Full Length Research Paper

Rapid detection of the avian influenza virus H5N1 subtype in Egypt

Shoman, S. A.[1]* and Khodier, M. H.[2]

[1]Microbiology Department, Faculty of Science, Ain Shams University, Abassia, Cairo, Egypt.
[2]Veterinary Serum and Vaccine Research Institute, Abassia, Cairo, Egypt.

Influenza A virus continue to cause widespread morbidity and mortality. The unprecedented spread of highly pathogenic avian influenza virus subtype H5N1 in Egypt is threatening poultry and public health systems. Effective diagnosis and control management are needed to control the disease. To this end, polyclonal antibodies (PAbs) were developed against the H5N1 avian influenza virus (AIV) and implemented an enzyme-linked immunosorbent assay (ELISA) to detect the H5 viral antigen. A group of rabbits was immunized with viral vaccine (H5N1) therefore, purified antibodies from rabbit serum, which secrete immunoglobulin G (IgG) was served as the detector antibody after conjugation with horse radish peroxidase and fluorescent isothiocyanate (FITC). The reactivity of the obtained peroxidase and fluorescent conjugated PAb of influenza virus revealed that they are specifically recognized H5N1 virus antigen. Specimens containing AIV subtypes collected from different Governments in Egypt yielded specific and strong signals with hemagglutination test. The detection limit of ELISA using the prepared peroxidae conjugated PAbs was 1:100000, while using fluorescent conjugated PAb was 1:10000. Reconstituted clinical samples consisting of H5 AIVs mixed with pharyngeal-tracheal mucus from healthy chickens also yielded positive signals in ELISA, and the results were confirmed using reference virus antigens. This investigation enhanced the usage of these PAbs in the surveillance and diagnosis of H5N1 AIV in Egypt.

Key words: Avian influenza virus, H5N1, fluorescent antibody enzyme-linked immunosorbent assay (ELISA) technique, polyclonal antibodies.

INTRODUCTION

Highly pathogenic avian influenza (HPAI) viruses have emerged in poultry and wildlife worldwide, causing sporadic but serious and devastating outbreaks. In Egypt, the

*Corresponding author. E-mail: karimbahaa2005@yahoo.com.

Abbreviations: HPAI, Highly pathogenic avian influenza; **MDCK,** Madin-Darby Canine kidney; **HA,** haemagglutinin; **RT-PCR,** reverse transcription polymerase chain reaction; **AIV,** avian influenza virus; **FLU OIA,** flu optical immunoassay; **PAbs,** polyclonal antibodies; **ELISA,** enzyme-linked immunosorbent assay; **FAT,** fluorescent antibody technique; **HI,** haemagglutination inhibition; **Abs,** antibodies; **HRP,** horse radish peroxidase; **OPD,** orthophenyldiamine.

Egyptian cabinet Information and Decision Support Center (IDSC) published in the issue no. 15 (2008) that Egypt was the third most affected country by the disease, where 12.7% of the total infections and 8.8% of the total death worldwide, that is, infection to mortality rate was 43.8% since the emergence of the disease in Egypt till April 8, 2008. The World Health Organization announced the death of 33 cases out of 60 in 2010 and there were 39 H5N1 confirmed cases in 2011 and the death of two men from H5N1 avian influenza in January, 2012 (WHO-Global influenza surveillance network, 12 April, 2012).

These viruses have been restricted to haemagglutinin (HA) subtypes H5 and H7, although not all viruses of these subtypes are highly pathogenic. An outbreak of

H5N1 HPAI in the live bird markets of Hong Kong in 1997 resulted in 18 human infections, 6 of them fatal (Sims, 2003). Similar H5N1 HPAI viruses have reemerged in several countries in Asia since 2001 and have continued to spread through Asia and into the Middle East and Eastern Europe (Swayne and Suarez, 2001). In the United State, it has been estimated that influenza A viruses are associated with approximately 31,000 death annually (Halvorson et al., 1998).

