



Comparison of ELISA and HI for detection of antibodies against Wesselsbron disease virus

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ABSTRACT

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A two-graph Receiver Operating Characteristic analysis was done to determine the optimal cut-off value of an indirect enzyme-linked immunosorbent assay (ELISA) for detection of antibodies to Wesselsbron disease (WSL) virus. When ELISA and haemagglutination-inhibition (HI) results of WSL-positive and WSL-negative control sheep sera were compared, the sensitivity of ELISA was 97,9% and that of HI, 87,5%, while the specificity of ELISA and HI were 95,7% and 100%, respectively. The ELISA's superior sensitivity was confirmed by the results of the two assays performed on a simulated range of low-positive sera, which showed that the ELISA was able to detect WSL-antibody levels at least ten times lower than those the HI could. The ELISA was also less cross-reactive than the HI to guinea-pig antisera against nine flaviviruses relevant to southern Africa. The combination of the ELISA's ability to test untreated sera in single dilution and its superior sensitivity and lower cross-reactivity as compared to that of the traditional HI, makes it the assay of choice for automation and large-scale screening of animals for antibodies to WSL virus.

Keywords: Antibodies, comparison, detection of antibodies, ELISA, enzyme-linked immunosorbent assay, haemagglutination inhibition, HI, Wesselsbron disease virus, WSL

INTRODUCTION

Wesselsbron disease (WSL) is caused by an arthropod-borne flavivirus originally isolated from a new-born lamb in the Wesselsbron district of the Orange Free State in 1955 (Weiss, Haig & Alexander 1956). Serological evidence has since shown the virus to be widespread in ruminants throughout southern Africa and West Africa and, although epidemiological data are lacking in many countries on the continent, the virus probably occurs widely in the rest of Africa if one considers the normal distribution of aedine mosquitoes associated with WSL. In spite of the widespread distribution of the virus, the incidence of actual clinical disease is quite low and is restricted to sporadic outbreaks at irregular intervals in years when exception-

ally heavy rains, which favour the breeding of mosquito vectors, trigger infection in susceptible animals. This usually occurs in the drier, temperate regions where the distribution of the virus is marginal (Blackburn & Swanepoel 1980; Swanepoel 1989).

Outbreaks of WSL are characterized by mortalities in newborn lambs and kids, occasional abortion in ewes, teratology of the ovine and bovine foetus and a non-fatal influenza-like illness in humans, while the disease is predominantly subclinical and inapparent in non-pregnant adult sheep, goats, cattle, horses, pigs and camels (Swanepoel & Coetzer 1994). Allwright, Geyer, Burger, Williams, Gerdes & Barnard (1995) recently recorded the first incidence of WSL infection in ostriches on a farm near Oudtshoorn in the Eastern Cape where mortalities occurred in 4-month-old ostriches.

Serodiagnosis of WSL virus infection has traditionally been based on haemagglutination-inhibition (HI), complement-fixation (CF) and neutralization (NT) tests (Shope & Sather 1979). Swanepoel (1989) found that antibodies can first be detected 4 d after infection with HI and NT tests, the titres rising to maximum levels in 1–3 weeks and then gradually declining until HI antibody becomes undetectable after 25 months, while VN antibody remains detectable. CF antibody can be detected from 10 d to approximately 12 weeks after infection.

Apart from WSL virus, which is regarded as the only flavivirus of any significance causing livestock disease in southern Africa (Weiss *et al.* 1956), the following nine flaviviruses are also known to be prevalent in this region (Barnard & Voges 1986):

- Banzi (BAN)
- Spondweni (SPO)
- Bagaza (BAG)
- Uganda-S (UG-S)
- Israel turkey meningo encephalitis (ITM)
- Usutu (USU)
- West Nile (WN)
- Ntaya (NTA)
- Yellow fever

Blackburn & Swanepoel (1980) found that antigenic relationships among the southern African flaviviruses conform to the general pattern described by Casals (1957) whereby sera were broadly cross-reactive in HI tests and less cross-reactive in CF and NT tests. Casals (1957) also showed that antibodies against flaviviruses are most specific for individual viruses after primary infection and then become more cross-reactive after reinfection, even with the same virus. In spite of this, homologous WSL titres greatly exceed heterologous flavivirus titres following primary WSL infection and can be distinguished relatively easily from other flaviviruses by quantitative NT tests (Blackburn & Swanepoel 1980).

