Avian influenza virus detection: sensitivity comparison of various in vitro and in OVO methods

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ABSTRACT

Avian influenza, especially highly pathogenic avian influenza (HPAI), poses global threat not only for poultry but also for public health. To control avian influenza expediently, rapid, sensitive, and specific detection method is therefore of high importance. Here we compared the sensitivity and specificity of real time reverse transcriptase polymerase chain reaction (rRT-PCR) and nucleic acid sequence based amplification assay - microplate detection method (NASBA-MDM), with chicken embryo infective dose 50 (EID_{50}). Additionally, we compared two cell culture systems, namely chicken embryo fibroblasts (CEF) and Madin-Darby canine kidney (MDCK) cells for virus detection. rRT-PCR and NASBA-MDM could detect about 0.1 EID_{50} virus. Both methods showed negative results for pathogen other than avian influenza viruses (AIVs) tested, indicating that they are sensitive and specific. In contrast, both cell systems could detect viruses about 1 EID50 in presence supplementary trypsin. rRT-PCR and NASBA-MDM could generate result within few hours. However, NASBA-MDM was more laborious than rRT-PCR. MDCK cells were found more sensitive when compared with CEF, but less sensitive than chicken embryo. However, cell cultures may serve as an alternate tool for virus isolation. Both molecular methods tested here may be applicable for rapid, early, and specific detection of AIVs. The usefulness of the various methods utilized here may be further appraised in terms of virus typing, antigenic subtyping and pathotyping.

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Introduction

Highly pathogenic avian influenza (HPAI) is a markedly contagious viral disease of domestic chickens and turkeys, characterized by severe illness with high morbidity and mortality (Easterday et al. 1997). Human infections with the HPAI virus H5N1 frequently cause death since 1997 (Class et al., 1998, Subbarao et al., 1998). Two surface glycoproteins, the haemagglutinin (HA) and neuraminidase (NA) form the basis of antigenic subtyping of avian influenza viruses (AIVs) (Wright and Webster, 2001). Currently sixteen HA plus nine NA subtypes have thereby been classified, thus far (Fouchier et al. 2005).

In response to continuing outbreak of avian influenza worldwide since the end of 2003, many countries strengthen surveillance studies in wild birds for early detection of avian influenza virus (AIV). To screen large number of samples rapidly, reliably, sensitively and specifically, an appropriate diagnostic test is a prime prerequisite. Different diagnostic tools are available and can be used, depending on the individual laboratory facilities. Among these, embryo inoculation or tissue cultures are the methods of choice for virus isolation. For the detection of viral nucleic acid, reverse transcriptase polymerase chain reaction (RT-PCR) is a commonly practiced method. Other techniques, including real time RT-PCR (rRT-PCR) and nucleic acid sequence based amplification assay (NASBA), are also developed to detect viral nucleic acid. Here we tested the sensitivity and specificity of rRT-PCR, NASBA-microplate

detection method (NASBA-MDM) and compared them with fifty percent embryo infective dose (EID_{50}) titer of virus. Additionally, through tissue culture infective dose fifty ($TCID_{50}$), we compared sensitivity of chicken embryo fibroblast (CEF) and Madin-Darby canine kidney (MDCK) cell cultures to AIV.

Materials and Methods

Viruses

Viruses, A/northern pintail/Aomori/395/04 H7N1 (H7N1) and A/northern pintail/Miyagi/258/05 H6N2 (H6N2) were taken from the repository of the Laboratory of Zoonosis, School of Veterinary Medicine, Kitasato University. These viruses (H7N1 and H6N2) were isolated from fecal materials of northern pintail (*Anas acuta*) through embryo inoculation and subtyped by RT-PCR and haemagglutination inhibition (HI) and neuraminidase inhibition (NI) tests (Jahangir et al. 2008). Stock viruses were cultured and titrated in 9-10 days old embryonated chicken egg to prepare working viruses.