In addition to their geographic spread, H5N1 HPAI viruses were found in multiple animal species, such as poultry, wild birds, tigers, and leopards (Suss et al., 1994; OIE, 2007). Besides these devastating consequences for animal health, H5N1 infections have resulted in 256 laboratories confirmed infected people, including 167 deaths (Subbarao et al., 1998). Human infections are generally the result of exposure to H5N1-infected poultry; so, reducing the prevalence of H5N1 in poultry would have a favorable impact on public health.

The accurate and prompt diagnosis of H5N1 infection in birds is a critical component of a disease control plan. Currently, virus isolation in embryonated eggs or in Madin-Darby Canine kidney (MDCK) cells and subsequent HA and neuraminidase subtyping by serological methods constitute the standard for avian influenza virus (AIV) detection and serological classification (Swayne et al., 1998). However, conventional culture methods require special collection and transport conditions to ensure virus viability, and the recovery of the results may take 1 to 2 weeks, by which time the results may no longer be relevant (Wanasawaeng et al., 2008).

Molecular detection methods, such as standard reverse transcription polymerase chain reaction (RT-PCR), have previously been applied for the diagnosis of AIV infections and HA subtype identification (Munch et al., 2001; Suarez et al., 2007). Additionally, real-time PCR assays and a DNA microarray analysis for the detection of influenza virus have been developed (Li et al., 2001; Aguero et al., 2007). However, these methods are technically demanding, and false-positive results may arise from cross contaminations between samples. Antigen detection methods have repeatedly shown their value in the diagnosis of various infectious diseases. The currently available antigen detection methods, such as the flu optical immunoassay (FLU OIA) test (Biostar) and the Directigen FLU A kit (BD, Biosciences) (Easterday et al., 1997; Wang et al., 2000), are based on the detection of the viral nucleoprotein, which is conserved in all influenza A viruses and which is therefore not specific for the H5 subtype influenza virus. Detection of the H5 antigen would provide strong evidence of AIV subtype H5 infection (Collins et al., 2002). This study describes the preparation and characterization of polyclonal antibodies (PAbs) against HA and the development of an enzyme-linked immunosorbent assay (ELISA) and fluorescent antibody technique (FAT) for the detection of AIV H5 in reconstituted clinical Egyptian samples. The specificity and sensitivity of the assay are evaluated.

MATERIALS AND METHODS

Viruses

Avian influenza vaccine

Inactivated avian influenza virus (H5N1) vaccine was supplied by Veterinary Serum and Vaccine Research Institute and used for rabbit immunization to prepare a specific polyclonal antiserum.

Standard antigen and antiserum

Avian influenza virus antigens (A/Ck/Scot/59/H5N1) and (A/OSt/Den/72420/96/H5N2) and their specific polyclonal antibodies were supplied by Veterinary Laboratories Agency-Weybridge and used as positive controls in ELIZA, haemagglutination inhibition (HI), and FAT.

Rabbits

Ten healthy boscat rabbits of about 3 kg body weight were used for preparation of avian influenza virus (H5N1) polyclonal antiserum. Another five rabbits were kept as control without immunization.

Rabbit immunization

Each rabbit received 2 ml of avian influenza (AI) vaccine injected subcutaneously (S/C) in different sites of the body and repeated 5 times/ week intervals using incomplete Freund's adjuvant with the first injection and complete Freund adjuvant with the last injection according to Benedict (1967). After another week, immunized rabbits were slaughtered and their blood was aseptically collected for serum separation.

Titration of AI H5N1 antibodies in the prepared antisera

Antibodies (Abs) were titrated for haemagglutination inhibition (HI) activity in a 96-well microtiter system. A/Ck/Scot/59(H5N1) subtype of AIV was used as the test antigens. Eight haemagglutainating (HA) units of viral antigen (final concentration 4HAU) were preincubated with serial twofold dilutions of Abs for 30 min at room temperature (RT) before the addition of 0.5% chicken red blood cells in phosphate buffered saline (PBS). The highest Ab dilution that completely inhibited haemagglutination was used as the antibody titer.

