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Immunological Detection of Avian Influenza Virus in Infected Ducks by Monoclonal Antibodies Against AIV-H5N1

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In order to establish a detection method for avian influenza virus (AIV) infection in ducks, monoclonal antibodies (MAbs) against the virus were produced. The virus used for the production of the monoclonal antibodies was AIV-H5N1 of Indonesian origin. Immortal mouse myeloma were fused with the lymphocytes derived from the spleen of mice immunized with the virus. The MAbs were tested for their specificity by enzyme linked immunosorbent assay (ELISA) and western blotting using formaldehyde inactivated virus and normal allantoic fluid as a negative control. Twelve MAbs which were specific against AIV were isolated and 8 of them were used for detecting of AIV antigen in duck's tissues. AIV antigen was detected in paraffin embedded tissues of AIV-infected ducks by immunohistochemistry using MAbs. AIV antigen was not detected in ducks, which were confirmed to be AIV negative. In the infected ducks, high intensity of AIV infection was detected in proventricle gland and small intestine. The AIV antigen with a lesser intensity was also detected in lungs, spleen, and bursa of Fabricius, but hardly detected in muscle, brain, and several other issues. This study shows a clear evidence that MAbs produced in this study are applicable for use in immunological detection of AIV in infected duck tissues.

Key words: avian influenza, H5N1, monoclonal antibodies, ducks, virus immunohistochemistry

The widespread outbreaks of avian influenza (AI) in South-East Asia in the recent years have led to the death of millions of domesticated birds and millions others have to be sacrificed in an effort to eradicate the disease (Swayne and Halvorson 2003; Stegeman and Bouma 2004). During this outbreak, many affected countries suffer a great deal of economic losses brought out by the collapse of their poultry industries (Perkins and Swayne 2002; Perkins and Swayne 2003; Lewis 2006). More importantly, the disease also affects human causing a great concern among health authorities in the world. The availabilities of accurate, simple, safe, and fast diagnostic methods are important for an effort to prevent and to control a future outbreak of AI in both animals and man. Most diagnostic methods developed in the recent years still require expensive facilities and reagents are slow to perform, lack of sensitivity and specificity, unsafe to perform, and unable to determine the virus subtype directly (Gough 2004).

Avian influenza viruses (AIVs) are a group of viruses with great genetic and antigenic diversities in nature. On the basis the antigenic characteristics of their two surface glycoproteins, haemagglitinin (HA) and neuraminidase (NA), AIVs are grouped into many subtypes. As many as 16 HA subtypes that can combine with 9 NA subtypes have been identified (Foucier *et al.* 2005). Such antigenic diversities have often caused a great difficulty in establishing an appropriate test for an accurate detection of AIV subtypes (Fouchier *et al*. 2005; Kida dan Sakoda 2006). MAbs which react only with a single epitope on an antigenic structure have been widely used to detect the viral antigen in the infected hosts and also to differentiate closely related viruses (Zheng *et al.* 2001; Vareckova *et al.* 2002; Ohnishi *et al.* 2005). In human influenza virus, for instance, the use of MAbs against the HA protein of the virus is reported to have 100% sensitivity and 99.1% specificity in determining the HA subtype of the virus (Vareckova *et al.* 2002). As in human influenza viruses, MAbs against AIV is very likely to have a similar degree of sensitivity and specificity when used in detecting of AIV antigen in the infected hosts including in determining the virus subtype.

Among many different hosts infected by AIV, aquatic birds including ducks are suggested to play an important role in the epidemiology of AI. Aquatic birds can serve as an important reservoir for low pathogenic avian influenza (LPAI) viruses (Alexander 2000). Evidences have shown that these LPAI viruses can mutate easily into highly pathogenic avian influenza (HPAI) virus in water fowls, especially if they carry AIV with H5 or H7 subtype (Banks and Plowright 2003). In addition, there are also evidences that many outbreaks of AI in susceptible commercial flocks originate from AIV-carrier waterfowls brought into live bird markets (Bulaga *et al.* 2003; Swayne and Halvorson 2003; Stegeman and Bouma 2004). In the carrier birds, the virus replicates mainly in the intestine of infected waterfowls, usually without showing a clear symptom. A large quantities of virus is usually excreted via feces into water, perpetuating the natural cycle of AIV infection (Stegeman and Bouma 2004). The detection of AIV infection in waterfowls such as ducks is therefore important both as confirmative diagnosis of clinically affected ducks and for monitoring of ducks subclinically carrying the virus. In our laboratory, several ducks with a severe clinical disease have been confirmed to be due to AIV infection (data not shown). The ducks were confirmed as AI positives by isolation of the virus in embryonated chicken eggs, identification of the virus by haemagglutination/ haemagglutination inhibition (HA/HI) test, and detection of viral nucleic acid by reverse transcriptase-polymerase chain reaction (RT-PCR). The availability of MAbs against

