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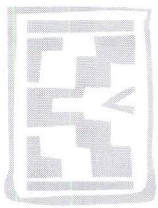

Development of solid phase antigen for indirect ELISA for the detection of specific antibody responses to infection with Newcastle disease virus

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ABSTRACT

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A simple and inexpensive method of antigen preparation by ultrafiltration was investigated using the V4 strain of Newcastle disease virus. The antigen designated XM300 was used in an indirect enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies to Newcastle disease virus in chicken serum. The assay was evaluated using both experimental and field sera, as well as reference control reactor and non-reactor sera. Antigen prepared by the ultrafiltration method was compared with antigen prepared by ultracentrifugation and the ultrafiltration antigen was found to react specifically with Newcastle disease virus antiserum in this ELISA system. This antigen preparation technique is also suitable for use in developing countries.

The ELISA provides an excellent method for measuring antibodies in the early stages of infection in serum samples from experimentally infected chickens. More than 14.58 % of the total serum samples which failed to be recognized as reactors by the conventional haemagglutination inhibition test were detected in the ELISA.

Keywords: Antigen preparation, indirect ELISA, Newcastle disease virus

INTRODUCTION

The poultry industry forms a major component of the livestock industry in many parts of the world. In most developing countries the poultry industry includes backyard types of operations where the meat and the eggs produced from this small-scale industry substantially contributes to the protein in the diet of many people. Disease is an important constraint to this type of farming. The success of such operations also depends on many factors including diagnosis of Newcastle disease virus and monitoring of the immune status of the flocks.

A range of antigen preparations for use in the enzyme-linked immunobsorbent assay (ELISA) for the detection of specific antibody to Newcastle disease virus (NDV) has been previously investigated. Crude virus from infected allantoic fluid (Charan, Rai & Mahajan 1981), alcohol precipitated virus (Miers, Bankowski & Zee 1983), sucrose gradient purified antigen, heat and formalin inactivated virus (Snyder, Marquardt, Mallinsion & Russel 1983) and semi-purified and Triton X-100 treated antigen (TTA) (Lamichhane 1988) have been used. Newcastle disease virus has also been concentrated and purified by microfiltration and exclusion liquid chromatography on macroporous glass (Krashenyuk & Goretskaya 1988). It has been noted that the

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degree of sensitivity varies with different antigen preparations and at all times requires standardization of the reagents used in the system. In all cases, despite the variety of antigen preparations, ELISA has proven to be at least as, or more sensitive, than the conventional haemagglutination inhibition (HI) test for detecting immune responses in chickens and ostriches (Miers *et al.* 1983; Snyder *et al.* 1983; Marquardt, Snyder, Savage, Kadavil & Yancey 1985; Thayer, Villegas & Fletcher 1987; Brown, Resurreccion & Dickson 1990; Reddy & Srinivasan 1992; Williams, Boshoff, Verwoerd, Schoeman, Van Wyk, Gerdes & Roos 1997; Koch, Czifra & Engstrom 1998; Verwoerd, Olivier, Gummow, Gerdes & Williams 1999). The preparations of antigens mentioned above require laboratory facilities with different levels of sophistication. Some of the antigen preparations require laboratory equipment that is not readily available in routine diagnostic laboratories in developing countries. This becomes an obstacle to antigen preparation and routine diagnosis of disease in many countries of the world. To overcome this problem may require a continuous effort in the area of investigation of simple and inexpensive methods for developing an assay that fulfils the requirements of the country in which it is to be used.

In this study trials of simple and inexpensive methods of antigen preparation for the use in ELISA are described. The antigen designated as XM300 is used in solid-phase in an ELISA to react with specific antibody to NDV.

MATERIALS AND METHODS

Control antigen preparation

The two different sources of stock virus were used in these preparations. The stock virus (Webster's vaccine strain) which had an infectivity titre of $10^{10.14}$ EID_{50} per mℓ was used for the preparation of the control semi-purified TTA by ultracentrifugation using the method of Lamichhane (1988), except that the casein concentration was reduced to 0.2%. This antigen was used as a control antigen preparation and for the standardisation of other reagents in the system. A second antigen was prepared in the same way from the apathogenic V4 strain (Simmons 1967) of NDV (Arthur Webster Pty. Ltd. Australia 1992). The pooled infected allantoic fluid had a titre of 10^9 EID_{50} per mℓ of stock virus and a 1:128 dilution of the virus contained 4 HA units per 50 mℓ of the stock virus. Antigen was prepared conventionally by ultracentrifugation as follows: Allantoic fluid infected with stock viruses was centrifuged for 3 h at 50 000 x *g*, the pellet was resuspended in 2% Triton X-100 overnight and centrifuged for a further 15 min at 50 000 x *g*. The supernatant was used as TTA. The antigen was concentrated to a titre of 10^9 EID_{50} per mℓ.

