followed by centrifugation at 140 000 g for 1 h through a 15 % sucrose gradient. The pellet was processed according to the sucrose-acetone extraction method of Clarke & Casals (1958). It was suspended in 8.5 % chilled sucrose, sonicated twice on ice for 30 s at 12 µM, and the resulting homogenate dehydrated by means of chilled acetone treatment using 20 x the volume (400 mℓ) of the original homogenate. After incubation for 30 min at 4 °C, the mixture was centrifuged at 1 100 g for 5 min, the supernatant decanted and the same amount of fresh acetone mixed with the pellet. The mixture was again incubated at 4 °C for 1 h, and then centrifuged at 1 100 g for 5 min. After centrifugation, the supernatant was decanted and the pellet ground to a fine powder. A volume of 100 mℓ of fresh acetone was briefly mixed with the powder, and the suspension centrifuged at 1 100 g for 5 min. The supernatant was decanted and the sediment stored overnight at 4 °C. The following day the sediment was rehydrated in 20 mℓ of 0.1M Tris pH 7.5. The same method was used to produce control antigen, from uninfected MDBK cells. Both antigens were irradiated at 25–30 kilo-

gray. The protein concentration of the positive and control antigen was 59.2 µg/mℓ and 150.2 µg/mℓ, respectively. Both antigens were diluted 1:10 in 50% glycerol/0,1M Tris buffer pH 7.5, aliquoted into 250 µℓ volumes and stored at –20 °C. These aliquots were subsequently tested for safety and shelf storage. The safety of the positive (RVF Ag Batch 3/2000) and the control (RVF Control Ag Batch 3/2000) antigen was tested in 2–3-day-old baby mice and Vero cells using standard inoculation procedures (Barnard & Gerdes 2000). Optimization of reagents for the I-ELISA was established by standard checkerboard titration (Crowther 1995).

TEST PROCEDURE

The procedure, with modifications, was based on the I-ELISA format developed by Paweska et al. (1995). The top half (rows A-D 1-12) of flat-bottom, 96-well immunoplates (NUNC C96 Polysorb, Cat # 4-46140) were coated with the positive, and the bottom half (rows E-G 1-12) with the control antigen. A volume of 50 µℓ/well of original RVFV antigen and control antigen, each diluted 1/1 000 in a carbonate-bicarbonate buffer pH 9.6, was used. After incubation at 4 °C overnight, unbound antigen was removed by washing the plate three times with 250 µℓ/well of TST buffer (Tris saline, Tween pH 8 ± 0.2). Thereafter, 100 µℓ of blocking buffer (used also as a diluting buffer) consisting of 3 % fat-free milk powder ("Elite", Clover SA, Pty Ltd) in TST was added to each well, and the plate incubated for 1 h at 37 °C. After washing the plate three times as before, 50 µℓ of control and test sera, diluted 1/100 in diluting buffer, were added in duplicates to wells pre-coated with positive and control antigen. Plates with diluted sera were then processed as follows:

1. After incubation at 37 °C for 60 min unbound antibody was removed by washing the plates three times with 250 µℓ/well of TST buffer.

2. A volume of 50 µℓ recombinant protein G conjugated with horse radish peroxidase (Cat No. 10-1223, Zymed) diluted 1/10 000 was added to each well and the plates incubated at 37 °C for 1 h.

3. Unbound conjugate was removed by washing the plates three times with 250 µℓ/well of TST buffer.

4. A volume of 50 µℓ substrate/chromogen (TMB, Cat No. 00-2023, Zymed) was added to each plate and the plates incubated in the dark for 20 min at room temperature (22–25 °C).

5. Reactions were stopped by adding 50 µℓ/well of 1M H_2SO_4 and colour development was immediately assessed in a spectrophotometer (EL 340, Bio-Tek Instruments) using 450 nm and 690 nm reference filters.

6. Optical density (OD) readings were converted to PP values (percentage of strong positive control serum) using the following equation:

$$\% \, PP = \frac{\text{Mean OD of test sample, positive antigen} - \text{Mean OD of test sample, negative antigen}}{\text{Mean OD of C++, positive antigen} - \text{Mean OD of C++, negative antigen}} \times 100$$

where % PP = Percentage positivity of C++.

Repeatability and internal quality control (IQC)

Each internal serum control was tested on five plates each with 12 repeats on five separate occasions (5 x 12 x 5 = 300 determinants). Means and standard deviations (SD) of I-ELISA OD and PP values were calculated from replicates of all samples in each plate and each run of the assay to assess intra- and inter-plate variation. Additionally, coefficients of variation (CV = standard deviation of replicates / mean of replicates x 100) were calculated for positive samples. Data obtained from this analysis were used to estimate the assay repeatability and to establish the upper (UCL = mean of 300 replicates plus 2 SD), and lower (LCL = mean of 300 replicates minus 2 SD) control limits for internal controls. UCL and LCL together with CV values (< 15 %) were applied as IQC rules for further analysis.

Frequency distributions of I-ELISA PP values in VN-negative animals

Statistical analysis of the distribution of I-ELISA PP values in RVFV-free subpopulations was done using the non-parametric Kruskal-Wallis one-way analysis of variance by ranks and Dunn's pairwise comparison method for comparing mean ranks (Siegel 1956) at the 5% level.

Selection of the cut-off

Selection and optimization of I-ELISA cut-off PP values was done using a Microsoft Excel template of the two-graph receiver operating characteristics (TG-ROC) including the misclassification costs term (MCT) and Youden index (J) as functions of the pre-selected diagnostic decision limits (Greiner 1995; Greiner, Sohr & Göbel 1995; Greiner 1996; Greiner & Gardner 2000b). In addition, the cut-off

values were determined as the mean plus two-fold standard deviation (SD) of the results observed with the RVF-free subpopulations (Jacobson 1998b).

Diagnostic accuracy

Diagnostic sensitivity (D-Sn), diagnostic specificity (D-Sp), and J were calculated according to methods described by Greiner & Gardner (2000b).

RESULTS

Test optimization

Optimization of test conditions and reagents is shown in Fig. 1.

IQC and repeatability

Data used to establish UCL and LCL for OD readings of C++ are shown in Fig. 2A. Within runs, the average CV for 25 plates was 6.03 % ± 2.8 SD for the high positive control (Fig. 2B) and 6.59 % ± 2.5 SD for the low positive control serum, respectively. Between runs, the average CV for the five runs was 9.2 % ± 2.2 SD for the high positive control and 8.2 % ± 0.74 SD for the low positive control serum respectively (Fig. 2C). Data used to establish UCL and LCL for PP values of all internal controls are shown in Fig. 3.