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of AIV-H5N1 is very likely to provide a relatively much simpler, quicker, cheaper, and safer diagnostic methods for the detection of AIV antigen in ducks. We have currently been able to produce MAbs against AIV-H5N1 of Indonesian isolate and the applicability of those MAbs for detecting AIV antigen in duck tissues was examined.

MATERIALS AND METHODS

Cells. Myeloma cells (P3-NS1/1-Ag4.1), used for the preparation of hybridomas were obtained from Murdoch University, Australia. The cells were grown in Dubelco's modified essential medium with 10% newborn calf serum (NBCS) and antibiotics penicillin, 200 IU ml^{-1}, streptomycin 200 $\mu g\ ml^{-1}$.

Virus. Formaldehyde inactivated AIV-H5N1 used in this study was an Indonesian isolate. The virus was isolated in 2005 from chicken with a severe clinical disease and the virus isolate was then designated as A/CK/Bali/2005. The virus was propagated in 10 days-old chicken embryonated eggs and harvested from allantoic fluids. The titer of the virus was determined by HA test (WHO 2002). The virus has been confirmed as H5N1 subtypes and PCR using H5 and N1 primers (data not shown).

Production of Monoclonal Antibodies. MAbs against the Indonesian isolate of AIV-H5N1 were produced by methods similar to those described by Ohnishi *et al.* (2005). Six to seven week-old female Balb/c mice were immunized with 0.2 ml (equivalent with approximately 2^7 HA units) virus emulsified in Freund's complete adjuvant. Fourteen and 28 days after the first immunization the mice were respectively immunized with the same antigen but emulsified in Freund's incomplete adjuvant. Fourteen, 15, and 16 days after the last immunization, the mice were boosted with the same antigen but without adjuvant. The mice were then sacrificed by cervical dislocation. The spleen was removed and used for the preparation of hybridomas.

As many as 2 x 10^7 immortal mouse myeloma cells prepared as described above were fused with 10^8 lymphocytes derived from the spleen of mice immunized with AIV-H5N1. The fusion of the two types of cells was carried out using polyethylene glycol (PEG) 45% (Sigma Co, USA) to produce hybridomas. The hybridomas were then screened by indirect ELISA (Campbell 1991) for the anti-AIV antibodies using formaldehyde inactivated AIV-H5N1 as antigen and normal allantoic fluid as negative control. The hybridomas producing MAbs reacted specifically with the virus were cloned by limiting dilution as described by McKearn (1980) and were then used in the production of MAbs against the AIV-H5N1.

Titration of MAbs. The titer of MAbs in hybridomas' supernatant fluid was determined by ELISA according to the procedure as described by Campbell (1991). ELISA microtitration plate was coated overnight with formaldehyde inactivated virus diluted in carbonate-bicarbonate coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$ pH.9.6). Each plate well was coated with 100 ml antigen containing of approximately 1 HA unit of the virus. After three times washes with 0.05% Tween-20 in phosphate buffered saline pH 7.2 (PBST), 100 ml blocking buffer (5% skim milk in PBST) was added and incubated for another 1 h at 37 °C. A serial two-fold dilution of MAbs was prepared and 100 ml MAb sample from each dilution were added to each well. The plate was incubated for 1 h at 37 °C. After three times washes as above, 100 ml anti-mouse IgG-conjugated with horseradish peroxidase (HRP) (Bio-Rad, USA) diluted 1:2 000 in PBS-T was added to each well. The microplate was then incubated for 1 h 37 °C and washed three times as above. One hundred µl of substrate solution (1 mM 2 2'-azinodi 3-ethylbenzthyazolin-6-sulfonic azide in 0.05% Na citrate, 0.15% Na phosphate, and 0.01% H_2O_2 was added to each well. After incubation for 15 min at room temperature, the absorbance of the substrate solution in each well was read by multiscan spectrophotometer using 405 nm filter. Titer of MAbs was determined as the antilog the highest dilution giving an absorbance reading of approximately 50% of its optimal reading.

