

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

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Abstract

A polyclonal antibody-based, group-specific, competitive ELISA (C-ELISA) for the detection of antibodies to equine encephalosis virus (EEV) was developed. The assay measures the competition between a specific guinea pig antiserum and a test serum, for a pre-titrated EEV antigen. The C-ELISA detected antibodies to the seven known EEV serotypes. Reference antisera raised against other arboviruses did not cross react with EEV antigen. Negative sera from horses in the United Kingdom were used to establish the baseline for a negative population. Negative and positive populations of South African horses, selected on the basis of virus neutralisation were assayed subsequently. Optimal test parameters, where sensitivity \cong specificity \cong 100%, were calculated by two-graph receiver operator characteristic (TG-ROC) analysis to be at a cut-off value of 29.5% inhibition. Results show the EEV C-ELISA described to be sensitive, specific and reliable. Used in conjunction with ELISAs available for African horse sickness virus (AHSV), differential serological diagnosis between EEV and AHSV can be achieved.

Keywords

equine encephalosis virus, EEV, competitive ELISA, serogroup-specific antibody

1. Introduction

Equine encephalosis (EE) is an acute arthropod-borne viral infection that infects all species of equids. The causal agent, Equine encephalosis virus (EEV) is classified as a distinct

species, one of 21 different virus species currently recognised within the genus Orbivirus, family Reoviridae (Mertens et al., 2005). Seven antigenically distinct serotypes of EEV have so far been identified (Howell et al., 2004). EEV is transmitted by the bites of certain *Culicoides* species. In horses EEV can cause mild or subclinical disease. Howell et al., (2004) have described clinical signs that ranged from a mild febrile disease to abortion and death with few preceding signs. Other clinical signs that have been observed less frequently included varying degrees of swelling of the eyelids, the supraorbital fossa and even the entire face. Signs of central nervous system involvement such as mild to severe ataxia, reluctance to walk and stiffness, a wild expression in the eyes, and changes in temperament and/or convulsions have also been attributed to EEV infections. Respiratory distress sometimes accompanied by a frothy, clear or slightly blood tinged nasal discharge, petechiae in the conjunctiva and signs of acute heart failure have also been reported. Many of these signs can be confused with the clinical signs caused by the related African horse sickness virus (AHSV) which is considered the most lethal and economically important viral disease affecting horses.

Several serological surveys for EEV have been carried out in recent years, although only limited numbers of sera from a small number of countries have been tested. The results to date suggest that EEV is endemic in equids in most parts of South Africa, Botswana, Namibia, Zimbabwe and Kenya (Barnard, 1997; Paweska et al., 1999). Recently EEV has been isolated from an outbreak in Israel (Personal communication, 2010); EEV was diagnosed using a novel DNA array with subsequent RT-PCR and sequence analysis (Mildenberg et al, 2009). The detection of EEV out of Africa highlights the risk of its spread to other areas where competent vectors and susceptible animals are present.

Traditionally, complement fixation and agar gel immunodiffusion tests have been used for the detection of group-specific antibody to EEV. Interpretation of the complement fixation test can be subjective and the anti-complementary effects that occur with zebra and donkey sera can seriously affect the reliability and efficiency of the test (Katz, 2008; Sánchez-Vizcaíno, 2008). The agar gel immunodiffusion test uses a concentrated cell derived antigen, which can react with antibodies against cellular proteins present in some animals vaccinated previously with vaccines containing antigens produced in cell culture e.g. equine influenza and AHS vaccines. There are also concerns about the lack of sensitivity of the agar gel immunodiffusion test, which is dependent on the concentration of antibody present in the test serum. The virus neutralisation test has also been used for the detection of antibody to EEV, however because it is serotype specific and requires tissue culture cells and live virus for each serotype, it is laborious and time-consuming to perform.

A group-specific indirect enzyme linked immunosorbent assay (ELISA) for the detection of EEV antibody has been described previously (Williams et al., 1993). This assay utilizes an anti-horse conjugate. As the binding capacity of this conjugate to donkey, mule and zebra antibody is not known; the assay can only be used with confidence for horse sera. This paper describes the development and validation of a competition ELISA (C-ELISA) for the detection of EEV antibody in equids that, when used in conjunction with AHSV ELISAs, will serologically differentiate between these two infections.