Preparation and estimation of the immunoglobulin from the prepared antiserum

A commercially available kit of Biomerieux Laboratory Reagent and products, Maroyl, France was used for this purpose, after precipitation of the immunoglobulins using ammonium sulphate solution according to Narin and Marrack (1964). The globulin concentration were detected and adjusted to be 20 mg/ml in normal saline.

Chemicals

Chemical reagents used for conjugation of the prepared AI H5N1 IgG with horse radish peroxidase (HRP): HRP product No. 8375 type VI lot 25C. 9510 was supplied by Sigma chemical company. It had an activity of 365 purogallin unit/μg. Sodium borohydride (NaBH4) was supplied by S. D Fine Chemical Limited Company,

Table 1. List of collected samples.

Locality	Chicken		Duck	
	In contact	Infected	In contact	Infected
Sharqueya,	10	35	3	-
Qalubia	5	20	7	-
Kafr Elsheikh	15	50	6	-

Chemical Manufacturing Division Fair, Lawa, New Jersey. Sodium periodate ($NaIO_4$) was supplied by Sigma Chemical Company.

Chemical reagents used for conjugation of the prepared AI H5N1 IgG with fluorescein isothiocyanate ($C_4H_{11}NO_5S$) E were supplied by Merk, Department of Microscopy, (M. Gew. 389.39).

Conjugation of the prepared AI-H5N1 IgG

With horse radish peroxidase

It was carried out according to Tijssen and Kurstak (1984).

With fluorescein isothiocyanate

It was carried out according to the method described by Narin (1969).

Field samples

Infected chickens with AIV and in contact chickens and ducks in Sharqueya, Qalubia, and Kafr Elsheikh Governments in Egypt were subjected to the present investigation. The collected samples included trachea, lungs, spleen, and brain of dead and slaughtered apparently healthy in contact birds (Table 1).

Preparation of the collected samples

Pooled samples from different organs from dead birds were placed in isotonic solution phosphate buffer saline pH 7.0 with antibiotic solution penicillin (2000 U/ml), streptomycin (2 mg/ml), gentamycin (50 μg/ml). These samples were homogenized mechanically to prepare 1/10 suspension in minimum essential medium plus antibiotics. Quantification of virus samples was achieved through haemagglutination (HA) test according to Allan et al. (1978) which provide a relative measure of antigen concentration.

ELISA and direct fluorescent antibody technique

Clarified field samples collected from AIV infected chickens and ducks were subjected to ELISA technique using antibodies in the prepared IgG conjugated with HRP antibodies according to Kemeny (1991) to detect the H5N1 AIV.

Basically in this test, the antigen was diluted in carbonate bicarbonate buffer, pH 9.6, and the serum to be tested was diluted in PBS with 0.05% tween-20 (PBS-T) with 5% of powder milk. Rabbit anti-H5N1 IgG conjugated with horse radish peroxidase was used diluted in PBS-T plus 5% of powder milk. The substrate used was orthophenyldiamine (OPD) and as stopper, a solution of 1NH2SO4 was used. Flat-bottomed microplates (Linbro, Flow laboratories, USA) were used throughout and read at optical density of 492 nm in a microplate reader (Titertek Multiskan, Flow laboratories).

The same field samples were subjected to the direct fluorescent antibody technique (FAT) using fluorescent conjugated-antibody and the slide glasses covered by the sample. They were washed with III PBS solution (pH 7.1), dried at room temperature and fixed in acetone at room temperature for 5 to 10 min. The fixed antigen was overlaid with the prepared antibodies labeled with fluorescent dye (fluorescent anit-H5N1 antibodies) which were diluted to 20 times in a physiological saline solution and absorbed in a moist chamber at room temperature for 30 min. They were washed three times with PBS for 5 min every time and mounted with 10 % PBS solution in glycerin. The specificity of the fluorescent staining was checked in the non-infected samples and in the infected samples treated with normal rabbit serum. The samples prepared stated above were examined under a fluorescent microscope, type ML-2 made in USSR, equipped with a dark field condenser.