The need to screen large numbers of domestic ruminants, game, ostriches and other species for specific WSL virus antibodies, and the various limitations of traditional serological techniques, initiated the development of an indirect enzyme-linked immunosorbent assay (ELISA). In this report we describe the use of purified whole-virus antigen in an WSL antibody detection ELISA and compare its performance as a diagnostic screening assay with the traditional HI test. ELISA test results of WSL-positive and -negative control sera were analysed by a two-graph Receiver Operating Characteristic (TG-ROC) computer program (Greiner, Sohr & Göring 1995) to determine a positive/negative cut-off value in order to compare the ELISA and HI in terms of sensitivity and specificity.

A panel of homologous guinea-pig antisera against ten flaviviruses found in southern Africa, were tested

with CF, HI and ELISA to determine and compare cross-reactivity of the different assays. Another panel of sera, simulating WSL-positive sheep sera ranging from very weak to strong positive, was used to compare the ability of the ELISA and HI to detect very low levels of antibody.

MATERIALS AND METHODS

Virus antigen

The original Van Tonder strain of WSL virus (Weiss *et al.* 1956) was first grown in CER cells, and maintained as monolayers in modified Eagles medium (Macpherson & Stoker 1962), supplemented with 5% bovine serum. Cells were infected and harvested for seven generations. Titrations were performed and virus infectivity was estimated at 1×10^{-4} plaque-forming units (pfu) per ml. Cell material containing the Wesselsbron virus was inoculated into mice brains. The brains were harvested and again inoculated into mice brains. This procedure was repeated nine times. A final concentration of 1×10^{-9} pfu was obtained from titrating the harvested mice-brain material. The mice-brain material containing the virus, was transferred to Roux flasks containing monolayers of CER cells. The virus was harvested after 96 h of incubation and transferred to roller bottles containing monolayers of CER cells. The cells were harvested after 96 h of incubation.

WSL virus was isolated by precipitating harvested cells overnight at room temperature with 8% (m/v) PEG 6 000 and 0.5 M of NaCl. The cell suspension containing the virus, was centrifuged at 3 000 rpm for 45 min at 4°C. The supernatant was discarded and the pellet resuspended in 2 mM of Tris-HCl (pH 9) and 10% Triton X-100. After sonication in an ultrasonic waterbath for 60 s, the suspension was precipitated by centrifugation at 6 000 rpm for 10 min at 4°C. The supernatant was kept on ice and the pellet treated once more with 2 mM of Tris-HCl (pH 9) and 10% Triton X-100, sonicated and precipitated as described above. The supernatants were layered on sucrose cushions of 35%, 45% and 55% in 2 mM of Tris-HCl (pH 9). The sucrose cushions were centrifuged in an ultra centrifuge with a SW 28 swing-out rotor at 4°C for 120 min at 28 000 rpm. Each different band was retrieved individually and layered onto a sucrose gradient of 30–65% in 2 mM of Tris-HCl (pH 9) at 5% intervals. The gradient was centrifuged at 28 000 rpm for 120 min at 4°C. The individual bands were easily discernible without the use of an artificial light source. The bands were retrieved by means of a glass syringe with a long, blunt needle and diluted in 2 mM of Tris-HCl (pH 9). The virus was precipitated by centrifugation at 28 000 rpm for 60 min at 4°C. The pellets were resuspended in 2 mM of Tris-HCl (pH 9). The purified virus served as the

antigen and this was titrated for optimal coating concentration.

Indirect ELISA

The procedure, with minor modifications, was based on an indirect ELISA as described for the detection of antibodies to Rift Valley fever virus (Paweska, Barnard & Williams 1995). A flat-bottomed, 96-well microtitre plate (NUNC polysorb) was coated overnight at 4 °C with purified WSL-virus antigen at a protein concentration of 1–2 µg per ml in phosphate-buffered saline (PBS), pH 9,6. The following day the plate was washed three times with TST buffer (50 mM of Tris-HCl pH 8,0, 150 mM of NaCl and 0,05% Tween-20). After it had been washed, the plate was blocked for 60 min at 37 °C with 100 µl per well of blocking solution consisting of 3% Elite fat-free milkpowder (Clover SA Ltd, Roodepoort, SA) dissolved in TST buffer. This solution also served as diluent for the sera and conjugate. After the plate had been washed once, 50 µl per well of a 1:100 dilution of each serum, including a positive and negative control serum were dispensed in duplicate wells and the plate incubated at 37 °C for 90 min. The plate was washed three times and 50 µl per well of a 1:5 000 dilution of recombinant peroxidase-conjugated protein G (Zymed Laboratories, San Francisco, CA, USA) was added to each well. After incubation of 60 min at 37 °C, the plate was washed three times and 50 µl per well of substrate solution consisting of o-phenylene diamine (OPD) in citrate buffer (pH 5,0) added to each well. The reaction was stopped after 20 min by adding 25 µl of a 2N sulphuric-acid solution to each well. Optical-density (OD) values were recorded with a microplate reader (Bio-Tek EL340, Bio-Tek Instruments, Winooski, VT, USA) at a wavelength of 490 nm.