Ultrafiltration

Amicon Diaflo Ultrafiltration (Amicon Scientific, Australia) membranes of size XM300 and YM100 with nominal molecular mass cut-offs of 300 000 and 100 000, respectively were used in the concentration and semi-purification process. After each batch, the membrane was washed by immersion in 1 % Decon-90 in distilled water. The membranes were stored in 10 % ethanol at 4 °C and before reuse they were soaked and washed with distilled water.

Procedure for XM300 antigen

The stock virus from pooled infected allantoic fluid was centrifuged at 1 000 x *g* for 10 min. The virus in the supernatant (50 mℓ) was then concentrated to the 5 mℓ using an Amicon XM300 filter membrane in a stirred cell apparatus. The retentate (5 mℓ) was resuspended in phosphate buffered saline A (PBS-A, pH 7.2) to 50 mℓ and concentrated again to 5 mℓ using the same size of filter membrane. The retentate was washed twice in PBS-A and concentrated to 5 mℓ with YM100. This was carried out to minimize soluble proteins such as albumin. After each steps of filtration both the filtrates and retentates were checked for the presence of soluble antigen using the haemagglutination test (HA) and infective virus on passage in the allantoic cavity of 10-days-old chick embryos. At final steps the retentate from YM100 was treated with Triton X-100 at a final concentration of 0.2 %. This was used as solid-phase antigen designated XM300 and stored at minus 70 °C.

Serum samples

Serum samples representing a range of HI antibody levels were collected. One hundred serum samples were from naturally infected birds provided by Dr M. MacKenzie, Ingham Enterprises, Queensland. Sixteen serum samples from a naturally infected adult broiler breeder flock and four non-reactive control sera from adult specific pathogen free layers were provided by the NSW Agriculture and Fisheries, Elizabeth Macarthur Agricultural

Institute. Twenty three known reactive and non-reactive sera from this laboratory serum bank. One hundred and forty two serum samples collected by Lamichhane (1988). This included serum samples from experimentally infected chickens collected before and after infection as follows: before inoculation, and 1, 2, 3 and 4 weeks after inoculation. The chickens were inoculated by the oronasal route with 10^7 EID_{50} of the V4 strain of NDV.

ELISA procedure

The indirect ELISA was developed in two types of round-bottomed plates, Titertek 96-well polyvinyl chloride (PVC) Immuno-Assay-plates (Flow Laboratories, The Netherlands) and polystyrene Linbro/Titertek plates (Flow General Company McLean, Virginia USA). ELISA procedure was carried out as follows.

1. The antigens designated TTA and XM300 were diluted in PBS A, pH 7.2 and aliquots of 50 µℓ of a range of dilutions were added to the wells of either the PVC or polystyrene plates. Plates were incubated overnight at room temperature.
2. Plates were washed with phosphate buffer saline and Tween 20 (PBS-T, pH 7.2) three times. Fifty microlitres of a 1/100 dilution of reactive or non-reactive serum per well were added and incubated for 1 h at room temperature.
3. The plates were then washed three times with PBS-T. Fifty microlitres per well of rabbit anti-chicken IgG HRPO conjugate (Jackson, USA) was added and incubated for 1 h at room temperature.
4. After washing the plates three times with PBS-T, 100 µℓ per well, the ELISA substrate solution containing the chromogen ABTS [(2-2'-azino-di-(3-ethyl benthiazoline sulfonic acid)] was added and incubated further for 1 h at room temperature.
5. The absorbance values were measured using a Titertek spectrophotometer at the dual wavelengths of 414 and 492 nm. The reader was blanked with 100 µℓ per well of unreacted substrate solution.

Optimization of concentration of antigen, conjugate and different ELISA reagents

The principle of checkerboard titration (Voller, Bidwell & Bartlett 1979; Spencer & Burgess 1984) was applied in standardization of immunoreactants. Phosphate buffered saline with 0.0005 % of Tween 20 (PBS-T, pH 7.2) was used for washing unbound reagents from the plate. Conjugate and serum were diluted in 0.4 % casein in TEN-T [Tris Tris- (Hydroxymethyl aminonethane), EDTA (Ethylenediamine tetra-acetic Acid), NaCl and Tween 20] buffer (TEN-TC). Preparation of standard serum dilutions and determination of ELISA titres from the standard curve were performed as general methods described by Voller *et al.* (1979), Spencer & Burgess (1984), Lamichhane (1988) and Malcolm (1990).

