# EVALUATION OF AN ENZYME IMMUNOSORBENT ASSAY FOR THE DIAGNOSIS OF ARGENTINE HAEMORRAGIC FEVER

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**Summary.** – To elaborate a set of serological tests for the diagnosis of Argentine haemorrhagic fever (AHF), an enzyme-linked immunosorbent assay (ELISA) for detection of specific anti-Junin virus (JV) IgG is described, and its performance is compared with that of the plaque reduction neutralization test (PRNT). The reproducibility, sensitivity, specificity, and confidence limits for positive and negative results for ELISA were statistically analysed. The value of 800 was demonstrated as the lowest positive titer. Titers ≥800 varied within one (two-fold) dilution in 95.6% of the tests, while the sensitivity and specificity were 99.2% and 98.8%, respectively. The assay yielded 1% of false positives and 0.05% of false negatives. A comparison of ELISA to PRNT in detecting the seroconversion for JV was studied by the chi square test (comparison of proportions in paired samples) and the K parameter for agreement proportion. Comparison of ELISA to PRNT showed no significant difference in the proportions of positive and negative results of these assays ($P < 0.01$), demonstrating an equivalent performance ($K = 0.98$) in the diagnosis of AHF. In addition, the simplicity and safety of the procedures involved make this ELISA the most suitable test to detect natural human JV infections.

**Key words:** Junin virus; arenavirus; ELISA; plaque neutralization test; Argentine haemorrhagic fever

## Introduction

AHF is an acute systemic disease caused by JV (*Arenaviridae* family, *Arenavirus* genus, Tacaribe complex). This disease, whose clinical features have been reported elsewhere (Maiztegui, 1975; Maiztegui *et al.*, 1986), is treated by means of transfusion of immune plasma from AHF-convalescent donors. This therapeutical approach reduces the mortality rates from 30% to less than 3%, when administered within the first eight days of the illness (Maiztegui *et al.*, 1979; Enria *et al.*, 1984). Etiological diagnosis of AHF is usually established by means of JV isolation and/or serological conversion. The demonstration of specific antibodies in the convalescence from AHF has a double relevance, because it allows confirmation of the diagnosis of the disease and selection of immune plasma donors for therapeutical purpose. Furthermore, recent reports (Calderón, 1996; Mills *et al.*, 1996), describing the activity in Argentina of new arenaviruses with unknown pathogenicity for man, emphasized the need for accurate etiological diagnosis in human diseases compatible with arenavirus infections. A suitable strategy to achieve this purpose is to use tests, whose sensitivity, specificity, and other features are statistically defined. Reliable diagnosis should be established on a set of tests in which the characteristic of one complements and/or improves that of the others.

For the serological diagnosis of AHF, PRNT (Webb *et al.*, 1969) in Vero cells is considered a very reliable assay that has been used in Argentina since the late 70s. On the other hand, an ELISA for detection of specific anti-JV IgG (Meegan *et al.*, 1986), modified by Riera *et al.* (1995), has been used simultaneously with the referred PRNT to detect seroconversion for JV. In this work, the main features of the ELISA modified by us are described, and both PRNT and ELISA were used simultaneously for the serological diagnosis of AHF to compare their performance and to improve the available methods for detection of human JV infections.

**Abbreviations:** AHF = Argentine haemorrhagic fever; ELISA = enzyme-linked immunosorbent assay; FBS = foetal bovine serum; JV = Junin virus; LCM = lymphocytic choriomeningitis virus; MOI = multiplicity of infection; PBS = phosphate-buffered saline; PRNT = plaque reduction neutralization test

## Materials and Methods

*Sera.* Paired serum samples from 280 patients, hospitalized during 1990 and 1991 with presumptive diagnosis of AHF, were randomly selected to compare the serological diagnosis of JV infection obtained by ELISA and PRNT. Paired sera were obtained from each patient at the time of admission and 60 to 90 days after the clinical onset. A separate group of 310 serum samples from AHF convalescents were used to determine the reproducibility (n = 143 sera) and the cut-off value (n = 167 sera) of the results obtained by ELISA.

