



## The use of chicken IgY in a double antibody sandwich ELISA for detecting African horsesickness virus

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### ABSTRACT

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An indirect sandwich ELISA that can detect as little as 8 ng of African horsesickness virus (AHSV) was developed. Viral antigen was captured from suspension using an immobilized monoclonal antibody specific for an epitope on VP7, a protein that is a major constituent of the virus core. Egg-yolk derived chicken IgY directed against AHSV (serotype 3) was used as the secondary antibody. Since IgY and mouse IgG do not cross-react serologically, the secondary antibody was not labelled, but was instead detected with enzyme-coupled sheep antibodies directed against avian immunoglobulins. The assay recognized all nine AHSV serotypes, but not the Cascara isolate of equine encephalosis virus, a related orbivirus that also infects horses. In addition to being able to detect and quantify whole AHSV, the ELISA could show the presence of VP7 produced by recombinant baculoviruses.

**Keywords:** African horsesickness, Antibody sandwich, chicken, egg-yolk, orbivirus

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African horsesickness virus (AHSV), an orbivirus (Reoviridae), causes a severe disease which can progress very rapidly, often resulting in death within a few days. Antibody detection is of limited diagnostic value since animals may die before measurable titres are attained. The presence of virus in spleen samples from dead animals or in cell cultures derived from isolated virus can, however, be shown in double antibody sandwich enzyme-linked immunoassays (DAS-ELISAs). In this assay format, viral antigen (the "filling" in the sandwich) is captured from suspension by an immobilized primary antibody and detected with a secondary immunoglobulin that recognizes the same antigen. The secondary antibody may either be directly labelled with an appropriate enzyme or it

may be detected indirectly using a labelled anti-immunoglobulin. Accordingly, ELISAs for AHSV antigen have used F(ab')<sub>2</sub> fragments in combination with intact IgG from a single animal species (Du Plessis, Van Wyngaardt & Bremer 1990), polyclonal antibodies from two different species (Hamblin, Mertens, Mellor, Burroughs & Crowther 1991) and a pair of monoclonal antibodies (Laviada, Babin, Dominguez, & Sánchez-Vizcaíno 1992) to form the respective capture and secondary antibody layers in the macromolecular sandwich.

While mammalian antibodies were used successfully in the assays referred to above, avian antibodies have several potential advantages which justify their being investigated as alternative immunoassay reagents. For example, chicken IgY is readily obtained from eggs laid by immunized hens (Polson, Von Wechmar & Van Regenmortel 1980) and because it does not cross-react with mammalian antibodies, is particularly suitable for use in immunoassays requiring antibodies from two species (Devergne, Cardin, Burckard & Van Regenmortel, 1981). Since chickens use gene conversion to generate antibody diversity

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(Reynaud, Dahan, Anquez & Weill 1987) it is not inconceivable that there may be subtle, and perhaps useful differences in the binding of their antibodies when compared to those of mice, for example. In addition, chickens require a minimum of specialized care and are well suited to being used as a source of immunological reagents in a resource-poor environment (Wallman, Staak & Luge 1990). This communication describes an indirect version of a DAS-ELISA which uses a monoclonal antibody (Mab) directed against an epitope on VP7 (Van Wyngaardt, Du Plessis, Van Wyngaardt & Verschoor 1992), a major structural protein of the AHSV core, to capture viral antigen from suspension. Egg-yolk derived chicken IgY raised against whole AHSV is used as the secondary antibody, which in turn is detected using enzyme-coupled sheep antibodies specific for chicken immunoglobulins. The assay was able to detect all nine AHSV serotypes as well as VP7 expressed by recombinant baculoviruses ("recombinant VP7").

AHSV (serotype 3) was propagated in CER cells and purified by Triton X-100 lysis followed by sucrose density-gradient centrifugation (Huisman, Van der Walt, Cloete & Erasmus 1987). Equine encephalosis virus (EEV) "Cascara" isolate, purified in the same way, was obtained from the Biochemistry Division at the Onderstepoort Veterinary Institute. Freeze-dried baby hamster kidney (BHK) cell cultures containing each of the nine AHSV serotypes were reconstituted in phosphate buffered saline pH 7.4 (PBS). The gene encoding VP7 of AHSV was expressed in *Spodoptera frugiperda* cells and purified by anion exchange chromatography as previously described (Bremer, Du Plessis & Van Dijk 1994).