Evaluation of indirect ELISA

An evaluation of ELISA was performed using sera collected from experimentally infected chickens (n = 142) and naturally infected chickens (n = 116). Serum from specific pathogen-free chickens (n = 4) as well as controls of known reactive (n = 11) and non-reactive sera (n = 12) were included in the evaluation of the assays. Haemagglutination inhibition was used as a reference test for the evaluation of the indirect ELISA. The micro-beta method of Allan & Gough (1974) was used for the HI test. Titres of > 8 in HI were considered to be reacting in the assay.

Analysis of the results

The results were analysed in two groups. The first group represented all serum samples collected from the field and control samples from the laboratory of James Cook University. The second group represented samples from experimentally infected chickens. The data comparing HI and the indirect ELISA were assessed by cross tabulating and the percentage of agreement was estimated. The correlation co-efficient between HI and indirect ELISA was calculated using the Statistic III statistical package (Statistic III, NH Analytical Software, USA).

RESULTS

Ultrafiltration prepared antigen

Infective virus and viral antigen detection

In the process of antigen preparation both filtrates and retentates were checked for the presence of soluble antigen (in ELISA) and infective virus (cell culture). The virus in the retentate from XM300 filter membrane was detected at up to eight ten-fold dilutions of retentate. Virus was also detected in some wells at the first dilution from the filtrate of XM300 membrane. No virus was detected in the filtrate from YM100 filter membrane after passage in

the allantoic cavity of 10-days-old chick embryos using HA test.

Optimization of concentration of antigen produced by ultrafiltration

The optimal dilution of 1:400 was selected for TTA antigen preparations. Dilutions of 1:1600 and 1:800 of the XM300 antigen were selected as the optimal dilutions for coating PVC and polystyrene plates, respectively. A minimum background signal for both antigen preparations was observed when 0.4 % of casein in TEN-T buffer was used and a conjugate dilution of 1:16 000 was used for PVC plates (Fig. 1). Final conjugate dilutions of 1:16 000 for PVC plates and 1:8 000 for polystyrene plates were found to result in minimal background signals when 0.4% of casein in TEN buffer was used as both conjugate and serum diluent (Table 1).

Serum samples

For serum samples collected before infection, the ELISA and HI agreed in detecting the samples as non-reactors. Larger differences between the two assays were observed for serum samples (n = 72) collected in the first and the second week after inoculation with V4 virus. A total of 21 (29.17 %) serum samples were detected as reactors in the ELISA and not in the HI test. There were no serum samples that were detected as reactors in the HI test and failed to react in the ELISA. In the third and fourth weeks after infection the two assays were in much closer agreement as high as 91.4% in detecting positive samples. For sera collected sequentially following experimental infection, an increase in titre with time was observed in both assays. Both tests detected the field serum samples of the uninfected control group as non-reactors. A total of 5.47 % (n = 37) of serum samples collected from naturally infected flocks and known reactive sera were not recognised as HI reactive sera, while in the indirect ELISA they were found to be reactive.

Analysis of all field serum samples indicated that the two assays correlate well (r = 0.942, n = 139). Overall analysis of the correlation co-efficient reveals comparatively less correlation (r = 0.631, n = 281) between the two assays.