RESULTS

In this study, different samples from different governments in Egypt (Sharqueya, Qalubia, and Kafr Elsheikh) were collected as infected samples and others as contact samples. Most samples were taken from dead chicken as a main reservoir of AIV (Table 1). Ducks, one of the avian species that was at last consider an impressive reservoir of AIV so; samples from ducks were implemented in this study.

Preliminary detection of AI viral antigen in the collected pooled samples was performed using haemagglutination activity. It was noted that the infected samples had appreciable HA) with 100% in Sharqueya samples, with 90% in Qalubia samples, and 96% in Kafr Elsheikh samples. While, in contact samples of chicken and ducks showed few positive HA as presented in Table 2.

Using H5N1 reference antiserum, the HA positive samples of the three governments (Sharqueya, Qalubia, and Kafr Elsheikh) chicken and ducks were subjected to HI technique. The antigen–antibody reaction revealed to present of 81% of the previously HA positive Sharqueya samples, containing virus. In Qalubia, and Kafr Elsheikh the percentage of viral detection in the previous positive HA samples were 76 and 75% (Table 3). Samples of in contact ducks showed no viral antigen except in Kafr Elsheikh samples, one of two samples give positive reaction.

According to the immunization protocol, the immunized rabbit's sera were investigated to determine the total protein, albumin and globulin. Table 4 shows the values of these protein concentrations (g/dl) in the immunized rabbits sera that are higher than normal ones.

Following up the immunization process, The H5N1

Table 2. Haemagglutination (HA) activity in pooled collected sample.

Locality	Chicken			Duck		
	Total sample	+ve sample	+ve %	Total sample	+ve sample	+ve %
Sharqueya	35- infected	35	100	-		
	10-incontact	2	20	3-in contact	0	0
Qalubia	20- infected	18	90	-		
	5-incontact	3	60	7-in contact	1	14
Kafr Elsheikh	50- infected	48	96			
	15-incontact	5	33	6-in contact	2	33

Table 3. Detection of AI- H5 N1 antigen using reference antiserum.

Locality	Chicken			Ducks		
	Total sample	+ve sample	+ve %	Total sample	+ve sample	+ve %
Sharqueya	37	30	81	0	0	0
Qalubia	21	16	76	1	0	0
Kafr Elsheikh	53	40	75	2	1	50

Table 4. Serum proteins in AI- H5 N1 immunized rabbits.

Tested serum	Total protein (g/dl)	Albumin (g/dl)	Globulin (g/dl)
Rabbit AI- H5 N1antiserum	5.8	2.5	3.3
Normal rabbit serum	4.6	2.0	2.6

Table 5. AI-H5N1 antibody in the serum of immunized rabbits using H1 and indirect ELISA.

Applied test	Pre-immunization	AI-H5N1 antibody titer/ weeks post immunization					
		1W	2W	3W	4W	5W	At slaughter
HI	>2	4	8	32	128	256	512
Indirect ELISA	0.27	0.50	0.750	0.91	1.03	1.09	1.154

antibody titration were detected weekly post immunization using HI and indirect ELISA. Table 5 indicates gradual increase in antibody titers. HI titer at the first week was 4 $\log_2$ and at the end of immunization (5 W) it was 256 log $_2$. EIISA was used to confirm the resulted data of HI where, OD of reaction increased in the same pattern (Table 5).

The antibodies were chemically conjugated with HRP and FITC to be used as diagnostic antibodies. The titer of H5N1 IgG conjugated antibodies was determined against reference antigen of H5N1 antigen. Table 6 shows that the HRP conjugated antibodies are sensitive as virus neutralization agent even to 1:100000 dilutions, while the FITC conjugated antibodies gave positive reaction with H5N1 as well as H5N2 reference antigens at dilution 1:10000 (Table 7).