*PRNT* was performed according to Webb *et al.* (1969) using a constant virus-variable serum technique. Two-fold dilutions of sera, starting at 1:5, were tested with JV XJC13 strain (2,000 PFU/ml). The virus-serum mixtures were inoculated on Vero C76 cells. The titers were expressed as the maximum serum dilution that inhibited plaque formation by 80%.

*ELISA.* The antigen consisted of Vero C76 cells infected with JV XJC13 at a multiplicity of infection (MOI) of 3 PFU/cell. An equal number of flasks inoculated with the virus diluent only was used as negative control antigen. Three days later, the cells were scraped from the flasks into maintenance medium and pelleted at 8,000 x g for 10 mins. The pellets were resuspended in borate saline pH 9.0 and sonicated until no visible cellular debris remained. These suspensions were centrifuged at 12,000 x g for 10 mins, and the supernatants were saved and stored at -70°C. ELISA was performed in 96-round-bottom-well-microplates (Dynatech). A mouse anti-human IgG (FC) serum conjugated with peroxidase (Accurate Chemical & Scientific Corp.) was used in every assay at a dilution determined in a pretitration. ABTS (2,2'-azino-bis(3-ethyl-benzothiazolin sulfonate) and hydrogen peroxide served as a substrate. The plates were coated with 100 µl of the cell lysate diluted in phosphate-buffered saline (PBS) pH 7.4: one half of the plate with the infected cell lysate and the other half with the uninfected cell lysate (normal control antigen). The plates were kept overnight at 4°C, and then washed six times with 0.1% Tween 20 in PBS. The wells were then filled with 100 µl of two-fold diluted test sera, starting at 1:100. As diluent, PBS with Tween 20 (0.1%) and foetal bovine serum (FBS) (5%) was used. The plates were incubated for 1 hr at 37°C. After six washes, 100 µl of the appropriate dilution of conjugate was placed in each well and the plates were incubated for 1 hr at 37°C. The six washes were repeated and 100 µl of the freshly prepared substrate was added to each well. The plates were kept for 30 mins at 37°C.

Objective reading of ELISA results was performed by determination of absorbancies (A) at 405 and 450 nm of the content of each well in a spectrophotometer by a computer programme. For each test the cut-off value was calculated by subtracting the A of negative antigen from that of positive antigen at each dilution in the group of negative control sera. The mean and the standard deviation (SD) were calculated for these differences, and the cut-off value was established as $\bar{x}$ + 3 SD.

The maximum serum dilution greater than the cut-off value were regarded as the titers, expressed as reciprocals of those dilutions.

*Reproducibility* of the ELISA was established by testing duplicate blind serum samples. Reproducibility levels of the titers obtained were expressed in % (White, 1973).

*Sensitivity and specificity of the ELISA.* Paired serum samples from 280 patients with AHF were used to determine the sensitivity and specificity of the ELISA in comparison with the presence or absence of the disease as determined by JV isolation and/or serological conversion by PRNT. The following formulas were used (Lilienfeld and Lilienfeld, 1980):

$$\text{Sensitivity (\%)} = \frac{\text{true positives}}{\text{true positives + false negatives}} \times 100$$

$$\text{Specificity (\%)} = \frac{\text{true negatives}}{\text{true negatives + false positives}} \times 100$$

True positives were defined as patients who showed seroconversion for JV by ELISA and actually had the disease; false positives were defined as patients who showed seroconversion by ELISA but did not have the disease; true negatives were defined as patients who did not show seroconversion by ELISA and did not have the disease; false negatives were negative by ELISA but had the disease.

*Analysis of the cut-off value of the ELISA* was made for further testing of cut-off values proposed from or defined by the analysis of reproducibility, sensitivity and specificity, to provide confidence limits for positive and negatives results, and to allow the estimation of false positives and false negatives for the proposed cut-off value. In this work, certain dilution determined by the reproducibility test was adopted to divide total results in two groups: positive and negative. The statistical parameters: mean, SD, confidence limits, and *t* value (Student test) for one-tailed test were calculated for ELISA data by the method of Steel and Torrie (1960) taken from Heck *et al.* (1980).