Safety and antigen stability

Inoculated mice were clinically normal for a period of 14 days and no CPE was observed in Vero cell cultures for a period of 10 days after inoculation with RVF Ag Batch 3/2000 and RVF Control Ag Batch 3/2000. Shelf-life test at −20 °C (Fig. 4A) and at 37 °C (Fig. 4B) storage showed that both antigens remained very stable over a period of time tested.

Frequency distributions of I-ELISA PP values in VN-negative animals

Kruskal-Wallis one-way analysis of variance by ranks showed that there were highly significant differences ($P < 0.001$) between the PP values of the five subpopulations of animal species tested. Dunn's pairwise comparison method for comparing mean ranks showed that panel V significantly differed from all other panels, panel III was significantly different from panels VII and IX. Panel I was related both to panel III and panels VII and IX (Table 1).
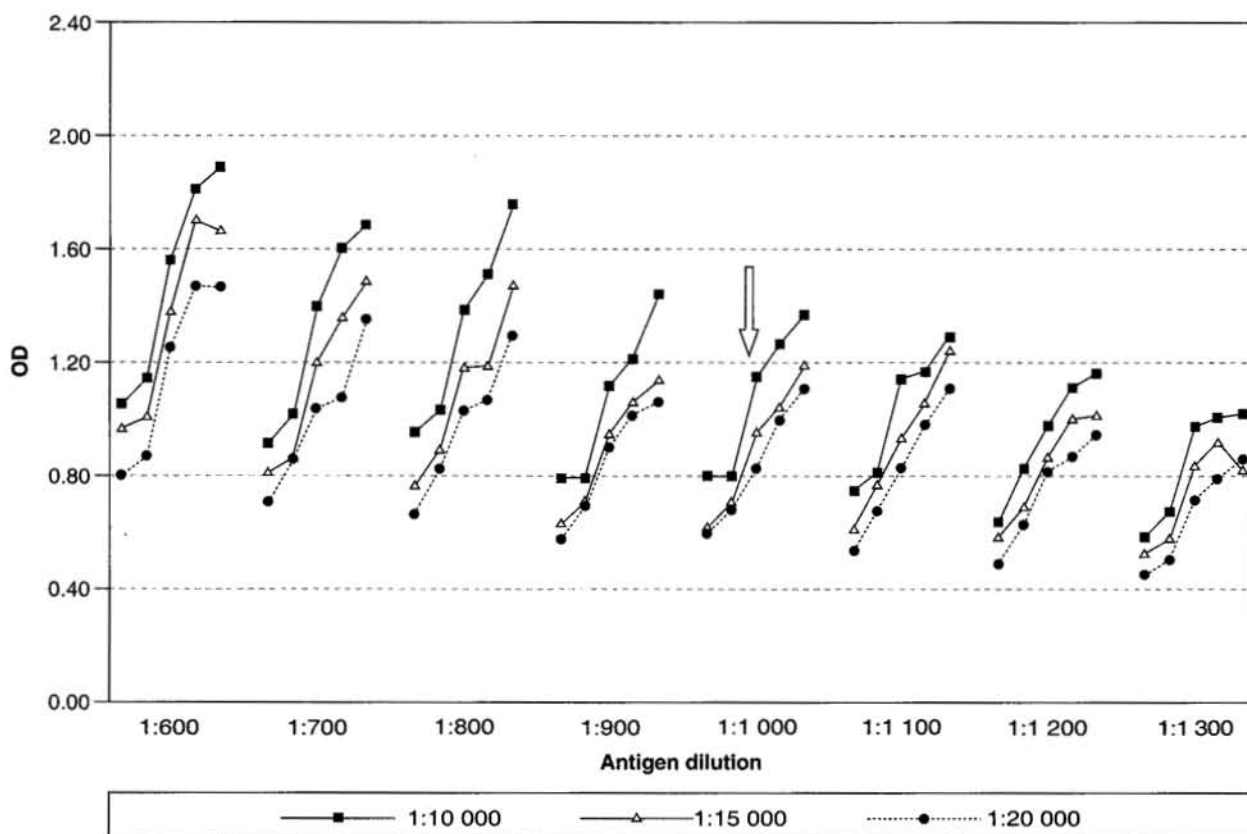
FIG. 1   Optimization of the I-ELISA reagents by checkerboard titration. Arrow indicates the conditions selected for the test

TABLE 1   Dunn's pairwise method for comparing mean ranks of the I-ELISA PP values in domestic and wild ruminants that tested negative for the presence of antibody against RVFV in the VN test

Panel	Number tested	Median	Mean Rank[a]	Result[b]
V (cattle)	152	−1.925	1 012.7	a
III (goats)	391	−1.430	1 216.7	b
I (sheep)	1 067	−1.190	1 313.6	bc
VII (buffalo)	874	−0.900	1 371.1	c
IX (other)	129	−0.754	1 559.6	c

[a]   At 5 % level
[b]   Mean ranks followed by the same letter do not differ significantly from each other

Cut-off and validity

The modality of I-ELISA PP values in sera from RVFV-free and RVFV-infected subpopulations shows an overlap between upper limit of negativity and lower limit of positivity: mostly, for panels I and II within a range of 3.50–8.15 PP (Fig. 5A and Fig. 5B); for panels III and IV within 5.90–11.10 (Fig. 5C and 5D); for panels V and VI within 0.00–3.31 PP (Fig. 5E and Fig. 5F); and for panels VII and VIII within 1.08–3.55 PP (Fig. 5G and Fig. 5H). Of the total of sera tested, the percentage of samples

falling within the area of overlap was: 2.60—panels I and II; 1.26—III and IV; 10.83—V and VI; and 3.77—VII and VIII. The observed overlap was primarily due to the deviation to the right in the distribution of PP values in panels I and III and the skewness to the left in the distribution of PP values in panels VI and VIII.