Determination of MAb Isotypes. The immunoglobulin (Ig) class and subclass of the MAbs were determined by indirect ELISA using rabbit antimouse subtyping isotyper kits (Bio-Rad Laboratory, USA) according to the procedures described by manufacturer. ELISA microtitration plate was firstly coated overnight with formaldehyde inactivated AIV-H5N1 as described above. Into each well, 100 µl MAb diluted 1:10 in PBST were added and incubated for 1 h at 37 °C. Following three times washes with PBS, rabbit anti-mouse Ig isotyper from the kit was added to the wells and incubated as above. After 3 times washes, 100 ml affinity purified goat anti-rabbit IgG conjugated with HRP (Bio-Rad, USA, diluted 1:1 000 in PBST) was added and incubated at 37 °C for 1 h. The plate was again washed as above and 100 ml substrate solution (1 mM 2.2'-azinodi 3-ethylbenzthyazoline-6-sulfonic acid in 0.005 Na citrate, 0.15 Na phosphate, and 0.01% H_2O_2) was added. The absorbance of the substrate solution was read in Multiscan spectrophotometer with a 405 nm filter.

Western Blotting. Western blotting assay was carried out according to procedure described by Zheng *et al.* (2001). Formaldehyde inactivated AIV-H5N1 were diluted in an equal volume of sample loading buffer (1.3% SDS, 5% mercaptoethanol, 0.0625 M Tris-HCl pH 6.8, 10% glycerol, 0.001% bromophenol blue). The viral proteins were analysed by sodium dodecyl sulfate-polyacrylamid gel electrophoresis (SDS-PAGE) using 3% loading gel and 10% separating gel. The proteins in the gel was then transferred onto nitrocellulose membrane (Bio-Rad, USA). Following 1 h blocking at room temperature with 3% skim milk in Tris-buffered saline (TBS/100 mM Tris pH 7.4 adjusted with 1 N HCl) and a brief washing with TBS, nitrocellulose membrane was then cut into 0.5 cm strips. Each strip was then soaked with hybridomas's supernatant fluid containing MAbs and incubated 24 h at room temperature. Following 3 times washes with TBS, anti-mouse IgG coupled with biotin (Bio-Rad USA, diluted 1:1 000 in TBS) was the added to the membrane. After 3 times washes with TBS, streptavidin-alkaline phosphatase (Promega, diluted 1:500 in TBS) was then added to the membrane. The membrane was washed 3 times as above and the AIV protein in the membrane which reacted with MAbs was visualized by adding 5-Bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) substrate kit (Bio-Rad, USA).

Detection of AIV Antigen in Duck Tissues by Immunoperoxidase Staining. The ducks used in this study were cordially provided by co-assistant students in the Faculty of Veterinary Medicine, Udayana University, Denpasar Bali. All ducks had been tested for AIV infection by the isolation of the virus in chicken embryonated eggs and identification of the virus by haemagglutination/ haemagglutination inhibition (HA/HI) test. The results of the test were then further confirmed by reverse trancriptase-polymerase chain reaction (RT-PCR) using primers specific to H5 and N1 subtypes. Two ducks that were confirmed positive to AIV-H5N1 infection and two ducks confirmed AIV negative (data not shown) were used in this study.

Several organs such as brain, proventricle, small intestine, liver, lung, bursa of Fabricius, spleen, and kidney derived from the ducks were preserved and fixed with 10% buffered formaldehyde. Paraffin embedded organs and thin sections of the organs were prepared by standard methods. Immunoperoxidase staining was then carried out according to the methods similar to those described by Ohnishi *et al.* (2005). Thin sections of tissues on microscope slides were de-paraffinized twice in xylol and twice with ethanol absolute. The tissue section was washed twice with PBS and treated with 0.05% trypsin for 1 min at 37 °C. The endogenous peroxidase of the tissues was then inactivated by treatment with 3% H_2O_2 in PBS for 20 min at room temperature. After blocking with 50% normal goat serum in PBS, MAbs against AIV-H5N1 was added onto the tissue section and incubated for 18 h at room temperature. The bound MAbs were detected by biotinylated goat anti-mouse IgG (Biodesign International) diluted 1:500 in PBS containing 10% normal goat serum and streptavidin-horse radish peroxidase (Sigma Co, USA) diluted 1:100 in PBS. A proper washing procedure was carried out using PBS in between each step. The AIV antigen bound with MAbs was then visualized by adding diazinobenzidine (DAB) substrate (Sigma Co, USA, 50 mg/ 50 ml PBS containing 0.07% H_2O_2).