Calculations were made by using as the variable (x) the reciprocal of the dilutions assayed, normalized as a series of values of the transformed variable ($\log_2 x/50$).

*Comparability of the ELISA to PRNT* was analyzed on the basis of seroconversion for JV as determined by both assays. Statistical analysis was performed by two methods. The chi square method for comparison of proportions in paired samples (Snedecor and Cochran, 1967) was used for the evaluation of the level of discrepancy between seroconversions for JV determined by PRNT and ELISA. Significance levels were assessed as follows:

| | PRNT+ | PRNT– |
|---|---|---|
| ELISA+ | a | b |
| ELISA– | c | d |

$$\chi^2 = \frac{(|b-c|-1)^2}{b+c}$$

The agreement proportion (kappa) method was used to estimate the level of coincidence between seroconversion by ELISA and PRNT, and was expressed in values of the K parameter calculated as follows (Fleiss, 1981):

**Table 1. Distribution and reproducibility of results of testing of the same serum samples two consecutive times by the ELISA**

| Results | Titers ≤ 400 | | Titers ≥ 800 | | Total | % |
|---|---|---|---|---|---|---|
| | Frequency | % | Frequency | % | | |
| Identical | 5 | 6.8 | 23 | 33.3 | 28 | 19.6 |
| Differing by one (two-fold) dilution | 51 | 68.9 | 43 | 62.3 | 94 | 65.7 |
| Differing by two (two-fold) dilutions | 18 | 24.3 | 3 | 4.4 | 21 | 14.7 |
| Total | 74 | 100.0 | 69 | 100.0 | 143 | 100.0 |

| | ELISA+ | ELISA– | |
|---|---|---|---|
| PRNT+ | a | b | $p_2$ |
| PRNT– | c | d | $q_2$ |
| | $p_1$ | $q_1$ | N |

$$K = \frac{I_o - I_e}{1 - I_e} \qquad I_o = \frac{a + d}{N} \qquad I_e = \frac{(p_1 \cdot p_2) + (q_1 \cdot q_2)}{N^2}$$

where $I_o$ is the observed value of the index (calculated from the proportions), $I_e$ is the value of the index expected on the basis of chance alone (calculated from the proportions), and K (kappa) is the measure of agreement with interval of values. If there is complete agreement, K = 1. If the observed agreement is greater than or equal to the chance agreement, K ≥ 0, and if the observed agreement is less than or equal to the chance agreement, K ≤ 0.The minimum value of K depends on the marginal proportions. K values range from –1 to +1, and it is considered that K < 0.4 indicates a low concordance, 0.4 < K < 0.7 indicates an intermediate concordance, and K > 0.7 indicates a high concordance.

## Results

### *Reproducibility of the ELISA*

The results of repeated testing of 143 serum samples by ELISA are shown in Table 1. The comparison of the titers obtained by two consecutive assays demonstrated that 85.3% of the results varied by one two-fold dilution or less. A higher variability (two two-fold dilutions) was found in 14.7% of the results. When the distribution of these percentages was compared in the two groups with lower (≤ 400) and higher (≥ 800) titers (Table 1), and tested by chi square values, a significant difference (P = 0.001) was found between the distribution of the variation by one dilution or less and of the variation by two dilutions, indicating that the higher titers were significantly more reproducible. Consequently, this conclusion defined the dilution of 1:800 as the lowest positive result to be used as the cut-off value.

### *Sensitivity and specificity of the ELISA*

Percentages of sensitivity and specificity of the ELISA are shown in Table 2. To explore the influence of different cut-off values on these percentages, they were calculated by use of two criteria of seroconversion: (a) titers raising from negative to ≥ 400, and (b) titers raising from negative to ≥800. The results demonstrate that the sensitivity of 99.2% remained unchanged for both seroconversion criteria, while there was an increase in specificity from 93.1% to 98.8% for the (b) criterium of seroconversion.