Selection and optimization of cut-off values by the TG-ROC (Fig. 6A–D) was based on the non-parametric programme option (Greiner *et al.* 1995) due to departure from a normal distribution of data sets
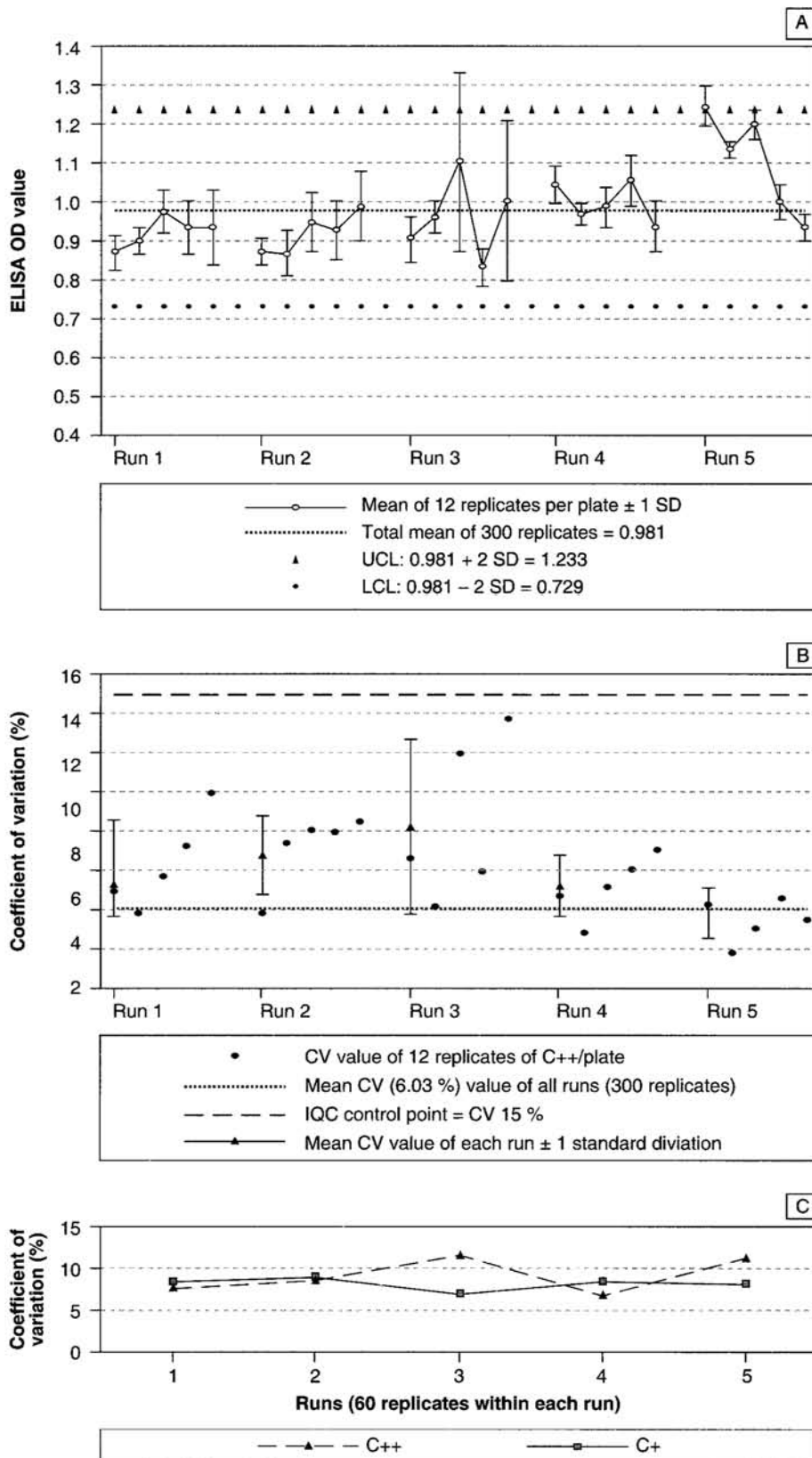
FIG. 2 Determination of (A) upper (UCL) and lower (LCL) control limits for optical density (OD) readings of high positive (C++) control serum, (B) coefficient of variation (CV) of C++ within runs and (C) CV of C++ and low positive (C+) control serum between runs of the assay
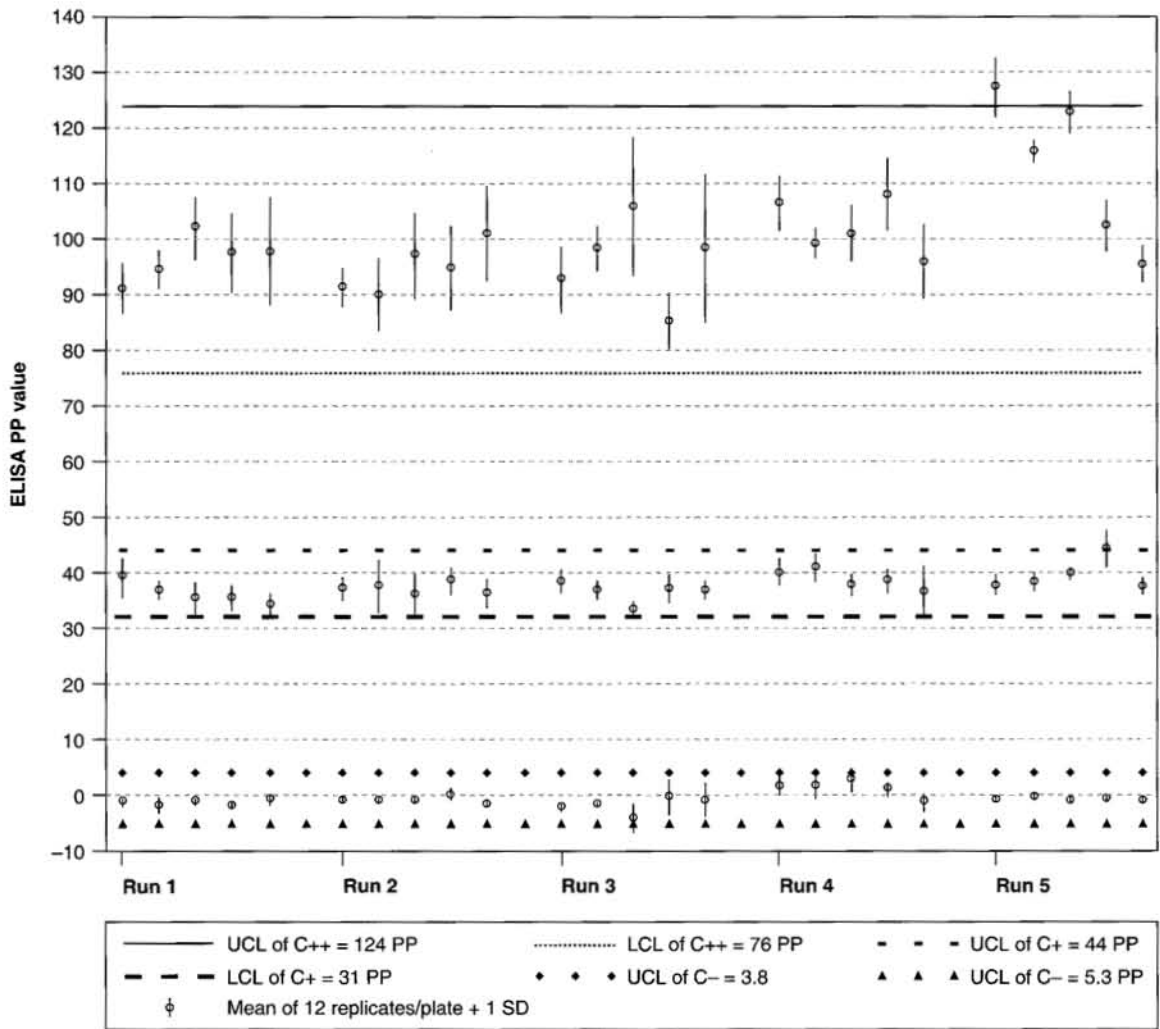
FIG. 3   Upper (UCL) and lower (LCL) control limits for percentage positivity (PP) readings of high positive (C++), low positive (C+) and negative (C–) control serum
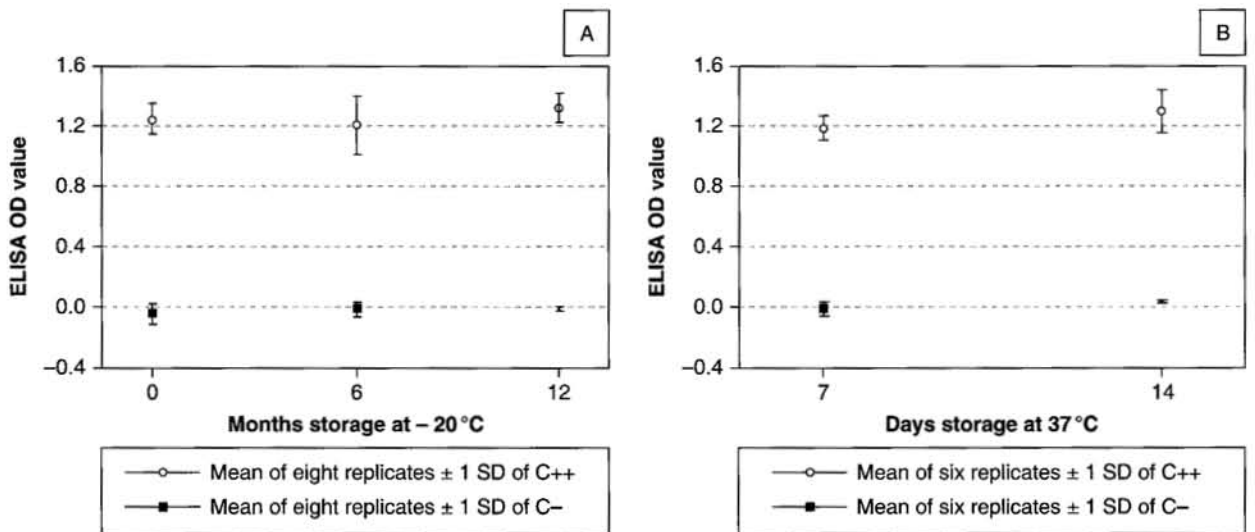