2. Materials and methods

2.1. Test sera

Sera tested included: reference antisera against EEV (serotypes 1-7) obtained from the Department of Veterinary Tropical Diseases University of Pretoria, South Africa; AHSV (serotypes 1-9), bluetongue virus (BTV) (serotypes 1-24), epizootic haemorrhagic disease of deer virus (EHDV) (serotypes 1-8 and isolate 318), Corriparta, Tilligery, Eubenangee, Pata and Palyam viruses obtained from the Institute for Animal Health, Woking, UK; 13 horse sera that were recorded positive in the virus neutralisation test to only one of the individual EEV serotypes; 517 horse sera collected at the 1999 National Yearling Sale in South Africa and 297 horse sera collected in the UK from horses that were presumed never to have been exposed to EEV.

2.2. Preparation of antigen

A concentrated cell-derived antigen of EEV-1 was prepared as described by Hamblin et al., (1990). The antigen was dispensed into 1 ml aliquots and stored at minus 70 °C.

Infectious sub viral particles of EEV-1 were purified as described (Crafford et al., 2003) and used as antigen.

2.3. Production of guinea pig antisera

Five guinea pigs were each inoculated subcutaneously, into four sites, with 0.25 ml/site of purified EEV-1 antigen at a concentration of 15 µg/ml in Freund's complete adjuvant

under the auspices of UK Home Office Licence 70/5010. Guinea pigs were exsanguinated after 28 days and the serum collected.

2.4. C-ELISA

The concentrations of cell-derived antigen required to coat the ELISA plates and of guinea pig antisera to just saturate the antigen adsorbed on the plate were determined by checkerboard titration and were selected to give an absorbance value of approximately 1.0 ± 0.5 .

The procedure used was based on the C-ELISA described previously for AHSV (Hamblin et al., 1990; Hamblin et al., 1992). Briefly 50 μ l/well of EEV antigen, optimally diluted in 0.05 M carbonate-bicarbonate buffer, pH 9.6 (Sigma, Gillingham, UK) was passively adsorbed onto ELISA plates (Dynex Technologies, East Grinstead, UK) overnight at 4 °C. Plates were washed by flooding with PBS three times and test sera diluted 1:5 in blocking buffer (PBS (pH = 7.2) supplemented with 0.05% Tween 20 (VWR, East Grinstead, UK), 5.0% skimmed milk powder (Marvel, Glenville, USA) and 1% bovine serum albumin (Sigma, Gillingham, UK) were added to duplicate wells of columns 1-10. A positive control serum was titrated across eight wells of column 11 from 1:5 to 1:640. Wells 12 A and B each received 100 μ l of blocking buffer and were used as the blank control wells. A negative control serum diluted 1:5 in blocking buffer was added to wells 12 C and D. Wells 12 E to H received 50 μ l/well of blocking buffer and were used as the guinea pig controls to provide the 100 % absorbance value.

Guinea-pig antiserum raised against EEV-1 optimally diluted was then added to all wells except 12 A and B (total volume in each well = 100 μ l). After incubation (37 °C for 1 hour)

and washing, rabbit anti-guinea pig immunoglobulin (DAKO, Ely, UK) conjugated to horseradish peroxidase optimally diluted 1/1000 in blocking buffer was added to all the wells. Plates were incubated and washed as before. Ortho-phenylenediamine (Sigma, Gillingham, UK) at a concentration of 0.04 mg/ml and containing 0.05% H₂O₂ (30% v/v) was then added to each well. The reaction was stopped after 10 minutes by addition of 1.0 M H₂SO₄ to all the wells. The plates were read spectrophotometrically at a wavelength of 492 nm.

The mean values for each sample were calculated using the formula:

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

A cut-off of 50 PI was used to distinguish between positive and negative sera during the development of the test parameters.