RESULT

Characteristic of Monoclonal Antibodies. As many as 12 clones of stable hybridomas secreting MAbs against the AIV-H5N1 of Indonesian isolate were produced. Screening by ELISA using formaldehyde inactivated AIV-H5N1as antigen showed that all of these 12 clones of hybridomas produced MAbs against the virus, not against the normal allantoic fluid. Eight MAbs were further characterized and they were designated as AG8, BC12, CC5, CG1, DD9, DF11, EA11, and EE8. All MAbs reacted strongly in ELISA test using formaldehyde inactivated AIV-H5N1. None of them reacted with normal egg allantoic fluids (negative control). The titer of MAbs varied from 2^6 to 2^9 (Table 1). Isotyping of MAbs using rabbit anti-mouse IgG subtyper isotyping kit showed that 3 MAbs (AG8, DF11, EA11) were of IgG1 subclass, 1 MAb (DF9) was of IgM subclass, 3 MAbs (CC5, CG1, DD9) were of IgG3 subclass, and 1 MAb (EE8) was of IgG2a subclass (Table 1).

Western Blotting with MAbs. In western blotting assay, a similar result was observed. All MAbs reacted only with formaldehyde inactivated AIV-H5N1. No MAb reacted with normal allantoic fluid. Two MAbs (DD9 and CC5) reacted with 2 protein bands with the molecular weight of approximately 76 and 58 kDa, 5 MAbs (AG8, CG1, DF11, EA11, and EE8) reacted with a single protein band of 76 kDa. One MAb (DF9) reacted with a diffuse protein band.

Detection of AIV Antigen in Duck Tissues. Three (CG1, EE8, AG8) produced a good and a strong result when used for the detection of AIV antigen in ducks. One MAb (DF11) did not react with AIV antigen in the duck tissues. AIV antigen was detected in the two infected ducks but not in uninfected ducks. AIV antigen with a high intensity was observed proventricle and in small intestine. AIV antigen at a lesser intensity was also observed in other organs such as lung, spleen, and bursa of Fabricius (Table 2, Fig 1). AIV antigen was difficult to observe in the brain, muscle tissue, and kidney. No clear difference on the distribution of infected tissues was observed between the two infected ducks.

DISCUSSION

Stable anti-AIV-H5N1 MAbs-secreting hybridomas were successfully produced by fusion of immortal myeloma cells with lymphocytes of mice immunized with the virus. The use of formaldehyde inactivated virus for immunization of mice in the preparation of MAbs appeared to be not an important factor for the production of hybridomas stably producing MAbs against AIV. This is evident as all of the isolated hybrodomas consistently produced MAbs against the virus and but not against the normal allantoic fluid. The use of relatively unpurified virus for immunization of mice in the preparation of MAbs has been reported (Wickramasinghe *et al.* 1993; Pantophlet *et al.* 2001).

Screening method appeared to be the more important factor for successful selection of hybridomas producing

Table 1 Characteristics of monoclonal antibodies prepared against avian influenza virus subtype H5N1 of Indonesian origin

MAbs	Isotypes	ELISA		Western blotting		IHC
		AIV-H5N1	NA	AIV-H5N1	NA	
AG8	IgG1	2^7	-	76	-	+++
BC12	IgG1	2^8	-	76	-	ND
CC5	IgM	2^7	-	Diffuse	-	ND
CG1	IgG3	2^8	-	76	-	+++
DD9	IgG3	2^6	-	Diffuse	-	+++
DF11	IgG1	2^9	-	76/58	-	ND
EA11	IgG1	2^8	-	76/58	-	+++
EE8	IgG2a	2^7	-	76	-	ND

NA: normal alantoic fluid, IHC: immunhistochemistry, -: negative, +++: strong positive, ND: not ditermined.

Table 2 Detection of avian influenza virus antigen in infected Ducks by immunostaining staining using monoclonal antibodies

Ducks organs	Infected ducks I				Infected duck II			
	AG8	DF11	EA11	EE8	AG8	DF11	EA11	EE8
Proventricle	+++	-	+++	+++	+++	-	+++	+++
Small intestine	+++	-	+++	+++	+++	-	+++	+++
Lung	++	-	++	++	++	-	++	++
Spleen	++	-	++	++	++	-	++	++
Muscle	+	-	+	+	+	-	+	+
Kidney	+	-	+	+	+	-	+	+
Liver	+	-	+	+	+	-	+	+
Brain	-	-	-	-	-	-	-	-

++: moderate positive, +++: strong positive, +: weak positive, -: negative.

anti-AIV-H5N1 MAbs. As in immunization, the antigen used in the ELISA test for screening MAbs was formaldehyde inactivated AIV-H5N1. The virus was originally propagated in the allantoic cavity of chicken embryonated eggs. The virus was then harvested from the allantoic fluid of the infected chicken embryo and was therefore expected to contain a plenty of normal allantoic fluid. It was therefore very likely that the immunization of mice with such antigen will stimulate the production of antibodies against both AIV and normal allantoic fluid. This then was confirmed when several MAbs which reacted with normal ascitic fluid were detected by ELISA using the unpurified AIV-H5N1 (data not shown). Such MAbs were excluded by further testing by ELISA using normal allantoic fluid as an antigen.