FIG. 4   Shelf-life of Rift Valley fever virus antigens for I-ELISA (A) at –20 °C for a period of 12 months and (B) at 37 °C for 14 days. The stability of antigen was tested using high positive control serum (C++) and negative control serum (C–)
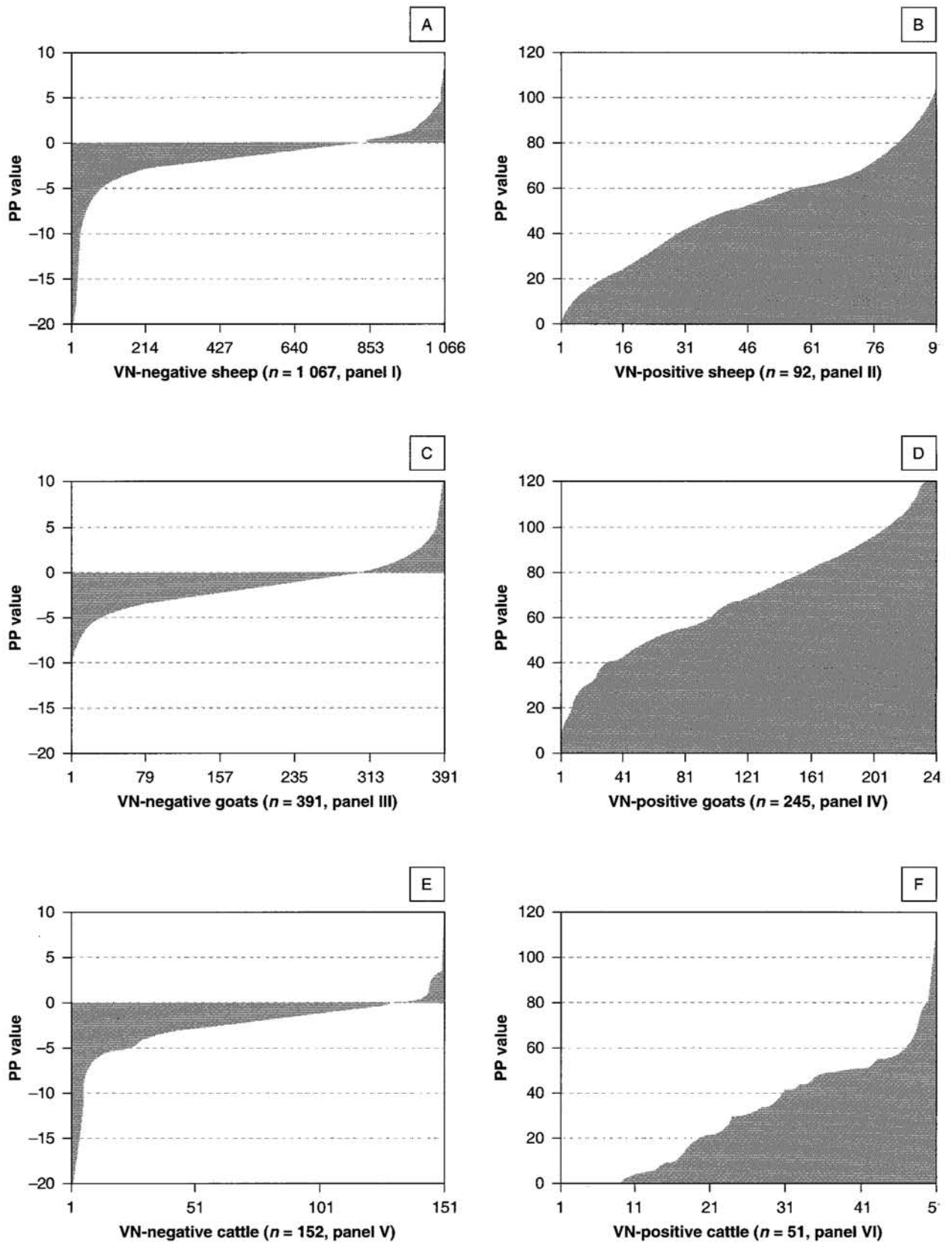
FIG. 5A–F Distribution of I-ELISA PP values in sera of domestic ruminants whose infectious status against RVFV was defined by virus neutralization test (VNT)
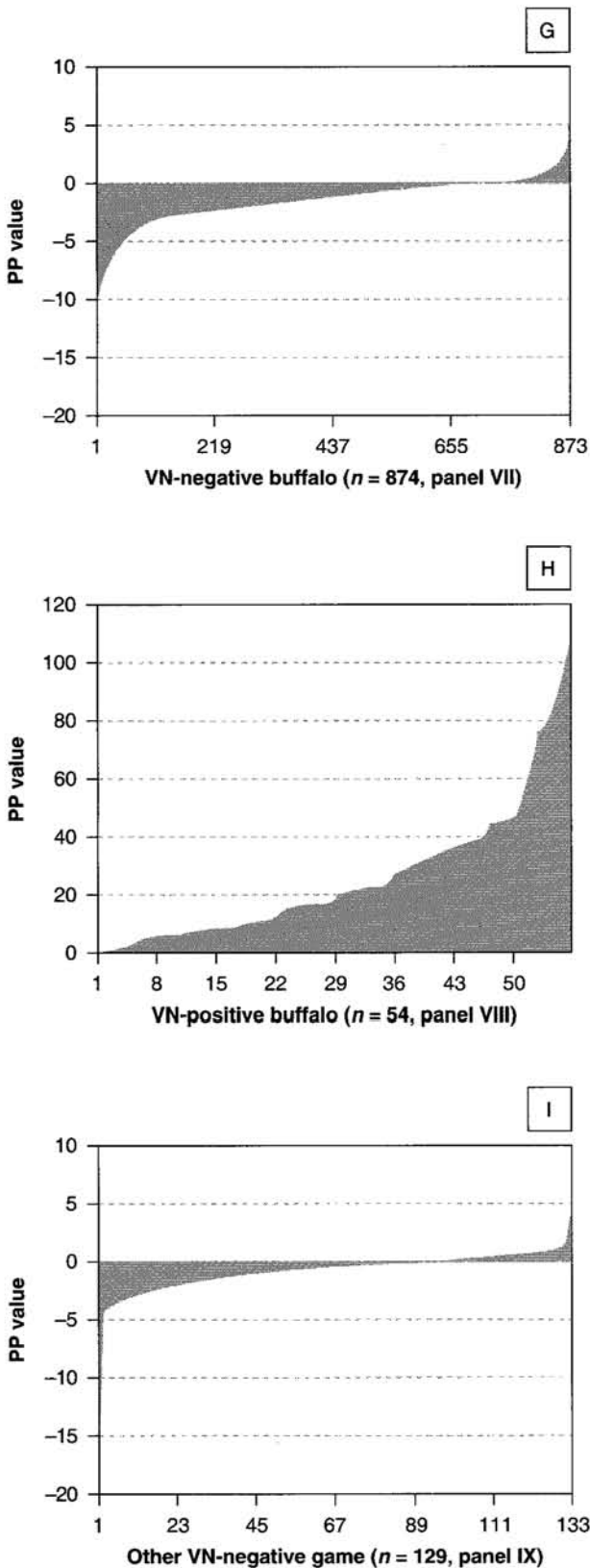
VN-negative buffalo (*n* = 874, panel VII)