For IgY production, a 24 week-old Amberlink laying hen (Golden Lay, South Africa) was injected at several sites in the pectoral muscle at 7 d intervals with purified AHSV suspended in 500  $\mu\text{l}$  PBS and emulsified with an equal volume of ISA 50 adjuvant (Seppic, France). The first injection contained 80  $\mu\text{g}$  of virus and the two boosters were of 40  $\mu\text{g}$  each. IgY was recovered from an egg collected 10 d after the final injection using selective precipitation with polyethylene glycol (Polson, Coetzer & Kruger 1985). The presence of AHSV-specific immunoglobulins was demonstrated by virus neutralization. Fifty per cent plaque reduction was achieved at an IgY dilution of 1/8 000 (not shown).

To capture AHSV from suspension, microtitre plates (Nunc Polysorp, Denmark) were coated for 2 h at 37°C with 50  $\mu\text{l}$ /well of the VP7-specific Mab 1F1 (Van Wyngaardt *et al.* 1992) at a concentration of 10  $\mu\text{g}/\text{m}\ell$  in PBS. The IgG for coating was prepared from ascitic fluid by ammonium sulphate precipitation and ion exchange chromatography (Clark & Adams 1977). Blocking was for 1 h at 37°C with 300  $\mu\text{l}$ /well

of 2% w/v milk powder (Elite) in PBS (MP/PBS). After washing with PBS containing 0.05% v/v Tween 20 (PBS-Tw), the antigen (50  $\mu\text{l}$ ) was added and incubated overnight at 4°C for 16 h. All subsequent incubations were for 1 h at 37°C. A wash followed before 50  $\mu\text{l}$ /well anti-AHSV chicken IgY at a concentration of 10  $\mu\text{g}/\text{m}\ell$  in MP/PBS was added. After washing to remove unbound IgY, 50  $\mu\text{l}$  horseradish peroxidase-labelled sheep antibodies directed against chicken immunoglobulins (The Binding Site, UK) diluted 1/5 000 in MP/PBS were introduced into the wells. After a final wash, 50  $\mu\text{l}$ /well of chromogen consisting of 1 mg/ml o-phenylene diamine and 0.5  $\mu\text{l}/\text{m}\ell$  of a 30% hydrogen peroxide substrate solution in 0.1M citrate buffer, pH 4.5 was added. After allowing 30 min for colour development, the reaction was stopped with 50  $\mu\text{l}$ /well 2N H<sub>2</sub>SO<sub>4</sub>. Absorbance at 492 nm was determined using a BDSL Immunoskan microtitre plate reader.

A dilution series of purified AHSV of the homologous serotype was used for constructing a titration curve to determine the sensitivity of the DAS-ELISA. In addition, a parallel experiment was performed with the related equine encephalosis virus (EEV) to establish whether the assay could specifically distinguish AHSV. Fig. 1 shows that as little as approximately 150 ng/ml, which is equivalent to about 8 ng per well of AHSV, produced an absorbance at least double that obtained in the absence of virus. In contrast, even at concentrations as high as 10  $\mu\text{g}/\text{m}\ell$ , the wells in which the Cascara isolate of EEV had been incubated showed no increase in absorbance above background. The assay thus has the potential to distinguish the two virus groups. Since the antigenic properties of the EEV serogroup have not been extensively studied, however, the remote possibility that both the capture Mab and secondary antibodies may find cross-reactive epitopes on one or more of the remaining EEV isolates cannot be excluded. Mab 1F1 binding can be blocked by antibodies to all AHSV serotypes in an inhibition ELISA (Van Wyngaardt *et al.* 1992). Not unexpectedly therefore, in its role as a sandwich ELISA capture antibody, it was able to recognize all nine serotypes as well as recombinant VP7 of the homologous serotype. The signals obtained confirm that egg-yolk IgY raised against AHSV serotype 3 and used as the secondary antibody also recognized each of the heterologous antigens. No significant absorbance was produced by a BHK cell lysate control (Fig. 2).

This study has demonstrated that egg-yolk IgY is indeed a practical immunological reagent for use in a DAS-ELISA that can detect AHSV. Its successful application depended, however, on the IgY being used as the secondary antibody. If the roles were reversed and it was used for capture with the Mab as the secondary antibody, no ELISA signals were obtained (not shown). A possible reason is that since