Specificity of the prepared conjugated antibodies for detection of AIV in the local viral infected samples was carried out using direct ELISA and FAT. It showed that

the HRP conjugated antibodies were specific binding with AIV antigen in Sharqueya samples with 93%, In Qalubia with 93%, and in Kafr Elsheikh with 87% in chicken and 100% in ducks (Table 8). On the other hand, the FITC conjugated antibodies detected the AIV in the collected samples with 100% in Sharqueya samples, 90%. In Qalubia samples, and in Kafr Elsheikh samples, 97% with chicken and 100% in ducks (Table 9). Both poaitive and negative FAT are shown in Figure 1.

DISCUSSION

The main goal of the present study is to prepare a specific standardized AI antiserum conjugated with enzymes and/or fluorescence to be used as diagnostic agent to help obtain rapid and confirmed results especially in emergency cases in Egypt. The presence of such material locally could save the time and cost where, it is usually unavailable and supplied in small amount of high

Table 6. Titer of the prepared anti AIV H5N1 IgG conjugated with HRP using OPD.

Test dilution	Optical Density (OD)	
	Positive result	Negative result
1:100	1.054	0.151
1:1000	1.038	0.144
1:10000	0.978	0.134
1:100000	0.832	0.131

Table 7. Titer of the prepared anti AIV H5N1 IgG conjugated with FITC using the reference antigens.

Tested antigen	Tested dilution				
	1:10	1:100	1:1000	1:10000	1:100000
H5N1 H5N2	4+	4+	3+	2+	-

Table 8. Detection of AIV H5N1 antigen in HA positive collected samples using the prepared IgG conjugated with HRP in direct ELISA.

Locality	Chicken			Ducks		
	Total sample	+ve sample	+ve %	Total sample	+ve sample	+ve %
Sharqueya	30	28	93	0	0	0
Qalubia	16	15	93	0	0	0
Kafr Elsheikh	40	35	87	1	1	100

+ve = positive

Table 9. Detection of AIV H5N1 antigen in HA positive collected samples using the prepared IgG fluorescent conjugated-antibody in the direct FAT.

Locality	Chicken			Ducks		
	Total sample	+ve sample	+ve %	Total sample	+ve sample	+ve %
Sharqueya	30	30	100	0	0	0
Qalubia	10	9	90	0	0	0
Kafr Elsheikh	40	39	97	1	1	100

+ve = positive

price. Laboratory identification of AIV is commonly carried out by direct antigen detection, isolation in cell culture, or detection of influenza RNA (Lee et al., 2001) when carried out following standard biosafety guidelines (Richmond et al., 1993). The standard method for detection of the influenza viruses is the HI test with reference antiserum to the known HA subtypes (Swayne et al., 1998). For the HI test, preparation of antiserum of good quality is essential. The polyclonal antibodies have the potential to attach to several antigenic sites on the protein, which should reduce the likelihood of a false-negative reaction by looking at only a single antigenic epitope. Polyclonal antibodies are also used more typically as immobilized capture reagents since their heterogeneity provides less potential for the

immobilization to impact the antigen binding efficiency (Tumpey et al., 2005).

In this investigation, the polyclonal antibodies were stimulated in injected rabbits. In inoculated rabbits, the total protein increased than normal rabbits which could be attributed to the formation of anti species antibodies which mainly formed globulins. Following titer of H5N1 antibodies in inoculated rabbits, it was found that these antibodies began to appear from the first week post inoculation to reach a peak titer by the fifth week as measured by HI and indirect ELISA, which indicate the high immunologically responses (Kataria and Sharma, 1993).