VN-positive buffalo (*n* = 54, panel VIII)

Other VN-negative game (*n* = 129, panel IX)

FIG. 5G–I Distribution of I-ELISA PP values in sera of wild ruminants whose infectious status against RVFV was defined by virus neutralization test (VNT)

analyzed. Examples of the graphical presentation of the TG-ROC, including its MCT and J options, are shown in Fig. 6A–C (for panels I and II) and Fig. 7A–C (for panels VII and VIII). A summary of cut-off values derived from the different statistical approaches used is given in Table 2. The optimal cut-off PP value was different for each of the subpopulations tested. The highest cut-off PP value based on TG-ROC analysis was derived from the results in panels III and VI (9.10 PP), and the lowest (0.28) from the results in panels V and VI (Table 2). Cut-off values calculated as mean plus 2 SD varied from 2.98 PP (panel IX) to 5.48 (panel I) (Table 2).

Estimates of I-ELISA diagnostic sensitivity and diagnostic specificity using cut-offs derived from TG-ROC analysis and calculated as mean plus two-fold SD of the PP values in RVF-free subpopulations are given in Table 2. At the optimum cut-off (giving the highest estimate of combined measure of diagnostic accuracy), the J value in the subpopulation of animals analyzed was: panels I and II—0.981; III and IV—0.984; V and VI—0.837; and VII and VIII—0.927.

Kinetics of RVFV antibody production in experimentally infected sheep

The dynamics of immunological response as measured by the HI, VN and I-ELISA in the group of eight RVF-experimentally infected sheep, is shown in Fig. 8 and Table 3. Seroconversion in all individuals was detected by VN on 4–6 dpi and by HI on 5–7 dpi. At the optimum I-ELISA cut-off of 5.37 PP derived from TG-ROC analysis (see Table 2), one sheep tested positive on 7 dpi, five on 8 dpi, and all on 9 dpi (Table 3). When applying this cut-off to the total of 136 experimental sera, 40 were both negative by VN and ELISA, and of 96 VN-positive sera, 70 (72.91 %) also tested positive by the I-ELISA. Amongst sera tested by the HI, 49 were both negative by the HI and I-ELISA, and of 87 HI-positive sera, 65 (74.71 %) were also I-ELISA positive. The mean plus 1 SD of the I-ELISA PP value in experimental sheep ranged from −0.81 ± 1.06 to −0.20 ± 1.85 on 0–6 dpi with marked increase in mean PP reading on 7 dpi (2.42 ± 3.75). The experimental data demonstrate that, to maximize the correlation of VN and HI-positive results in individual animals with those of the I-ELISA lower than 5.37 PP cut-off value would have to be used. The reaction profiles of the test values for individual experimental sheep clearly show that, in fact, seroconversion was detectable in one animal on 6 dpi,
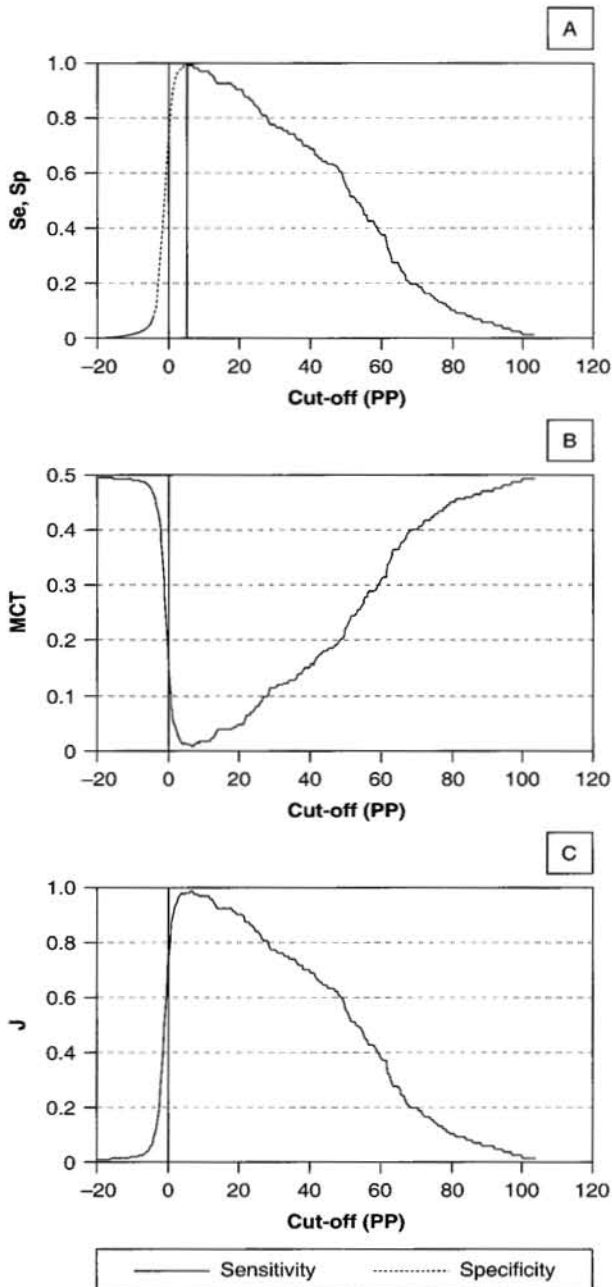
FIG. 6   Selection and optimization of cut-off for the I-ELISA by (A) TG-ROC analysis, (B) the misclassification cost term (MCT), and (C) Youden index (J) based on results from sheep that tested negative (panel III) and positive (panel IV) in the VN test