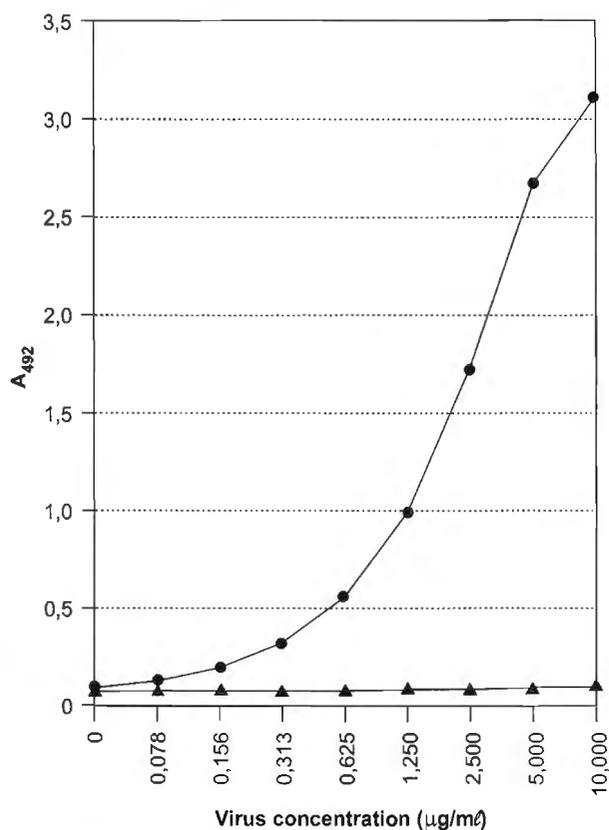


FIG. 1 Absorbance values obtained in the IgY DAS-ELISA with different dilutions of purified AHSV serotype 3 (▲) and EEV Cascara (●). Each point represents the mean of three determinations

the hen used for IgY production had been previously immunized against several avian diseases, only a relatively small fraction of its egg-yolk antibodies was likely to have been specific for AHSV. As a result, the affinity matrix in the microtitre well consisting of polyclonal IgY may have been unable to efficiently capture virus particles owing to the relative abundance of other antibody specificities. In addition, the core protein-specific Mab may have had only a limited number of accessible epitopes per particle to which it could bind, thus making it a poor secondary reagent. By contrast, its utility as a capture antibody can be explained by the immobilized Mabs providing a high density matrix of identical paratopes which would favour multivalent interactions with these same epitopes.

In addition to detecting and quantifying AHSV with its array of repeating subunits, the sandwich ELISA could detect the comparatively low molecular mass VP7 core particle subunit. With intact virus, determinants recognized by the secondary immunoglobulins could potentially include multiple copies of the capture epitope as well as those on other structural proteins. On the other hand, antibodies to epitopes on the same polypeptide, but which are distinct from the

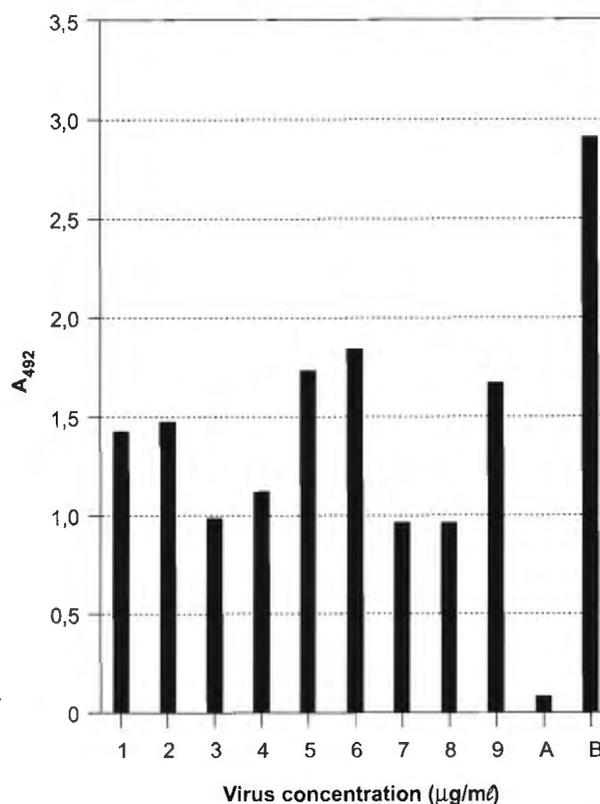


FIG. 2 Bar chart showing DAS-ELISA absorbance values produced by each of the nine AHSV serotypes (1–9), BHK cell homogenate (A) and recombinant VP7 (B). Each bar represents the average of three readings

one that binds the primary antibody, are required for a captured VP7 subunit to be detectable. Although whole AHSV was used for immunization, the chicken's immune system nonetheless produced an antibody population that, in combination with a capture Mab from its murine counterpart, allowed both types of antigen to be detected.

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