Virus-neutralization test

Twofold serum dilutions starting at 1:4 were made in microtitre plates. An equal volume of a virus suspension was added to each well. The serum mixtures were left at room temperature for 60 min before they were seeded with sufficient cells to form a confluent monolayer within 8–12 h at an incubation temperature of 35–37 °C at 5% CO₂. The titre was expressed as the reciprocal of the serum dilution that completely or almost completely inhibited viral cytopathogenic effect (CPE).

Complement-fixation test

A six-volume CF test, comprising one volume of serum, one volume of antigen, two volumes of complement and two volumes of sensitized sheep red-blood cells, was used. The antigen, complement and haemolysin were diluted to contain two units each. The sucrose-acetone-extracted antigen was prepared according to the method of Clarke & Casals (1958).

Haemagglutination inhibition

The HI technique described by Clark & Casals (1958) was used to determine antibody titres. A sucrose-acetone extract of infected mouse brain was used in the tests, which were performed at pre-determined pH values. The positive/negative cut-off titre of the HI test was 1:10.

Sera

Control sera

A group of 46 Australian sheep sera served as a negative control group. The positive control group consisted of 48 post-vaccinated sera from South African sheep vaccinated with Onderstepoort live attenuated WSL vaccine.

Guinea-pig sera

Monovalent guinea-pig antisera against WSL, Banzi, Usutu, Spondweni, West Nile, Bagaza, Ntaya, Uganda-S, AR 5189 and Turkey meningo encephalitis were used to determine and compare the extent of cross-reactions of ELISA, CF and HI.

Simulated panel of positive sera

A panel of 12 sera, simulating a range of low-positive sera, was prepared by diluting a known WSL-positive sheep serum with a WSL-negative sheep serum in dilutions ranging from 0,25–40% of the original positive.

RESULTS

Sera from the WSL-positive control group ($n = 48$) and the WSL-negative control group ($n = 46$) were tested with ELISA. The test results were submitted to the TG-ROC analysis and are displayed graphically in Fig. 1. The TG-ROC is a plot of the test

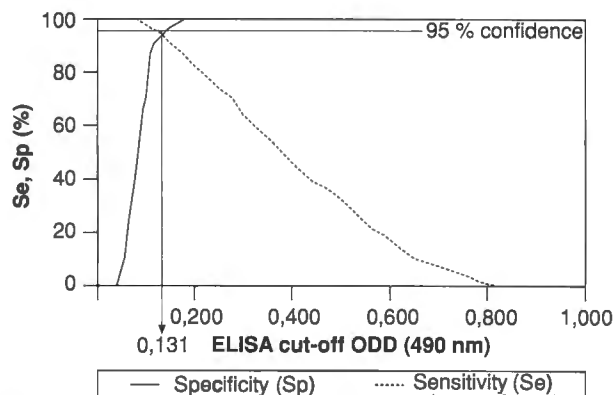


FIG. 1 TG-ROC plot of the WSL-ELISA with an indicated 95% accuracy level. The intersection point of the Se and Sp graphs indicates the cut-off value of 0,131 at which $Se = Sp$, which is 93,6%

sensitivity (Se) and specificity (Sp) against the cut-off (threshold) value, the latter being an independent variable. Se and Sp obtained with each cut-off value were calculated as the proportion of positive results in the positive and negative results, in the negative control population respectively. From this multiple x-y plot which represents the two observed parameters (Se and Sp) over the specified range of cut-off values, valid pairs of Se and Sp can be read for any specified cut-off value. At the intersection point of the two graphs, known as the point of equivalence,

the assay Se is equal to the assay Sp. For the ELISA, this point was at a cut-off OD value of 0,131, where the Se and Sp were both equal to 93,6%. By adjusting the cut-off value on the TG-ROC, the assay Se and Sp could be modified to suit specific circumstances. By increasing the cut-off value to 0,168, the Sp will increase to 100% while the Se will decrease to 88,3%.