     A The insertion point of the sensitivity (Se) and specificity (Sp) graphs represents a cut-off PP value (5.37) at which equivalent test parameters (Se = Sp) are achieved at 95 % accuracy level

     B Within a range of cut-off values of 6.36–6.86 PP, MCT becomes minimal (0.008) under assumption of 50 % prevalence and equal costs of false-positive and false-negative results

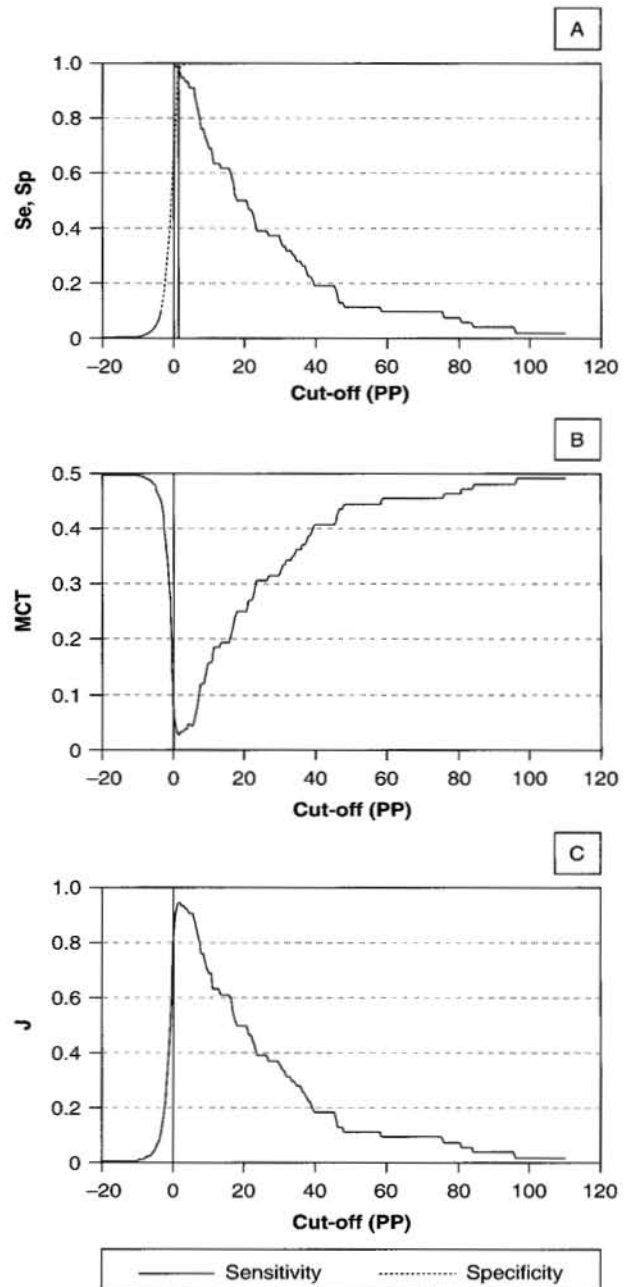   C Within a range of cut-off values of 6.36–6.86, J becomes the highest (0.984)

FIG. 7   Selection and optimization of cut-off for the I-ELISA by (A) TG-ROC analysis, (B) the misclassification cost term (MCT), and (C) Youden index (J) based on results from buffalo that tested negative (panel VII) and positive (Panel VIII) in the VN test

     A The insertion point of the sensitivity (Se) and specificity (Sp) graphs represents a cut-off PP value (1.65) at which equivalent test parameters (Se = Sp) are achieved at 95 % accuracy level

     B At cut-off value of 1.65 PP, MCT becomes minimal (0.028) under assumption of 50 % prevalence and equal costs of false-positive and false-negative results

