DEVELOPMENT OF INDIRECT AVIAN INFLUENZA ELISA TEST USING MALAYSIA LOCAL ISOLATE H5N2

ONG G.H.[1], SURIANI M.N.[1], SYAMSIAH A.S.[1], KHAIRUL A.M.[1], RAMLAN M.[1], CHANDRAWATHANI P.[1] AND MAIZAN M.[2]

[1] Veterinary Research Institute, 59 Jalan Sultan Azlan Shah, 31400 Ipoh, Perak.

[2] Institute for Research in Molecular Medicine (INFORMM), Kampus Kesihatan, Universiti Sains Malaysia, Kubang Kerian, Kelantan.

Corresponding author: *Ongghuai@yahoo.com*

ABSTRACT. Serological test such as Hemagglutination Inhibition (HI) is used to detect Avian Influenza (AI) subtype specific antibodies. Agar gel precipitin test (AGPT) and Enzyme-Linked Immunosorbent Assay (ELISA) are used to detect AI group specific antibodies. These tests are used for AI poultry monitoring purposes. Indirect (AI) ELISA has been successfully developed in Veterinary Research Institute (VRI), Ipoh, Malaysia. Two types of antigens were prepared for this development to coat them onto the microtiter wells of MaxiSorb flat bottom plate (Nunc). They are from infected allantoic fluid (AF) of chicken embryonated eggs and infected tissue culture fluid (TCF) of Madin Darby Cannie Kidney cells using local isolate of H5N2 designated A/duck/Malaysia/8443/04. Conjugate of anti-chicken Horseradish Peroxidase and chromogen of o-Phenylenediamine (oPD) and Substrate 30% Hydrogen Peroxide were used. Results were read using ELISA reader of OD_{490} with reference OD_{630}.

Keywords: Avian Influenza (AI), ELISA, MDCK cells.

INTRODUCTION

Avian Influenza (AI) is a viral disease caused by type A Orthomyxovirus which only infect avian. Highly Pathogenic Avian Influenza (HPAI) is the most devasting viral disease of poultry caused by the virus strains of H5 and H7 subtypes. It is important to monitor the immune responses for controlling the disease spread.

All Influenza A viruses have similar nucleocapsid (NP) and matrix (M) antigens or proteins. ELISA, AGPT and HI tests are used to detect antibodies to these antigens. Not all birds develop demonstrable antibodies because hemagglutinin is subtyped specific. Therefore, ELISA test which is accepted as a screening tool by OIE was developed. Low Pathogenic Avian Influenza (LPAI) H5N2 designated A/duck/Malaysia/8443/04 was used as the coated antigen in this development to detect AI group specific antibodies against type A Influenza virus. ELISA test which is a much faster screening test which takes only approximately 5 hours to get the results compared to AGPT which takes 3

days. With ELISA test more samples can be screened in a shorter length of time.

MATERIALS AND METHODS

Antigens

Two types of antigen were prepared for the use in this development. Local isolate of H5N2 designated A/duck/Malaysia/8443/04 were grown in 9 to 10 days old Specific Pathogenic Free (SPF) chicken embryonated eggs and Madin-Darby Canine Kidney (MDCK) cells line. Infected Allantoic Fluid (AF) and tissue culture fluid (TCF) were harvested, inactivated using Binary ethylenimine (BEI) and purified by ultra centrifugation. Ultra centrifuged antigen was then solubilised with 1% Sodium Dodecyl Sulfate (SDS). Dilution of antigens to the amount of Hemagglutination unit (HA) were used in this case.They were then coated onto microtiter wells of MaxiSorb flat bottom plate (Nunc).

Reference Sera

Negative and positive control sera were prepared using inhouse bred specific pathogenic free (SPF) birds of White Leg Horn breed. Positive sera were prepared using the local isolate designated A/duck/Malaysia/8443/04. They were diluted twofold starting from 1/100 in diluting buffer for the optimization.

Conjugate and Substrate

Anti-chicken IgG Horseradish Peroxidase (HRPO) and o-Phenylenediamine (oPD) substrate from SIGMA were used. HRPO were diluted 2-fold starting from 1/2000 for the optimization. Chromogen oPD 2 mg with substrate of 30% H_2O_2 was used for the color development.