Sample preparation

Viruses in allantoic fluid were mixed with tracheal swab. Tracheal swab was prepared as follows. Tracheas of chickens were collected from slaughter house, cut into small pieces and mixed with 3.5ml of brain heart infusion (BHI) broth. Stomaching was carried out with high speed for 2 minutes. Liquid (BHI-Tracheal mixture later referred to as tracheal swab) was collected to the maximum possible. The virus in allantoic fluid was subjected to ten-fold serial dilutions with this tracheal

swab from 10^{-1} to 10^{-10} and used to detect EID_{50}, $TCID_{50}$ and extract RNA. RNA was extracted from diluted samples using trizol, a mono-phasic solution of phenol and guanidine isothiocyanate (Trizol$^{®}$LS, Invitrogen, USA) according to manufacturer's instructions and implemented in both rRT-PCR and NASBA-MDM.

Realtime reverse transcription polymerase chain reaction (rRT-PCR)

One-step rRT-PCR was conducted as described (Spackman et al. 2002) with minor modifications at thermal cycling conditions. Qiagen one-step RT-PCR kit except RNase inhibitor (Promega 40U/µL) was used in the study. No template control (NTC) which contains all components except template RNA was run always with the test sample. The thermal cycling conditions were one cycle of 50^0C 30 min and 95^0C 15 min followed by 45 cycles of 94^0C 5 sec (denaturation) and 60^0C 20 sec (annealing). Thermal cycling was performed using ABI Prism 7700 system (Applied Biosystems). At the end of each annealing step, fluorescent signal along with data (referred to as cycle threshold-Ct) were acquired and analyzed using sequence detection software (Applied Biosystems). Data for rRT-PCR presented in this report are the averages of two or more replicates. The Ct value up to 35 was considered as positive for AIV, whereas Ct value above 35 was considered as negative.

Nucleic acid sequence based amplification assay-microplate detection method (NASBA-MDM)

NASBA-MDM was performed according to manufacturer's instructions (Hong Kong DNA Chips Ltd). Briefly, for amplification, 5µl of extracted RNA was mixed with 10µl of amplification solution, incubated at 65^0C for 5 min, and cooled at 41^0C for 5 min. After adding 5µl of enzyme solution, the mixture was incubated at 41^0C for 5 min, and thereafter spinned again at 41^0C for 90 min. For detection, a hybridization mixture (2µl probe solution, 43µl hybridization buffer and 5µl NASBA product) was added to the microtiter strip obtained from the test kit, and incubated at 41^0C for 1 hour. Washing was performed with 250µl of 1XTBS (pH7.0), followed by air drying. Then 100µl detection solution was added into each well of the strip and incubation for 30 minutes at room temperature. After washing as mentioned above, 100µl of substrate solution was added and incubated in darkness at room temperature. To stop color development at the end of incubation period, 100µl of stop solution was added and mixed gently. The reading was taken using a microplate spectrophotometer at 405 nm wavelength. To get background (negative control) a mixture of substrate and stop solution (without hybridization and detection reagents) was used along with known positive samples. Cutoff value was determined according to instructions (i.e mean of negative controls plus 10 standard deviations). Data for NASBA-MDM presented in this report are the averages of two or more replicates. Newcastle disease virus (NDV) was also employed in the test to check the specificity of rRT-PCR and NASBA-MDM.

Cell culture and titration of virus

Primary culture of chicken embryo fibroblast (CEF) was prepared from 10-day-old embryonating chicken eggs (Koiwai Farm, Iwate, Japan) and grown in growth medium (GM), in Eagle's minimum essential medium (MEM: Nissui Pharmaceutical Co. Ltd., Tokyo, Japan), supplemented with penicillin 100 IU/ml, streptomycin 100 µg/ml, and amphotericin B 0.5 µg/ml, 4mM L-glutamine and 10% FCS, in an environment of 5% CO_2. Madin Darby canine kidney (MDCK)

cells were also grown in GM. In occasion, cells were maintained in maintenance medium (MM) obtained by depletion of FCS from GM. Titrations of these two viruses were performed in presence or absence of supplementary trypsin. We found by comparison that final concentrations of trypsin 0.3 and 2 µg/ml (TPCK treated bovine pancreatic trypsin, Sigma, USA) were suitable for CEF and MDCK cells, respectively, hence used throughout the study.