2.5. Diagnostic sensitivity and diagnostic specificity

Horse sera (n = 297) from the UK, were used to determine the negative baseline for a population outside an EEV endemic area. Horse sera (n = 517) from South Africa were tested to determine the distribution of PI values for an endemic population. A representative sample of 207 sera from the South African collection were also assayed for antibody against each of the known EEV serotypes by virus neutralisation test. These data were then subjected to a two-graph receiver operating characteristic (TG-ROC) analysis (Greiner et al., 1995). In contrast to conventional ROC analysis, valid pairs of sensitivity and specificity can be read for pre-assigned threshold values directly from the TG-ROC plots.

2.6. Analytical specificity and sensitivity

The analytical specificity of the assay was determined by comparing the PI values obtained for a 1:5 dilution of reference antisera against each of the seven EEV serotypes and 47 related Orbiviruses. Sera from 13 horses, shown previously by virus neutralisation test to be positive to only one of the EEV serotypes, were each titrated in duplicate in a two-fold dilution series from 1:5 to 1:640.

2.7. Repeatability

Strong positive, weak positive and negative sera were tested on 16 separate days to determine the day-to-day variation. Plate to plate variation was determined by comparing the absorbance values recorded for the same samples located in the same positions on 13 different plates and assayed on a single day.

3. Results

3.1. Diagnostic sensitivity and diagnostic specificity

C-ELISA results obtained for negative horse sera (n=297) collected in the UK gave a mean PI value of minus 26.6% with a range between minus 100.0% and plus 31.8% (Figure 1). The results of testing 517 sera collected from horses at the South African yearling sales are presented as a frequency distribution plot in Figure 2. The plot shows a clear discrimination between positive and negative sera. All 148 sera from the South African yearling sales that recorded a PI > 50% in the C-ELISA were confirmed positive to EEV-6 by virus neutralisation test. Five sera with a PI of between 30% and 50%, five sera with a

PI of between 20% and 30% and a representative sample comprising 49 of the remaining 359 sera with a PI < 20% were also tested by virus neutralisation test. All sera with a PI between 30% and 50% tested positive for EEV-6 by virus neutralisation test, whilst all sera with a PI between 20% and 30% tested negative on virus neutralisation test. Of the remainder 49 sera (with a PI <20%), one tested positive for EEV-6 on virus neutralisation test.

The C-ELISA data generated for the 154 virus neutralisation test positive and 53 virus neutralisation test negative samples were then subjected to a two-graph receiver operating characteristic (TG-ROC) analysis (Greiner et al., 1995). The software indicated that data from the negative population showed departure from a normal distribution. Therefore, the non-parametric (distribution-free) method for the estimation of the measures described was used. A cut-off as established by the non-parametric option in the software with sensitivity \cong specificity \cong 100% was 29.5% with 95% accuracy (Figure 3). Summary statistics for the positive and negative reference population C-ELISA results are presented in Table 1.

Using the conventional method of determining the baseline between positive and negative, calculated as two standard deviations above the mean, the cut-off values would be at 33.6% and 23.4% inhibition for the South African (n=54) and UK (n=297) horses, respectively. With a PI cut-off set at 30% only one serum tested negative on C-ELISA whilst been shown to be positive to serotype 6 by virus neutralisation test.

3.2. Analytical specificity

The PI values recorded from EEV reference antisera prepared in sheep against the seven known serotypes of EEV at a 1:5 dilution were between 70.0% and 80.5 % and were

therefore deemed positive in the C-ELISA. Antisera prepared against the other 47 related Orbiviruses recorded inhibition values below 20.0% and were therefore recorded as negative and deemed not to cross react with EEV.

Sera from the thirteen horses that were positive to individual EEV serotypes as determined by virus neutralisation test were all also positive by C-ELISA recording PI values between 72 and 93% at a 1:5 dilution. The ELISA titres of these sera based on a 50% end-point were between 1:15 and 1:220.

3.3. Repeatability

Analyses of the data obtained after repeat testing of three standard sera by C-ELISA are presented in Table 2. The coefficients of variation (CV) calculated for the normalised data obtained from replicates of the strong and weak positive sera were all below 7%, which is below the acceptable value of 10%. The CV for the replicates of the negative sera was not calculated because it is considered not to be significant when the mean absorbance value approaches zero.