### *Analysis of the cut-off value of the ELISA*

On the basis of the reproducibility, sensitivity and specificity studies, the dilution of 1:800 appeared as the most suitable to be proposed as the first positive result, splitting the population of the results into two groups.: positive (titers ≥ 800) and negative (titers <800). The mean for the negatives was 189, ranging between 100 and 400, and the mean for the positives was 64,313 with values between 800 and 3,276,800 (Table 3).

The confidence limits calculated by using the one-tailed Student's *t* test are depicted on Fig. 1. The estimated probability for a titer of a positive serum being ≤162 resulted in P <0.0005, and for a titer of a negative serum being ≥788 in P < 0.01. These confidence intervals determined an over-

**Table 2. Sensitivity and specificity of the ELISA by use of two different cut-off values**

| Cut-off value | Sensitivity (%) | Specificity (%) |
|---|---|---|
| ≥ 400 | 99.2 | 93.1 |
| ≥ 800 | 99.2 | 98.8 |

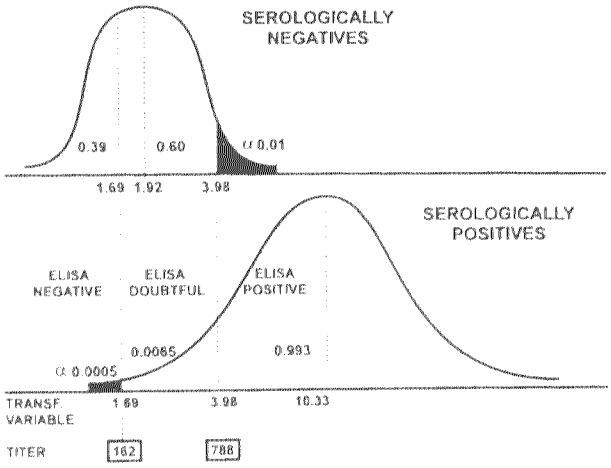


**Fig. 1**
**Intervals and confidence limits for positive, negative and doubtful results from sera tested by the ELISA**

lapping range of titers between 162 and 788 that were considered doubtful.

For the serologically negative sera (Fig. 1), 39% of the population could be expected to have a titer ≤162, and 1% ($\alpha = 0.01$) to have a titer ≥788 and to be erroneously classified as positive, leaving 60% of the population doubtful.

For the serologically positive sera, 99.30% of the population could be expected to have a titer ≥788, and 0.05% ($\alpha = 0.0005$) to have a titer ≤162, and therefore to be erroneously classified as negative. The remaining 0.65% of the population could be expected doubtful.

*Comparability of the ELISA and PRNT*

The ELISA and PRNT were compared considering agreement and discrepancy between the seroconversions for JV detected by these methods.

Criteria applied to define the seroconversion were as follows: for PRNT, the seroconversion for JV consisted of an increase in the specific antibody titer from a negative result (titer <5) at the time of admission during the acute phase of AHF to a titer ≥10 at 60 days after the onset of the disease. For ELISA with serum samples obtained at the described intervals, two criteria for seroconversion were used: an increase in antibody titer from ≤200 to ≥400 (criterium 1), and from ≤400 to ≥800 (criterium 2).

| | Criterium 1 | Criterium 2 |
|---|---|---|
| Pairs of proportions | ELISA+ / PRNT- = 11/280 = 0.039 | ELISA+ / PRNT- = 2/280 = 0.0071 |
| | ELISA- / PRNT+ = 1/280 = 0.0036 | ELISA- / PRNT+ = 1/280 = 0.0036 |
| | $\chi^2 = 6.75$ (P < 0.01) | $\chi^2 = 0.0$ (P > 0.05) |
| | K = 0.91 | K = 0.98 |

**Fig. 2**
**Distribution of serological diagnosis of AHF by the ELISA and PRNT in 280 patients according to two different criteria for seroconversion to JV**

The results obtained from the comparison of the ELISA and PRNT by use of proportions in paired samples are shown in Fig. 2. When ELISA values ≥400 were considered positive (the criterium 1), a significantly higher proportion of seroconversions was determined ($P < 0.01$). However, this difference disappeared when the criterium 2 (values ≥800 considered positive) was followed ($P > 0.05$). High K values, namely 0.91 and 0.98, were obtained by using the criteria 1 and 2, respectively.