To detect EID_{50}, ten-fold diluted virus (10^{-1}-10^{-10} as mentioned above) was inoculated to 10-days-old embryonating chicken eggs at the dose rate of 100µl /egg and 5 eggs for each dilution were inoculated. Three days post inoculation allantoic fluid was harvested and tested by haemagglutination test. EID_{50} was calculated from results of haemagglutination test according to Behrens-Kaerber`s method (Matumoto, 1949). For $TCID_{50}$, monolayer of either CEF or MDCK cells in 96-well tissue culture plate was washed thrice with PBS. Thereafter, 100 µl of ten-fold diluted virus (10^{-1}-10^{-10} as mentioned above) was inoculated per well. For each dilution four wells were used. Then equal volume of MM containing twice of final concentration of trypsin was added. The plates were incubated at 37 C at 5% CO_2 incubator for 3 days. Growth of virus was confirmed by haemagglutination test. The titer of virus was calculated from haemagglutination test result according to Behrens-Kaerber`s method (Matumoto, 1949).

Results and Discussion

To mimic field condition, tracheas were collected from slaughter house and swab was prepared. To check whether tracheal swab has any effect on virus titer yiled, viruses (H7N1 and H6N2) in allantoic fluids were serially diluted in both tracheal swab and phosphate buffered saline (PBS pH7.0) and titrated in embryonated chicken egg. After dilution with tracheal swab, the EID_{50}/100 µl of the tested viruses were found to be $10^{7.9}$ and $10^{6.5}$ for H7N1 and H6N2 viruses, respectively (Table 1), similar to those determined by dilutions of these viruses with PBS. It indicates, then, that tracheal swab has no negative effect on virus titer yield in embryonated chicken eggs. When the same sample was titrated in CEF or MDCK cells in the presence or absence of supplemental trypsin, the titers of these viruses were found to be variable and lower than EID_{50}. Mean $TCID_{50}$/100 µl of H7N1 in CEF and MDCK cells were found to be $10^{3.5}$ and $10^{5.0}$ per 100 µl, respectively, without supplementation of trypsin. However, with added trypsin, the titers were increased about 100 fold in both of the tissue culture systems. On the other hand, the mean titer of the virus H6N2 was found 100 and 1000 times higher in CEF and MDCK cells, respectively, when supplementary trypsin was added to culture medium (Table 1). These data indicate that the sensitivity of CEF and MDCK cells is variable, whether or not in association with subtype of virus. However, to get better insight, it needs further investigation with various subtypes of AIVs. For example, in the case of H7N1, the titer was found almost the same in both of the cell culture systems in presence of trypsin. Under the same conditions, the titer of H6N2 in MDCK cells was found at least 10 times higher than in CEF, though. When compared with EID_{50}, the sensitivity of MDCK cells with supplementary trypsin increased and was almost same as embryonated eggs. However, the sensitivity of the same cells was found 2.5-3 folds lower than embryonated eggs without supplementary trypsin. On the other hand, the sensitivity of CEF was found to be about 1 and 4 folds lower than EID_{50} with or without additional trypsin, respectively (Table 1). For detection of avian influenza A virus, MDCK cells

are used more frequently, as it is established that trypsin from external sources can enhance virus-cell interaction resulting in plaque formation with high efficiency (Wright and Webster, 2001). Although less sensitive when compared with MDCK cells, CEF may be a choice for virus isolation. It is documented, for instance, that H5N1 virus can replicate and produce large, well defined plaques in CEF without addition of trypsin (Lee et al. 2005).