The correlation of ELISA and HI results for the negative and positive control groups is shown in the form

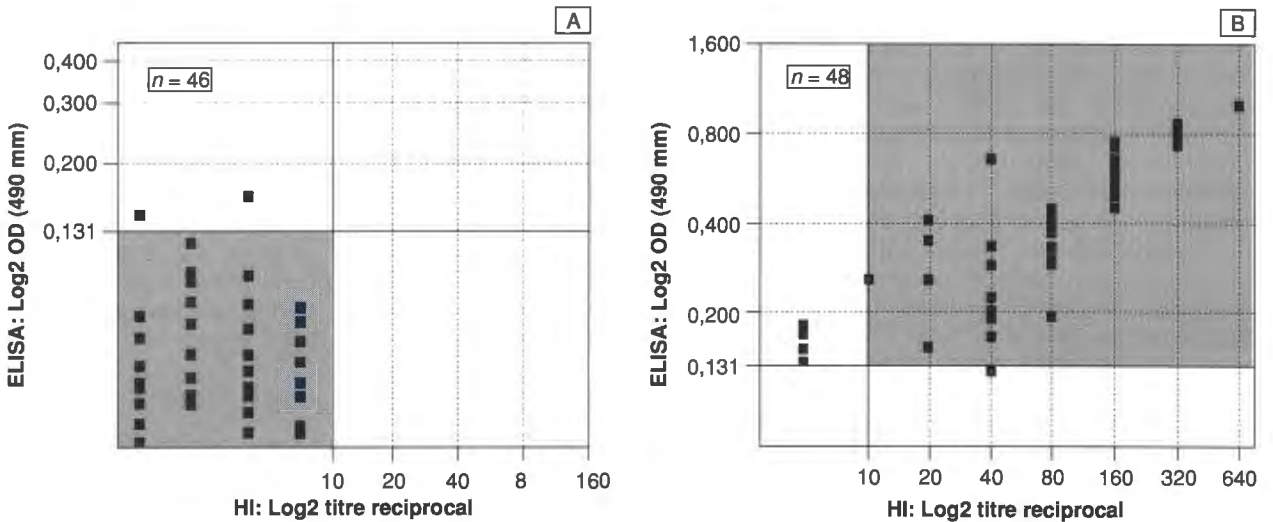


FIG. 2 Scatter diagrams showing the correlation between ELISA and HI test results recorded for WSL-negative (A) and WSL-positive (B) control groups of sheep. The ELISA cut-off OD value is 0,131

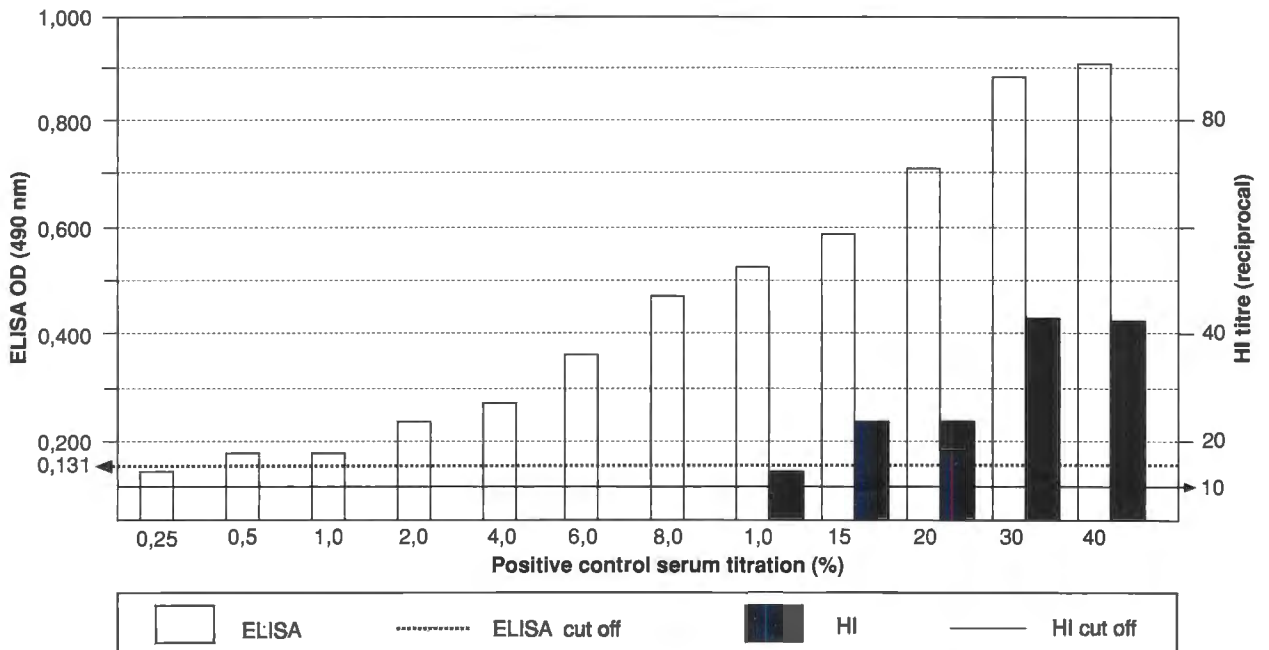


FIG. 3 ELISA and HI test results plotted for a range of simulated WSL-positive serums ranging from 0,25–40% of a known positive. The ELISA cut-off OD value is 0,131 and the HI cut-off titre is 10