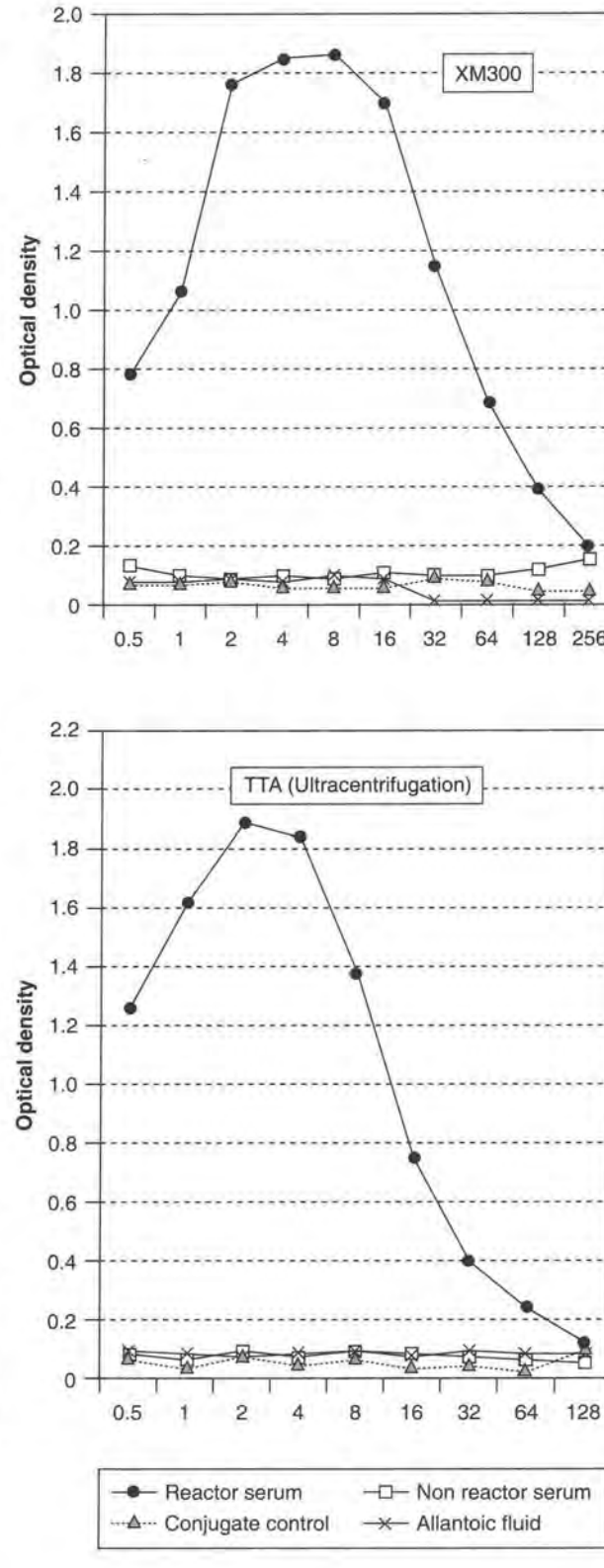

FIG. 1 Optical density reading of serial dilution of two different solid-phase antigens (XM300 & TTA), in indirect ELISA using selected reagents on PVC plates (X-axis = antigen dilution times 100)

DISCUSSION

Antigen preparation techniques normally require expensive equipment (e.g. ultracentrifugation) and require considerable operator skills. These tech-

TABLE 1 Optical density reading ± SD of controls for optimization of XM300 antigen

	TTA (1:400)*	XM300	
		PVC (1:1600)	POL (1:800)
Reactor serum	1.83 ± 0.05	1.73 ± 0.03	1.80 ± 0.04
Non-reactor serum	0.07 ± 0.04	0.09 ± 0.01	0.06 ± 0.03
Conjugate control	0.04 ± 0.02	0.06 ± 0.02	0.04 ± 0.03
Allantoic fluid	0.06 ± 0.03	0.04 ± 0.03	0.05

* The highest peak of antigen dilution selected

niques are not always suitable for developing countries. A method that is easy to perform using inexpensive equipment is required. In this method of antigen preparation infected allantoic fluid was forced through a membrane where the size of the pores was sufficient to retain the macromolecules. The concentration step therefore depends only on the diameter of the pores.

In each step of the ultrafiltration process the retentate was washed thrice with PBS to reduce the concentration of contaminating proteins. Each concentration step should result in the removal of 90 % of the contaminants and would have a theoretical purification factor of 99.9 % after three cycles.

This preparation has additional advantages over other methods of antigen preparation. There is no involvement of other detergents during the preparation of the antigen, which may interfere with the viability of the antigen. In this preparation there is no need for pH adjustment, addition of polyvalent cations or organic compounds which may have an effect on the reactivity of either the virus or its antigenic component.

The effectiveness of casein as a protein blocker has been widely investigated (Kenna, Major & Williams 1985). Lamichhane (1988) found that 0.2 % casein in TEN buffer effectively reduced the background signal when it is used as conjugate and serum diluent. It was found that 0.4 % of casein in TEN buffer significantly reduced the background signal. However, it may be necessary to standardize ELISA reagents for each type of antigen preparation.

This study demonstrated that very little intact NDV passed through an XM300 filter membrane. Reasons might be that either the virus is larger than the pores or the virus may exist in an aggregated form. This may be further explained by the observation that the haemagglutinin neuraminidase (HN) antigen with a molecular mass less than 300 000 (before treatment with detergent) did not readily pass through this filter.

In this ELISA a comparatively unpurified antigen (XM300) can be used and antibody will possibly be detected to a wider range of epitopes than would more highly purified antigen preparations. Higher optical density readings for the controls were observed when using the XM300 antigen compare to the semi-purified Triton X-100 treated antigen (Table 1). However, these differences were not significant and were within acceptable limits.

More than 81 % of field serum samples from naturally infected properties reacted in the two assays. Early detection of immunity to NDV, however, influences the management of disease control and the profiling of the immune status of the flocks. This study also shows that the ELISA can be used to assess immunity to NDV in early stages of infection and the results are consistent with the finding of other studies in chickens and ostriches (Miers *et al.* 1983; Snyder *et al.* 1983; Marquardt *et al.* 1985; Thayer *et al.* 1987; Brown *et al.* 1990; Reddy & Srinivasan 1992; Verwoerd *et al.* 1999; Williams *et al.* 1997; Koch *et al.* 1998).

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