The mean absorbance value recorded for the guinea pig control wells of the 13 plates assayed on a single day was 0.85 (SD 0.158; range 0.52-1.23). Similarly, the mean absorbance value for the 16 plates assayed on different days was 1.08 (SD 0.207; range 0.52-1.23).

4. Discussion

Competitive ELISAs have been used successfully for many years for the detection of antibodies to different Orbivirus species, including AHSV (Hamblin et al., 1990; Hamblin

et al., 1992), BTV (Anderson, 1984) and EHDV (Thevasagayam et al., 1995). The assay described here measures the competition between an EEV specific guinea pig antiserum and a test serum for a pre-titrated EEV antigen. The amount of competition observed in the test wells (inhibition of the colour reaction), when compared to control values in the absence of test serum, was used to determine either positivity or negativity. Optimising the dilutions of antigen and specific antibody is essential when developing a new ELISA to ensure an acceptable balance between sensitivity and specificity. By selecting a concentration of EEV specific guinea pig antiserum that just saturates the antigen on the plate, the optimal conditions for competition between the test sera and guinea pig antiserum is ensured.

The expression of C-ELISA results as a PI of the competing antibody, is a continuous scale from 0 to 100 PI and is directly proportional to antibody activity (Wright et al., 1993).

Interestingly, the amount of colour that developed in some test wells containing negative equine sera was higher than that observed in the guinea pig control wells where equine serum was absent. This has been noted previously by other researchers (personal communication); however, the cause remains unknown. This phenomenon may be due to conformational changes in the antigen induced by these negative sera accidentally exposing more specific sites for the guinea pig antibody and thereby increasing the amount of colour. This is not observed with positive sera because of the high affinity of the EEV antibodies in the positive serum, which effectively counteracts this phenomenon.

Sera from two separate populations of horses that were assumed to have never been infected with EEV were used to establish the baseline for a negative population. These included sera from horses resident in the UK (n=297), where there have been no reports of

EEV, and sera from South African yearlings (n=53) that had been tested and reported negative by the traditional virus neutralisation test. The latter population were older than six months, when maternal antibody should have declined. The frequency plot of the PI values obtained for the UK sera was distributed slightly to the left of the South African sera. This difference was also reflected when using the conventional method of determining the cut-off between positive and negative samples (mean plus two SD) i.e. 23.4% and 33.6% calculated for the respective populations. It is not uncommon to find differences in the negative base-line between populations from naïve and endemic areas. The cut-off value between positive and negative samples calculated by the TG-ROC analysis (sensitivity \cong specificity \cong 100%) was 29.5% inhibition (Figure 3). This compares favourably with recommended method of determining the cut off value, calculated as two SD above the mean of the negative population (PI = 33.6%). Previous workers have set similar cut-off values for C-ELISA: i.e. 45% for a EHDV C-ELISA (Thevasagayam et al., 1995), 40% for a BTV C-ELISA (Anderson, 1984) and 50% for a AHSV C-ELISA (Hamblin et al., 1990).

Like other C-ELISA designed to detect antibody to specific Orbivirus species, this assay is serogroup specific, identifying all seven known EEV serotypes. The absence of any significant cross-reactions with the other 47 Orbivirus reference antisera confirms the assay as being highly specific. There was some variation between plates within and between assays (CV < 20%), although the absorbance values recorded for identical samples on individual plates were very similar. The C-ELISA described here has been shown to be sensitive, specific and reliable.

In areas where both AHSV and EEV are present, the EEV C-ELISA can be used in parallel with the AHSV ELISA thereby allowing simple parallel testing of equid sera for the detection of EEV and AHSV antibodies.

Although EEVs are often considered to be viruses of Southern Africa they are highly likely to be far more widespread and have recently been isolated in Israel. As they are transmitted by Culicoides species that are themselves widespread throughout Africa, Europe and the Middle East it is possible EEV could emerge in these areas. Clearly there is a need for further work to be undertaken to establish the true distribution of these viruses. Currently there are no vaccines against EEV and there is no requirement for EEV testing outside South Africa. Because mortality is considered to be <5% it is possible that these viruses are already resident in countries where other equine Orbivirus infections (AHSV) have either occurred or are currently present.