Isotyping showed that the MAbs produced in this experiment were of IgM, IgG1, IgG2a, and IgG3 subclasses. The information on the isotype of MAbs is important in the selection of techniques used for the purification of MAbs. In addition, the information on the MAbs' isotype is also important in the selection of techniques to be developed using MAbs. The MAbs with IgG isotype generally produces a more specific and sensitive result and can also be used in a relatively wider range of serological tests then those of MAbs of IgM isotype. In some immunodetection systems, however, antibody with IgM isotype is preferred as it will produce a better and a stronger reaction than MAbs of IgG isotype. The main problem working with IgM is that it is more difficult to purify than IgG. In addition, the availability of MAbs of IgG isotypes will also enable the purification of the MAbs using Protein A or G (de Masi *et al*. 2005) which is often required for the development of a particular test such as capture ELISA (Ohnishi *et al.* 2005).

In western blotting assay, all isolated MAbs reacted specifically only with AIV-H5N1 antigen. None of them reacted with normal allantoic fluid which was used as an antigen for AIV negative control. The result confirmed that MAbs specific to AIV-H5N1 can be produced by immunization of mice with relatively unpurified virus. The protein bands recognized by MAbs were around 76 kDa, 58 kDa, and several other diffuse bands. The protein band with 76 kDa detected by most MAbs (CG1, AG8, EA11, EE8, BC12) is likely to be uncleaved haemagglutinin (HA0) of AIV-H5N1. The HA protein of AIV is a surface glycoprotein encoded by segment 4 (HA) of the viral segmented RNA gemomes. The protein is initially translated as uncleaved precursor of HA0 protein with the molecular weight of around 76 kDa. It is then post-translationally cleaved by host cellular proteases into two sub units, HA1 (56 kDa) and HA2 (25 kDa) (Skehel and Waterfield 1975; Zhirnov *et al.* 2002). However, other workers on influenza A virus reported the molecular weight of HA1 varied from 50-61 kDa and HA2 varied from 25-30 kDa (Bucher *et al*. 1976; Boulay *et al*. 1987; Jaspers *et al*. 2005). The protein contains sialic acid which plays an important role in the binding of the virus into the receptor molecules on the surface of susceptible cells (Hulse *et al.* 2004) and such cleavage step is necessary for the infection of the virus into not react with AIV antigen in the infected ducks, suggesting that this MAbs did not recognized the AIV epitope in formadehyde fixed and paraffin embedded tissues. The reason behind this is unknown. It is possible that the epitope recognized by this MAb has been destroyed or hidened during the tissue proccecing. When the 4 MAbs were used to immunostain tissues or organs of normal uninfected ducks, none of them produced a positive result. This showed that three of the selected MAbs are applicable for use in development of specific test for the detection AIV infection in ducks. The use of MAbs in the immunochemistry staining for the detection of viral antigen in the infected host has been widely reported (Ohnishi *et al.* 2005; Astawa *et al.* 2006).

In the infected ducks, high intensity of AIV antigen was detected in organs such as preventricle and intestine villi (Fig 1), suggesting that the virus replicates very efficiently in these two gastrointestinal organs. This is in accord with the finding that, in waterfowl, influenza viruses replicate preferentially in the intestinal tract, resulting in excretion of high-titer viruses in the feces (Horimoto and Kawaoka 2001). The combination of the availability of cells bearing the receptor for AIV and the presence of abundant proteolytic enzymes may contribute to the efficient replication of the virus in the intestine and proventricle of ducks. Unlike those which originate from chicken, many AIVs isolated from ducks have a strong binding activity to gangliosides with short sugar chains that were found abundant in duck gastrointestinal tissues (Slemons and Easterday 1978; Gambaryan *et al*. 2003). In addition, gastrointestinal tract is rich in proteolytic enzymes (Banks and Plowright 2003) which are responsible for post translational cleaving of HA0 of into HA1 and HA2 (Garten and Klenk 1999). The cleavage of HA protein is required for the efficient replication of the virus in the two organs.