Enzyme-linked Immunosorbent Assay

Optimization on both types of antigens, positive and negative sera, anti-chicken IgG Horseradish Peroxidase (HRPO) were carried out to minimize the amount of non-specific binding to the antigen-coated wells and to obtain the best cut-off value. Polystyrene microtiter plates (NUNC) were coated overnight at 4°C and at 37°C 1 hour with 100 µl diluted antigens. Plates were washed with PBS Tween 20 for 3 times. Plates were then blocked at 37°C for 30 minutes with skim milk followed by washing. Diluted reference serum samples were added to each well and incubated for 1 hour at 37°C. After washing, Horseradish Peroxidase (HRPO) conjugated with rabbit anti-chicken IgG (SIGMA chemical) was diluted and added to the plates which were subsequently incubated for 1 hour and washed. 20 mg of the chromogen o-Phenylenediamine (oPD) and substrate 30% of hydrogen peroxide (H_2O_2) were added and incubated in darkness and optical density was read at OD_{490} with reference $OD_{630.}$

RESULTS

Among the two antigens used, only the infected tissue culture fluid showed good response to the expected optical density values towards both the negative and positive sera. Antigens from infected allantoic fluid did not show result of expected OD values in the negative sera although optimization had been done several times against the conjugate, infected AF and negative serum.

The best conditions were obtained when infected tissue culture fluid concentration was diluted to contain 4 HA incubated at 37°C 1 hour reacted to serum 1/100 and with conjugated HRPO diluted to 1/2400.

The positive reference serum diluted 1:100 using Infected tissue culture fluid had ELISA OD value 0.8645 and negative reference serum 0.1295 respectively. Test on specifivity and sensitivity against both the ELISA and AGPT are yet to be carried out on at least 500 chicken sera samples.

CONCLUSION

HI test is still the best choice to confirm the presence of specific subtype antibodies. ELISA test is an alternative test for AI group specific screening antibodies directed against the viral matrix and nucleoprotein antigens where they can perform test on more sera samples in a shorter time approximately 5 hours for rapid screening results compared to AGPT which takes 3 days.

REFERENCES

1. Aarthi D, Ananda Rao K, Robinson R and Srinivasan V.A. (2004). Validation of binary ethyleneimine (BEI) used as an inactivant for foot and mouth disease tissue culture vaccine. *Biologicals.* **32(3):**153-6
2. By passing eggs, flu vaccine grown in infects cells shows promise. *Virus Weekly.* http://www.newsrx.com.
3. Gough R.E. (2004). Diagnosis of Avian Influenza. *11th International Conference of theAssociation of Institution for Tropical Veterinary Medicine and 16th Vet. Asso. Malaysia Congress.* 144-146.
4. Habib Mudasser, Hussain Iflikhan, Irshad Hamid, Yang Zong-Zhao, Shuai Jiang-bing and Chen Ning. Imuunogenicity of formaldehyde and binary ethylenimine inactivated infectious bursal disease tissue culture vaccine. *Journal of Zhejiang University Science B.* ISSN 1673-1581.
5. Harder T.C. and Werner O. Avian Influenza. In Kamps B.S., Hoffmann C. and Preiser, *W Influenza Report, Influenza Report 2006.* http://www.influenzareport.com/ir/ai.htm
6. Highly Pathogenic Avian Influenza (Chapter 2.1.14). From *OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals.* 2004.
7. J.S. Malik Peiris, Menno D. de Jong and Yi Guan (2007). Avian Influenza Virus (H5N1): a threat to human health. *Clinical Microbiology Reviews,* **20 (2)**, 243-267.
8. Livestock and Poultry. December 2005. Newsletter.
9. Perez D.R., Sorrell E.M. and Donis R.O. (2005). Avian Influenza: an omnipresent pandemic threat. *The Pediatric Infectious Disease Journal.* **24 (11)**. S208-S216.
10. Thermo Scientific Inc. TPCK trypsin and immobilized TPCK trypsin. http://www.piercenet.com/Products/Browse.cfm?fldID=02040710
11. Yovil R., Su Q., Toner T.J., Szykowiak C., Kwan W.S., Rubin B., Petrukhin L., Kiselova I., Shaw A.R. and DiStefano D. Comparative study of influenza virus replication in vero and MDCK cell lines. *Journal of Virological Methods* **121 (1)**, 23-31.