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Figure 1 A frequency distribution plot showing EEV group-specific antibody C-ELISA results obtained for horse sera (n = 297) collected in the UK. The mean PI value was minus 26.6% with a range between minus 100.0% and 31.6%. Two of the specimens were severely contaminated and gave inhibition values of 38% and 60%, respectively, and therefore these results were not included.

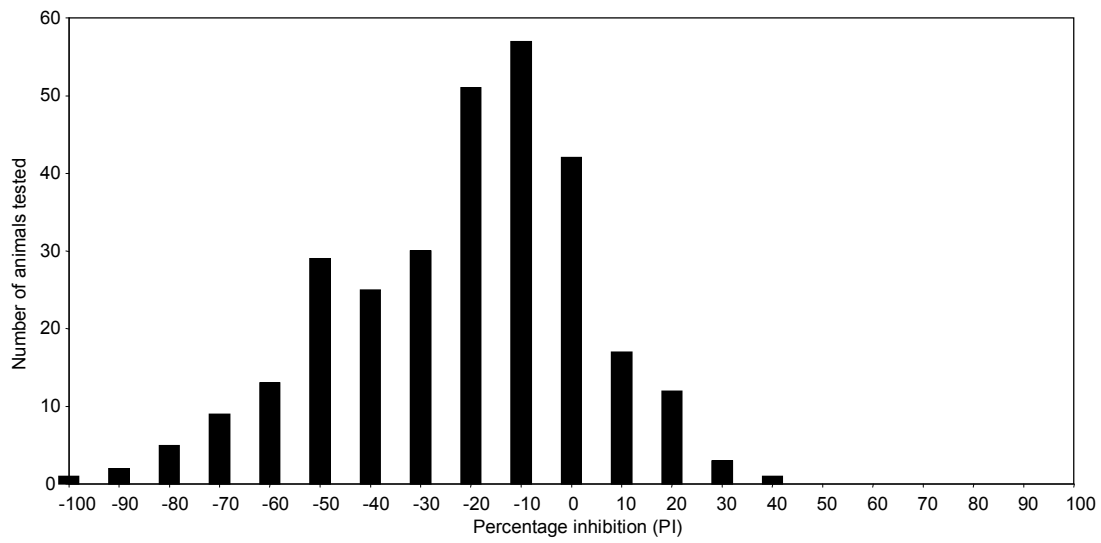


Figure 2 Frequency distribution plot showing EEV group-specific antibody C-ELISA results obtained from horse sera (n = 517) collected at the 1999 National Yearling Sale in South Africa. The distribution shows a clear discrimination between positive and negative sera.