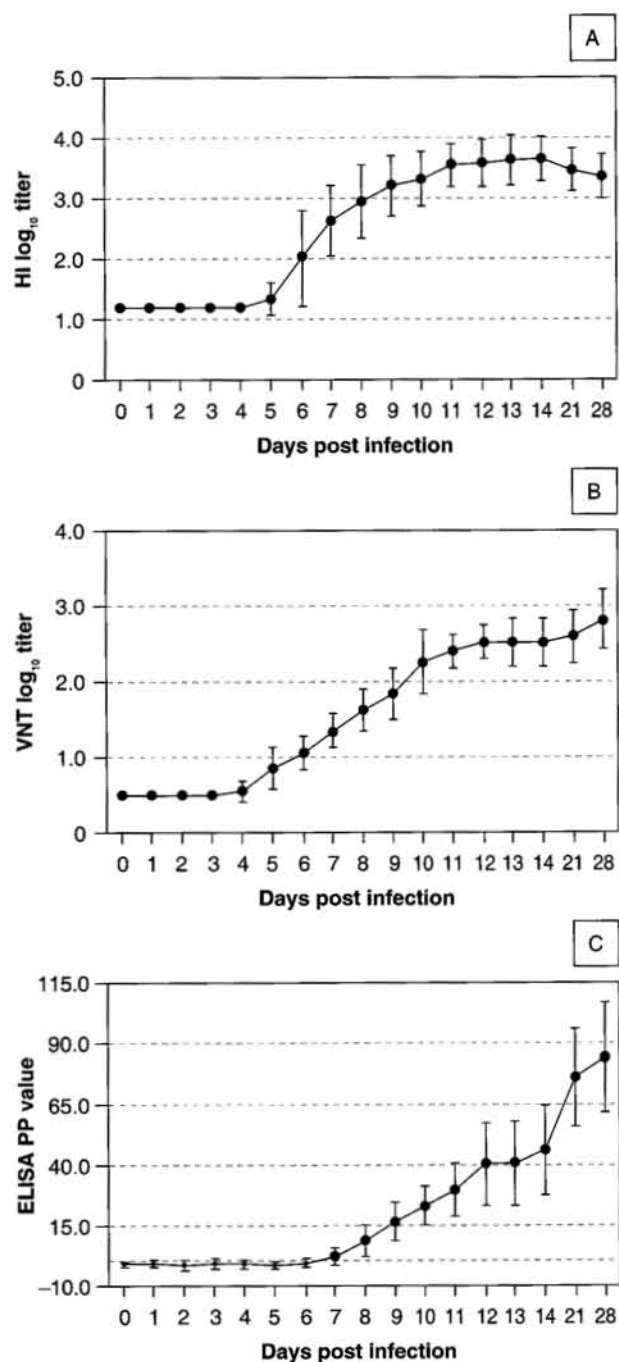   C At cut-off value of 1.65 PP, J becomes the highest (0.943)

FIG. 8   The immune response of eight sheep inoculated sub-
cutaneously with AR 20368 isolate of Rift Valley fever
virus as measured by (A) HI, (B) VN, and (C) I-ELISA

• Mean response of eight sheep

⊥ ± 1 SD

and in all animals on 7–8 dpi at the lowest I-ELISA
PP values ranging from 1.22–3.87 PP.

All tests showed the same kinetic pattern of the
immunological response to the experimental infec-
tion with RVFV. This response was very rapid and
after a very short period of low antibody levels
detected first by the VN test, high-titer antibody pro-
duction developed and it was easily detectable by
both the HI and I-ELISA.

DISCUSSION

Central to any serological assay is the determina-
tion of the diagnostic threshold or cut-off. The cut-
off represents the test result value selected for dis-
tinguishing between negative and positive results.
By inference, serological results are used to deter-
mine the infection or vaccination status of animals
against a particular agent of disease. Appropriate-
ness of data underlying the selection of the cut-off
consequently impacts on diagnostic sensitivity and
specificity and other measures of test performance
(Jacobson 1998b).

The first consideration in determining the cut-off in
an assay is to select sera from animals that are
unequivocally infected and sera from animals that
have never experienced an infection with the agent
in question. Criteria for selection of truly infected
and uninfected individuals are well defined (Jacob-
son 2000). Secondly, in order to account for the
distribution of covariate factors (such as genetic,
nutritional, geographical and stage of infection) that
may impact on the diagnostic sensitivity and speci-
ficity, the target population should preferably be
sampled, using simple random, systematic or strat-
ified sampling methods (Greiner & Gardner 2000a).
These ideal conditions could not be applied during
this study. However, the use of the current serolog-
ical gold standard to classify the infection status of
animals and the numbers of individuals tested with-
in each subpopulation, provides the means for
establishing at least the initial estimates of test
parameters (Jacobson 2000).

The influence of the referral patterns on the char-
acteristics of diagnostic tests suggests that results
from submission-based collections cannot easily
be extrapolated to other situations. This, however,
seems not to apply to laboratories that are involved
in large-scale testing in the context of prescribed
test procedures (Greiner & Gardner 2000b). From
the point of using the VN test in classifying animals
as infected (exposed) or non-infected (non-exposed)
with RVFV, it is worth noting that infection with this
virus induces life long immunity (Barnard 1979).
There is also no evidence of serological subgroups
or major antigenic variation between RVF virus iso-

TABLE 2 Diagnostic accuracy of the I-ELISA for the detection of antibody against RVFV in domestic and wild ruminant species

Animal species targeted: Cut-off	D-Sn[a] (%) (No. TP[1], no. FN[2])	D-Sp[b] (%) (No. TN[3], no. FP[4])	J[c]
Sheep: panels I and II			
5.37[d]	98.91 (TP = 91, FN =1)	99.16 (TN = 1 058, FP = 9)	0.981
5.48[e]	98.91 (TP = 91, FN = 1)	99.16 (TN = 1 058, FP = 9)	0.981
Goat: panels III and IV			
9.09	99.18 (TP = 243, FN = 2)	99.23 (TN = 388, FP = 3)	0.984
4.19	100.0 (TP = 245, FN = 0)	96.93 (TN = 379, FP = 12)	0.969
Cattle: panels V and VI			
0.28	88.23 (TP = 45, FN = 6)	94.73 (TN = 144, FP = 8)	0.829
4.69	84.31 (TP = 43, FN = 8)	99.34 (TN = 151, FP = 1)	0.837
Buffalo: panels VII and VIII			
1.65	94.44 (TP = 51, FN = 3)	98.28 (TN = 859, FP = 15)	0.983
3.43	92.59 (TP = 50, FN = 4)	99.31 (TN = 868, FP = 6)	0.993
Other wild ruminants: panel IX			
–	–	–	–
2.98		99.22 (TN = 128, FP = 1)	–

Formulas used for calculation of diagnostic accuracy of the I-ELISA (Greiner & Gardner 2000b):

[a] D-Sn (Diagnostic sensitivity) = $TP^1/(TP + FN^2)$
[b] D-Sp (Diagnostic specificity) = $TN^3/(TN + FP^4)$
[c] J (Youden index)   = D-Sn + (D-Sp1)
[d] Cut-off derived from TG-ROC analysis
[e] Cut-off calculated as mean plus two SD of the results from VN-negative serum panels

Where TP[1] = True positive (VN-positive)
     FN[2]   = False negative (VN-positive)
     TN[3]   = True negative (VN-negative)
     FP[4]   = False positive (VN-negative)

lates of disparate chronologic or geographic origins (Swanepoel & Coetzer 1994). Although the possibility of cross-reacting antibody in the I-ELISA was not addressed here, previous antigenic cross-reactivity studies in sheep (Swanepoel *et al.* 1986b) and field studies in cattle (Davies 1975; Swanepoel 1976, 1981) failed to provide any evidence that other African phleboviruses could influence the diagnosis of RVF.