## Discussion

In this study, to define a set of serological tests for detection of human JV infection, the features of a direct ELISA for specific anti-JV IgG were studied, and its performance was compared with that of PRNT. The results of repeated testing of the same serum samples by the ELISA demonstrated a variation by one (two-fold) dilution or less in 85% of the cases. This percentage increased to 95.6% for titers ≥800, leaving a variation by two (two-fold) dilutions to only 4.4% of the repetitions. This high reproducibility not only proved the reliability of the assay, but also established that an increase in the titer of antibodies between 2 consecutive serum samples from an AHF patient, equal to or higher than two (two-fold) dilution factors, should be considered a specific seroconversion.

The percentages of sensitivity and specificity of the ELISA for titers ≥800 were 99.2% and 98.8%, respectively.

Testing of the reproducibility, sensitivity and specificity of the ELISA in this way provided a starting point for the definition of seroconversion as well as a cut-off value to be statistically tested. By use of the Student's *t* test we demonstrated that with 800 as the first positive titer, the assay yielded 1% of false positives and 0.05% of false negatives.

The specificity of 98.8% demonstrated for ELISA in this study was emphasized by the absence of cross-reaction with other arenaviruses coexisting in the JV endemic area. For the newly described Oliveros (Mills *et al.*, 1996) and Pampa (Calderón, 1996) agents, serological methods can complement a test detecting immunofluorescent antibodies in the search for human infections that have not yet been demonstrated with the currently available serology. Lymphocytic choriomeningitis virus (LCMV), the prototype virus of the *Arenaviridae* family, has been causing human infections in the same area as JV since the early 70s. In over 5% of the total confirmed cases of AHF, a previous infection with LCMV can be detected by specific serological tests (Barre-

**Table 3. Parameters of distribution of serum titers determined by the ELISA for the analysis of confidence limits for positive and negative results**

| Serological status | n | Parameters | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Mean | | SD | Range | | Confidence limit | |
| | | Titer | Transf. variable | | Titer | Transf. variable | Titer | Transf. variable |
| Negative | 38 | 189 | 1.92 | 0.85 | (100-400) | (1-3) | 788 (0.99) | 3.98 |
| Positive | 129 | 64,313 | 10.33 | 2.56 | (800-3,276,800) | (4-16) | 162 (0.9995) | 1.69 |

ra Oro *et al.*, 1977). A cross-reaction with LCMV in the ELISA described herein was ruled out in a number of paired serum samples in which preexistence of anti-LCMV antibodies had been demonstrated (data not shown). In those cases, like in the rest of samples in this study, the serum sample taken during the acute phase of AHF was negative for anti-JV antibody.

The comparison of the seroconversions for JV determined by two different assays, ELISA and PRNT, showed no significant difference in the proportions of positive and negative results, demonstrating an equivalence (K = 0.98) in their performance. This high K value has broader implications, because PRNT has been used for many years to demonstrate the presence of antibodies associated with protection against JV infection, indicating that those antibodies are mainly specific IgG, the type of Ig detected by the ELISA described herein. Since this ELISA has been used only recently, there are no studies on the persistence of the specific IgG detected by this assay. However, previous work on AHF, reporting that anti-JV antibodies appearing in early convalescence can be detected by PRNT for long periods of time (Damilano *et al.*, 1982), suggests that its equivalent ELISA may be suitable for the detection of recent and remote human JV infections.

The simplicity of performance and the results of the ELISA described herein indicate that this may be the most suitable method of detection of natural human JV infections for diagnostic and epidemiological surveillance studies. To cover every JV infection, the ELISA should be combined with PRNT, which will also disclose non-specific and doubtful ELISA results.

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