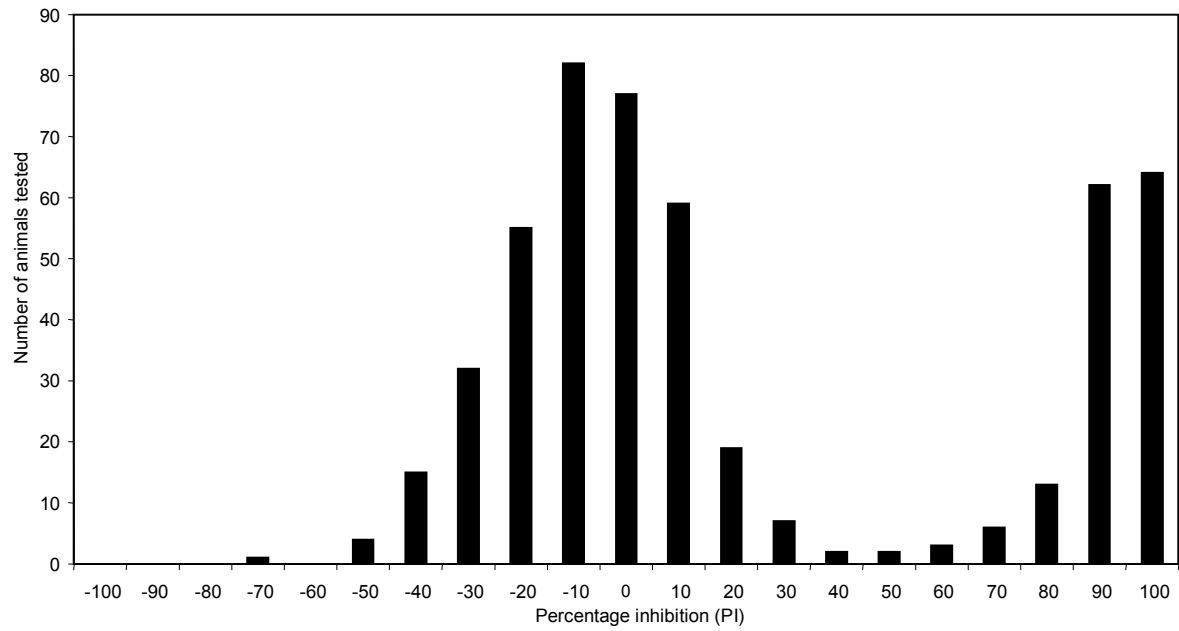


Figure 3 TG-ROC analysis of an EEV group-specific antibody C-ELISA. The intersection point of the two graphs indicates the cut-off point (29.5%) at which equivalence (sensitivity (Se)~ specificity (Sp)) can be achieved. The dotted line indicates sensitivity and specificity with an accuracy of 95%.

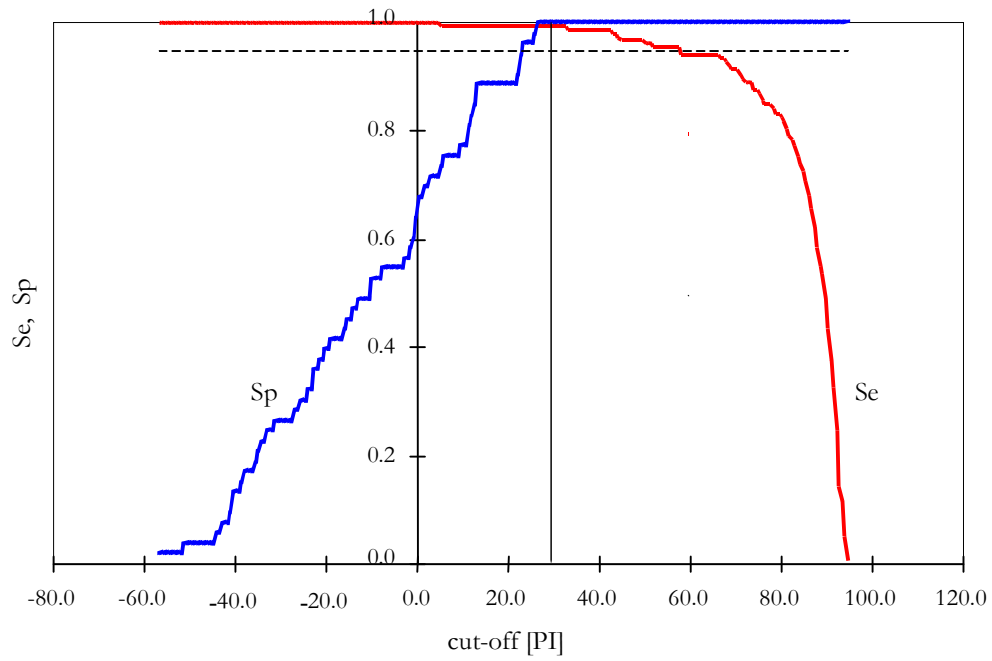


Table 1: Descriptive measures for the results of an EEV group-specific antibody C-ELISA after testing positive and negative reference populations (South African yearlings) that were confirmed by VNT. Data for sera from horses in the UK presumed negative is also shown.

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

VNT: Virus neutralisation test

Table 2: Descriptive measures for the results of an EEV-specific, antibody C-ELISA that was

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

repeated several times for a strong positive, a weak positive and a negative serum specimen, respectively.

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