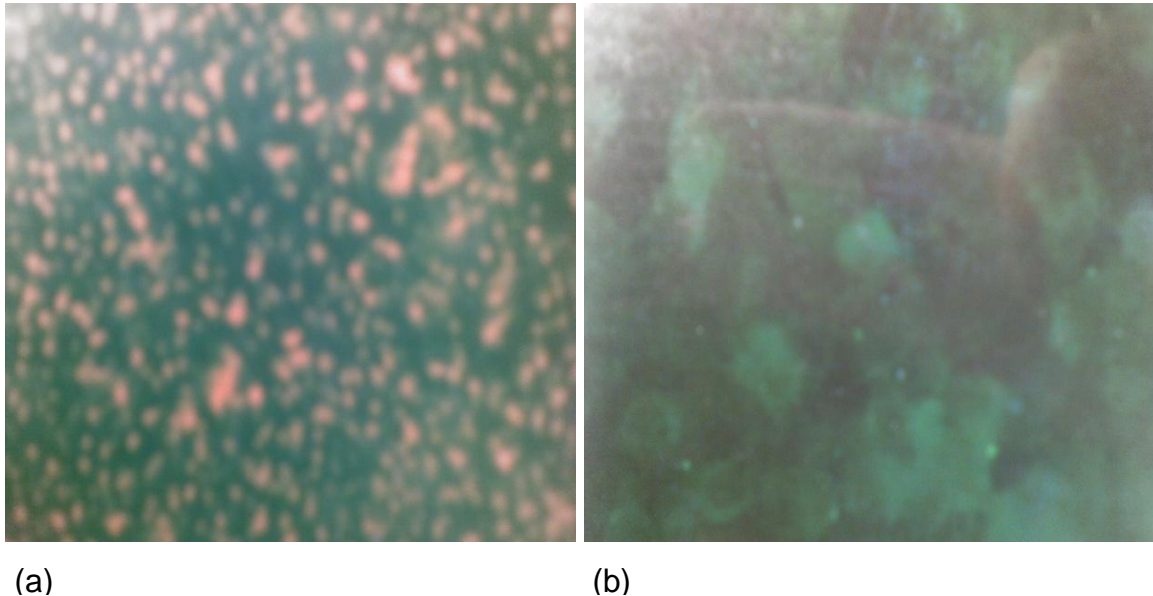The emergence of avian H5N1 virus in humans prompted

(a)   (b)

Figure 1. (**a**) Positive fluorescent antibody reaction (**b**) negative fluorescent antibody reaction.

a series of seroepidemiological studies. The FAT assay and ELISA were the most sensitive and specific assays for detecting avian influenza A virus (Shafer et al., 1998; OIE, 2008). In comparison, the traditional HI assay detected only H5-specific antibody in sera with high neutralizing-antibody titers and did not detect the relatively low serum antibody titers that developed in many individuals following primary infection with avian H5N1 virus (Lu et al., 1982).

To optimize the utility of the prepared antibodies in different serological applications in diagnostic laboratory, it must be conjugated. The use of FITC labeled antibody helps in such rapid diagnosis (Brian and Hillor, 1996) where, fluorescent antibody technique has gained a wide acceptance in virology.

ELISA also showed greater sensitivity; the use of specific antiserum against viral antigens offers great possibilities of the attainment of more accurate information by diagnostic virological serology (Hornsleth et al., 1981; He et al., 2007).

Specificity of the both conjugated prepared antibodies was tested against standard antigen (H5N1) and the results indicate that the H5N1 antiserum is highly specific and valuable experimental tool in detecting virus in the infected samples. Moreover, their titrations were enough for detection and identification, especially in HRP-conjugate antibodies.

Testing samples collected from three different governments (Sharqueya, Qalubia, and Kafr Elsheikh) using conjugated antibodies revealed that these locally conjugated antibodies were successfully prepared and its effectiveness were determined showing clear positive results with H5N1 antigen. The FITC Also have strong reactivity against H5 antigen (H5N1 and H5N2) and both conjugated antibodies (FITC and HRP) have the same potential for detecting viral antigens in chicken or duck samples infected or in contact.

Continuous outbreaks of the highly pathogenic H5N1 avian influenza A in Asia has resulted in an urgent effort to improve current diagnostics to aid containment of the virus and lower the threat of a influenza pandemic. A one-step reverse-transcription PCR assay was developed to detect the H5N1 avian influenza A virus. The specificity of the assay was shown by testing sub-types of influenza A virus and other viral and bacterial pathogens, and on field samples.