TABLE 1 Comparison of assay sensitivity and specificity of ELISA and HI, by means of test results of a WSL-positive and WSL-negative control group of sheep

Tests		ELISA	HI
Negative control sera (<i>n</i> = 46) Specificity	WSL-negative	44/46	46/46
		95,7%	100%
Positive control sera (<i>n</i> = 48)	WSL-positive	47/48	42/48
	Sensitivity	97,9%	87,5%

TABLE 2 Comparison of HI, CF and ELISA test results done on guinea-pig antisera against ten flaviviruses relevant to southern Africa

Flavivirus	HI (titre)	CF (titre)	ELISA (OD)
Wesselsbron	160	> 32	0,365
Spondweni	10	–	0,027
Usutu	–	–	0,029
West Nile	–	–	0,027
Banzi	80	8	0,110
Bagaza	–	4	0,033
Turkey meningoencephalitis	80	8	0,142
Ntaya	–	–	0,053
Uganda-S	–	–	0,098
Yellow fever	80	–	0,020

of scatter diagrams in Fig. 2a and b, respectively. Samples in the overlapping shaded area of Fig. 2a represent the samples which tested both HI-negative and ELISA-negative (44 out of 46). Similarly, samples in the overlapping shaded area of Fig. 2b represent the samples which tested positive with both assays (42 out of 48).

Table 1 is a summary of the data used in Fig. 2a and b. The specificity of the ELISA was 95,7% (44 out of 46) compared with 100% (46 out of 46) for the HI test, while the ELISA had an assay sensitivity of 97,9% (47 out of 48) compared with 87,5% (42 out of 48) for the HI.

Results of the HI and ELISA performed on the panel of 12 serums simulating a range of positive sera, are shown in Fig. 3. With an OD cut-off value of 0,131, the ELISA was able to detect samples with a relative antibody level of 0,5% or higher, while the HI could only detect positive samples higher than 10%.

Table 2 shows the results of HI, CF and ELISA tests done on guinea-pig antisera against ten flaviviruses relevant to southern Africa. Each of the three assays reacted most strongly to the homologous WSL guinea-pig serum. In addition to this, the HI test cross-reacted strongly with heterologous Banzi, ITM and Yellow Fever serums. The CF and ELISA showed only mild cross-reaction with Banzi and ITM, with

the ELISA reacting very weakly with Uganda S, and the CF very weakly with Bagaza.

DISCUSSION

Comparison of ELISA and HI test results of WSL-positive and -negative control sheep sera (Table 1, Fig. 2) showed the ELISA to be at least 10% more sensitive than the HI, while the ELISA had a specificity of 95,7% compared with the 100% of the HI. Adjusting the cut-off OD value of the ELISA on the TG-ROC to obtain a specificity of 100%, decreased the ELISA sensitivity to 88,3%, which still compared favourably with the HI. The superior sensitivity of the ELISA was further demonstrated by the results of the two assays performed on a simulated range of low-positive WSL antisera (Fig. 3). The ELISA was able to detect WSL-specific antibodies in sheep sera at a level at least ten times lower than that at which the HI could.

Cross-reactivity of the CF and HI tests to guinea-pig antisera against nine flaviviruses relevant to southern Africa, was in accordance with previous findings (Casals 1957) where the CF was less cross-reactive than the HI (Table 2). Blackburn & Swanepoel (1980) found that this pattern also applied to the sera of cattle and sheep infected with WSL virus. It can be surmised therefore that the ELISA, which was less cross-reactive than the HI in guinea-pig sera, while corresponding well with CF cross-reactivity, will also be less cross-reactive than the HI in WSL antisera of sheep and cattle.

The growing international export of domestic ruminants, game and ostriches has created a specific need for efficient and reliable routine screening of large numbers of sera for the presence of antibodies to WSL virus. The necessity to pre-treat sera and to titrate individual samples with the traditional HI test, restricts its use for this purpose. In comparison, the ELISA can utilize untreated sera in single dilution, making it very suitable for large-scale screening and automation, while its superior sensitivity is of significant importance in the screening of animals for export purposes.

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