An indirect ELISA is a test format that can be difficult to validate because of signal amplification of both specific and non-specific components (Crow-

ther 1995). Due to inherent differences amongst assay systems, binding-antibody levels should be expressed in relative rather than absolute terms. One of the distinct advantages of using PP values as a measure of antibody activity in the indirect ELISA is that this method does not assume uniform background activity, and therefore it is also preferred for inter-laboratory standardization (Wright *et al.* 1993).

Various statistical analyses were used to obtain the most accurate selection of the cut-off values. Our results demonstrate that, depending on the sub-

TABLE 3 Detection of antidoby against Rift Valley fever virus in sheep sera by the HI, VN and I-ELISA after experimental challenge with the AR 20368 isolate of the virus

Dpi[a]	Test	Sheep no.								Mean of ELISA PP[b] value ± 1 SD[c]
		6 154	6 962	6 963	69 67	6 145	6 537	6 572	6 650	
0	HI	< 1.3[d]	< 1.3	< 1.3	< 1.3	< 1.3	< 1.3	< 1.3	< 1.3	
	VN	< 0.6[e]	< 0.6	< 0.6	< 0.6	< 0.6	< 0.6	< 0.6	< 0.6	
	I-ELISA	−0.42	−0.99	−0.75	−0.62	−0.55	−1.66	−2.61	−1.08	−0.81 ± 1.06
1	HI	< 1.3	< 1.3	< 1.3	< 1.3	< 1.3	< 1.3	< 1.3	< 1.3	
	VNT	< 0.6	< 0.6	< 0.6	< 0.6	< 0.6	< 0.6	< 0.6	< 0.6	
	I-ELISA	0.69	0.57	−0.71	−1.52	0.22	−3.37	−1.90	−0.34	−0.80 ± 1.40
2	HI	< 1.3	< 1.3	< 1.3	< 1.3	< 1.3	< 1.3	< 1.3	< 1.3	
	VN	< 0.6	< 0.6	< 0.6	< 0.6	< 0.6	< 0.6	< 0.6	< 0.6	
	I-ELISA	−0.31	0.97	−1.11	−1.81	0.16	−2.01	−5.84	0.97	−1.12 ± 2.22
3	HI	< 1.3	< 1.3	< 1.3	< 1.3	< 1.3	< 1.3	< 1.3	< 1.3	
	VNT	< 0.6	< 0.6	< 0.6	< 0.6	< 0.6	< 0.6	< 0.6	< 0.6	
	I-ELISA	−0.65	0.49	0.58	−1.89	1.04	−3.24	−3.94	1.51	−0.76 ± 2.05
4	HI	< 1.3	< 1.3	< 1.3	< 1.3	< 1.3	< 1.3	< 1.3	< 1.3	
	VN	< 0.6	< 0.6	< 0.6	< 0.6	< 0.6	0.9	< 0.6	< 0.6	
	I-ELISA	−0.77	−0.88	−0.40	−0.49	−0.71	−1.09	−3.86	2.45	−0.72 ± 1.7
5	HI	< 1.3	1.9	< 1.3	1.6	< 1.3	< 1.3	< 1.3	< 1.3	
	VN	0.6	0.6	0.9	0.9	< 0.6	1.2	1.2	0.9	
	I-ELISA	−0.38	−0.75	−0.98	−1.65	−1.42	−2.36	−3.71	0.11	−1.39 ± 1.21
6	HI	1.9	2.8	< 1.3	2.8	< 1.3	1.9	3.1	< 1.3	
	VN	0.9	0.9	1.2	0.9	0.9	1.2	1.5	0.9	
	I-ELISA	0.19	−1.16	−0.58	−0.58	0.27	−1.36	−2.28	3.87	−0.20 ± 1.85
7	HI	2.2	3.1	2.1	2.8	1.9	2.5	3.7	2.8	
	VN	1.2	1.2	1.5	1.2	1.2	1.5	1.8	1.2	
	I-ELISA	1.26	0.31	1.46	5.27	0.25	1.49	−1.19	10.49	2.42 ± 3.75
8	HI	2.5	3.4	2.8	2.8	2.2	2.5	4.0	3.4	
	VN	1.5	1.5	1.8	1.5	1.2	1.5	2.1	1.8	
	I-ELISA	4.71	6.84	4.70	16.75	1.22	7.75	6.98	20.51	8.68 ± 6.54
9	HI	3.4	3.4	3.1	2.8	2.5	2.8	4.0	3.7	
	VN	1.8	1.8	1.8	1.8	1.2	1.8	2.4	2.1	
	I-ELISA	20.43	14.61	9.75	31.98	6.57	13.13	14.75	21.65	16.61 ± 7.96

[a] Days post infection
[b] Percentage positivity of high positive control serum
[c] Standard deviation
[d] Sera with HI-antibody titre $< \log_{10}1.3$ = negative, $\geq \log_{10}1.3$ = positive
[e] Sera with VN-antibody titre $< \log_{10}0.6$ = negative, $\geq \log_{10}0.6$ = positive

populations tested, different cut-off values should be applied to maximize test sensitivity and specificity, and to minimize false results. The statistically significant difference found in the distribution of I-ELISA PP values in the VN-defined negative subpopulations also indicates that different cut-off values should be used when distinct animal species are tested.

RVF infection status of animals in this study was classified according to the VN test reactions. Calculations of diagnostic sensitivity and specificity are most reliable when a gold standard of comparison is available. When a relative standard of comparison is used, estimates of diagnostic sensitivity and specificity for the new assay may be compromised because the error in the estimates of diagnostic

accuracy for relative standards is carried over into those estimates for the new assay. Therefore, using other serological tests to define sera can affect the optimization of the cut-off values of the assay being validated. On the other hand, because a true golden standard is practically unachievable, relative standards often remain the only possible option for test validation (Jacobson 2000). VN techniques are regarded as extremely sensitive and, under African settings, cross-reactivity issues in serological assays have been addressed. However, the possibility exists that new unrecognized phleboviruses may hamper the serological diagnosis of RVF, especially in countries outside Africa (Tesh, Peters & Meegan 1982). For this reason, laboratories that are not involved in routine or reference serological diagnosis of RVF should consider the use of the I-ELISA with caution.

All serological tests used in this study showed a very similar kinetic pattern of immunological response to experimental infection with RVFV. This response was very rapid and, after a very short period with low antibody titres first detectable by the VN test, its high level production followed, and was easily detectable by the HI and I-ELISA. While the results of experimental infection provide important information on the kinetics of RVFV antibody production, it is not known whether the reaction profile seen can be expected under field conditions. Compared to the VN and HI tests, the slightly lower sensitivity of I-ELISA in detection of the earliest stages of experimentally induced immunological responses is practically insignificant when it is used in population-based, disease-surveillance programmes.

We recommend, however, that test parameters established in this study be regarded as in-house diagnostic decision limits, for which further updating is recommended—particularly for international use, and preferably by applying a standardized method for sampling of new subpopulations of animals to be targeted by the assay.

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