The Asian H5N1 highly pathogenic avian influenza (HPAI) virus causes a systemic disease with high mortality of poultry and is potentially zoonotic. The virus was also detected in tissues by real-time reverse transcription–polymerase chain reaction (RRT-PCR) and virus isolation, and in the trachea by RRT-PCR and a commercial AI viral antigen detection test (Lisa et al., 2006; Hagag et al., 2012). The availability of these antibodies of avian influenza virus as local preparation is very clinically important for laboratories dealing with influenza A virus in Egypt where, it save time and cost exhausted for importing of such antisera, it was successfully used for the specific diagnosis of H5N1 infections in submitted clinical cases and also in an ongoing surveillance program for avian influenza.

REFERENCES

Allan WI, Lancasker JE, Toth B (1978). Newcastle disease vaccines. Their production and use. FAO animal production and health series No. 10. FAO, Rone.
Aguero M, San Miguel E, Sanchez A, Gomez-Tejedor C, Jimenez-Clavero MA (2007). A fully automated procedure for the high-

throughout detection of avain influenza virus by real-time reverse transcription-polymerase chain reaction. Avian Dis. 51:235-241.

Benedict A (1967). Methods in immunology and immunochemistry Ed. Williams and M. R. Chase, p 229, Academic press, London and New York.

Brian WJ, Hillor O (1996). Virology methods manual P 318. Academic Press, Harcourt Brace and company publishers London, San Diego, New York, Sydney, Tokyo.

Collins RN, Ko LS, Ellis T, Lau LT, Yu ACH (2002). Detection of highly pathogenic and low pathogenic avian influenza subtype H5 (Eurasian lineage) using NASBA. J. Virol. Methods 103:213-225.

Easterday BC, Hinshaw VS, Halvorson DA (1997). Influenza. In: Diseases of Poultry, 10th ed, by Calnek, BW, Barnes HJ, Beard CW, McDougald LR,, and. Saif YM, Iowa State University Press, Ames. pp. 583-605.

Hagag NM, Arafa A, Shalaby MA, El sanousi AA, Aly MM (2012). Comparative detection of H5N1 avian influenza virus using convention and Real time Rt-PCR. Int. J. Virol. 8(2):203-013.

Halvorson DA, Frame DD, Friendshuh AJ Shaw DP (1998). Outbreaks of low pathogenicity avian influenza in USA. Proceeding of the fourth international Symposium on avian influenza, Athens, Georgia, USA. Swayne DE & Slemons RD., eds. US Anim. health assoc. pp. 36-46.

He QS, Velumani S1, Du Q1, Lim CW, Kheong FN, Donis R, Kwang 1J (2007). Detection of H5 Avian Influenza Viruses by Antigen-Capture Enzyme-Linked Immunosorbent Assay Using H5-Specific Monoclonantibody Clin. Vaccine Immunol. 14(5):617-623.

Hornsleth A, Grauballer PC, Friis B, Genner J, Pedersen, IR (1981). Production of antiserum to respiratory syncytial virus polypeptides: Application in Enzyme –Linked Immunosorbent Assay. J. Clin. Microbiol. Nov. pp. 501-509.

Kataria AK, Sharma KN (1993). Serum electrophoretic studies in sheep vaccinated with live sheep pox virus (Rumaniam strain) Ind. Vet. J. 70(2):191-192.

Kemeny DM (1991). A practical guide to ELISA. Bergman Press Oxford 1st ED.

Lee SP, Chang J, Shien M, Cheng, Shieh H (2001). Identification and subtyping of avian influenza viruses by reverse transcription-PCR. J. Virol. Methods 97:13-22.

Li J, Chen S, Evans DH (2001). Typing and subtyping influenza virus using DNA microarrays and multiplex reverse transctiptase PCR. J. Clin. Microbiol. 39:696-704.

Lisa FP Ng, Barr I, Nguyen T, Noor SM, Rosemary S, Tan, P, Agathe LV, Gupta S, Khalil H, Long TT, Syed SH, Ren EC (2006). Specific detection of H5N1 avian influenza A virus in field specimens by a one-step RT-PCR assay Infect. Dis. 6:40

Lu B, Webster RG, Hinshaw VS (1982). Failure to detect hemagglutination-inhibiting antibodies with intact avian influenza virions. Infect. Immun. 38:530–535.