The availability of MAbs against AIV-H5N1 has enabled the detection of AIV antigen in duck tissues. The duck used study was previously confirmed to be infected by AIV-H5N1 but the tests used still require expensive facilities and reagents such as PCR. It is also unsafe to perform as isolation of AIV in chicken embryonated eggs and identification by HA/HI test require the use of live virus (Gough 2004). The use of MAbs on formaldehyde fixed and paraffin embedded tissues has made it possible to develop a relatively simpler and safer test which can be performed in laboratory with simple facilities and low biosecurity level. The immunological detection system developed in this experiment also safe to perform on daily basis as it uses formaldehyde fixed tissues which inactivates the AIV. As ducks and other aquatic birds play an important role in the transmission of AIV into susceptible hosts (Matrosovich *et al.* 1999), the availability of test to detect ducks carrying the virus will be important in preventing the AI outbreaks brought out by this carrier water fowls.

It is also important to note that most MAbs produced in this experiment appeared to react with the HA protein of AIV-H5N1. This was further confirmed by HI test that MAb AG8 that react with at the protein band of around 76 kDa (Fig 2) did exhibit inhibition of HA activity of the virus (data not shown). At this stage, however, it is still not possible to determine which MAbs react specifically to H5 subtypes and which MAbs cross-react AIV subtypes other than H5. If a panel of AIV isolates with several different H5 subtypes is available for study, it will be likely to be able to evaluate

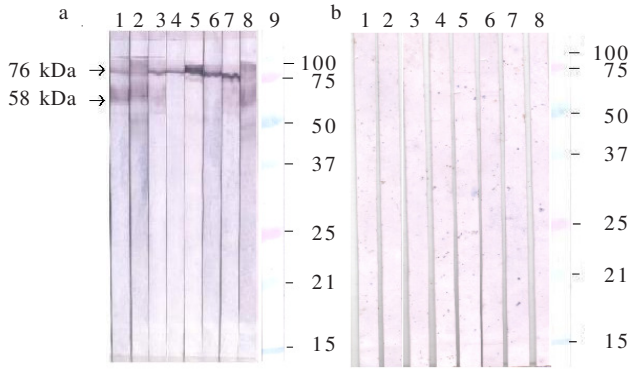

Fig 2 Reactivity of monoclonal antibodies with AIV-H5N1 and normal allantoic antigens analysed by western blotting. a: antigen: formaldehyde inactivated AIV-H5N1, and b: normal allantoic fluid. Strip no. 1-8 MAbs: 1: DD9, 2: CC5, 3: AG8, 4: CG1, 5: DF11, 6: EA11, 7: EE8, and 8: DF9, strip no. 9: standard marker.

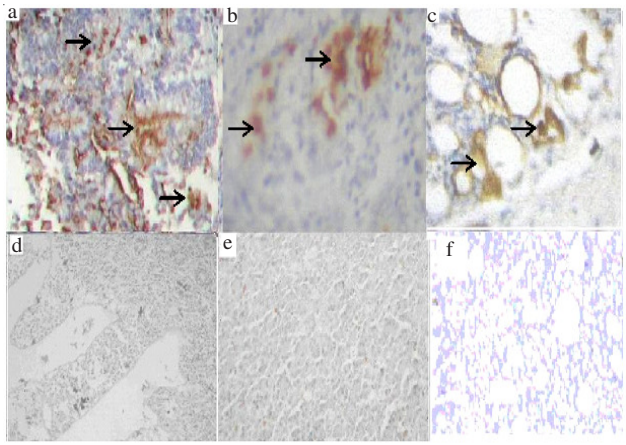

Fig 1 Detection of avian influenza virus antigen in various duck tissues by immunoperoxidase staining using MAbs anti-AIV H5N1. Infected cells (⟶). Uninfeted (purplish blue). Infected small intestine (a), spleen (b), and lung (c). Normal unifected small intestine (d), spleen (e), and lung (f).

the cross-reactivity of several MAbs with many different H subtypes. When MAbs react specifically only with AIV of H5 subtype are available then determination of AIV-H5 subtypes can be carried out directly by immunohistrochemistry staining using MAbs. This is important as AIV-H5 virus is one subype that causes most fatal infection in avian species and in mammal including human (Swayne and Suarez 2000). A further investigation is required in order to confirm this suggestion, especially when AIV of many different H subtypes are available for study.

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