Munch M, Nielsen L, Handberg K, Jorgensen P (2001). Detection and subtyping (H5 and H7) of avian type A influenza virus by reverse transcription PCR and PCR ELISA. Arch. Virol., 146:87-97.

Narin RC (1969). Fluorescent protein tracing 3rd ed., Edinburgh and London Livingstone.

Narin RC, Marrack JR (1964). Fluorescent protein tracing 2nd ed., Livingstone, Edinburgh and London.

Richmond JY, McKinney RW (1993). Biosafety in microbiological and biomedical laboratories, 3rd ed. U.S. Department of Health and Human Services, CDC/NIH. Atlanta, Georgia.

Shafer AL, Katz JB, Eearnisse KA (1998). Development and validation of a competitive enzyme-linked immunosorbent assay for detection of type A influenza antibodies in avian sera. Avian Dis. 42:28-34.

Sims LD (2003). Avian influenza in Hong Kong. Proceeding of the fifth international Symposium on avian influenza. Athens, Georgia, USA, Avian Dis. 47:832-838.

Subbarao K, Klimov A, Katz J, Regenery H, Lim W, Hall H, Perdue M, Swayne D, Bender C, Huang J, Hemphill M, Rowe T, Shaw M, Xu X, Fukuda K, Cox N (1998). Characterization of an avian influenza A (H5N1) virus isolated from a child with a fatal respiratory illness. Science 279:393-396.

Suss J, Schafer J, Sinnecker H, Webster RG (1994). Influenza virus subtypes in aquatic birds of eastern Germany. Arch. Virol. 135:101-114.

Swayne DE, Senne DA, Beard CW (1998). Influenza. In: isolation and identification of avian pathogens, Fourth Edition, Swayne, D. E., Gilsson, J. R., Jackwood, M. W., Pearson, J. E. and Read W. M., eds. American Association of Avian Pathologists, Kennett Square, Pennsylvania, USA. pp. 150-155.

Swayne DE, Suarez DL (2001). Avian influenza in Europe, Asia and central America during 2001. In: Proceeding of the 104th annual meeting of the US Animal Healyh Association, USAHA, Richmond, Virgina, USA, pp. 465-470.

Suarez DL, Das A, Ellis E (2007). Review of rapid molecular diagnostic tools for avian influenza virus. Avian Dis. 51:201-208.

Tijssen P, Kurstak E (1984). Progress in enzyme immunoassays: production of reagents, experimental design, and interpretation Bull World Health Organ. 1985 63(4):793-811.

Tumpey TM, Alvarez R, Swayne DE, Suarez DL (2005). A diagnostic aid for differentiating infected from vaccinated poultry based on antibodies to the nonstructural (NSI) protein of influenza A virus. J. Clin. Microbiol. 43:676-683.

Wanasawaeng W, BunpapongN, Thanawongnuwech R (2008). Growth Characteristics of the Thai H5N1 Avian Influenza Virus in Chicken Embryonic Eggs and MDCK Cells Proceedings, The 15th Congress of FAVA 27-30 October FAVA - OIE Joint Symposium on Emerging Diseases Bangkok, Thailand. p. 95.

Wang X, Castro AE, Castro MD, Lu H, Weinstock D, Soyster N, Scheuchenzuber W, Perdue M (2000). Production and evaluation criteria of specific monoclonal antibodies to the haemagglutinin of the H7N2 subtype of avian influenza virus. J. Vet. Diagn. Invest. 12(6):503-509.

World Organization for Animal Health (OIE) (2007). Chapter 2.7.12 Avian influenza. In: Terrestrial Animal Health code, Sixteenth Edition. OIE, Paris, France. pp. 279-285.

World Organization for Animal Health (OIE) (2008). Chapter 2.3.4 Avian influenza. In: OIE Terrestrial Manual. Seventeenth Edition. OIE, Paris, France pp. 465-481.