RNA extracted from the viruses diluted with tracheal swab was subjected to detection by both rRT-PCR and NASBA-MDM. Based on Ct values obtained through fluorescent signal, it was found that samples having higher concentrations of nucleic acid had been detected earlier than those having lower concentrations. Inversely, the Ct values of higher diluted samples (meaning lower concentration of nucleic acid) were higher than those of lower diluted samples. The Ct values along with their corresponding dilutions are depicted in Table 2. The Ct values for dilutions with positive results range from 18.39-32.29 for H7N1 and 22.06-31.46 for H6N2. We used primers and probes targeting the matrix gene of AIVs as described (Spackman et al., 2002). Both viruses, H7N1 and H6N2, were detected efficiently up to 0.1 EID_{50} virus. Contrastingly, no amplification was observed in case of no template control (NTC) and pathogen other than AIVs. The NASBA-MDM also found as efficient as rRT-PCR in terms of sensitivity and specificity. We did not find any regular pattern of optical density (OD) value (ascending or descending order in relation to concentrations of nucleic acid in the test sample). The data derived as OD value at final step of detection technique for NASBA-MDM are also presented in Table 2. As was the case in rRT-PCR, the method could not amplify non AIV sample. These data indicate that these tests are sensitive and specific. Table 3 represents the comparative analysis of in ovo, cell culture method and nucleic acid detection method in terms of their sensitivity. We found both methods can detect 0.1 EID_{50} of AIV (Table 3), thus indicating high sensitivity.

Our findings comply with the findings of Spackman et al., (2002) but differ from the result of Lau et al., (2004). The detection limit of matrix gene of AIVs by NASBA is reported $10^{1.92}EID_{50}/100$ μl whereas we found $10^{0.1}EID_{50}/100$μl. The detection method may have some effect in the variations of results we found. In NASBA technique, there are two steps- one is amplification of nucleic acid and the other is detection of amplified product. We use microplate detection method which differ from Lau`s detection method. From the Table 3, it is evident that embryonated chicken egg is still supreme whenever the objective is virus isolation. However, cell culture methods constitute a feasible alternative. On the other hand, if the purpose is only detection of presence or absence of viral nucleic acid, both techniques, rRT-PCR and NASBA-MDM may be suitable. However, each technique has draw back. For example, virus isolation and identification through embryo inoculation, the gold standard method, is laborious and requires long time (CDC, 1997; Steininger et al. 2002; Templeton et al. 2004). Several kits like FLU OIA kit and Directigen FLU A kit have been developed for rapid avian influenza virus detection but are less sensitive and specific (Fouchier et al. 2005; Shan et al. 2003; Swierkosz et al. 1989; Tobita et al. 1975). Molecular diagnosis of influenza by RT-PCR has provided improved sensitivity and shorter time to achieve results than cell culture (Atmar et al. 1996; Storch, 2003; van Elden et al. 2002) and has facilitated the typing and subtyping of influenza viruses (Poddar

et al. 2002). However, it needs, although not always, RT and PCR steps in separate tubes followed by post PCR processing. So there is a chance of contamination after reverse transcription step. Two molecular methods, rRT-PCR and NASBA for rapid detection of AIV and subtyping H5, H7 (the two subtypes that occasionally include highly pathogenic avian influenza viruses) were described (Lau et al. 2004; Spackman et al. 2002). We found both methods could generate result within few hours. Moreover, rRT-PCR does not require running in gel or any post-PCR processing (Heid et al. 1996; Quinlivan et al. 2004; Templeton et al. 2004) thus reducing the chance of contamination. Precautions against cross-contamination and RNase contamination still need to be applied during the RNA extraction step and RT-PCR setup (Habib-Bein et al. 2003). The drawback of rRT-PCR is in that it is not suitable for cloacal swab, whereas the NASBA method can detect virus in tissue, blood, cloacal and tracheal swabs (Spackman et al. 2002). NASBA is a continuous isothermal reaction; a molecular technique for RNA detection, which does not require thermocycler. NASBA-MDM only requires spectrophotometer, which is available in most of the laboratories for detecting OD value. For specificity test, in both cases only Newcastle disease virus was included - thereby resembling the pitfalls of the present study - and it was found that both methods showed negative results, hence indicating adequate specificity.

Nevertheless, any methodology involving infection – whether in vivo, in ovo, or in vitro - is fundamentally advantageous in that it evidences the presence of viable virus, whereas any other methodology does not, tracing merely the presence of genetic material (or another viral component), which is indicative of either current or past infection, perhaps even just an abortive infection. Moreover, in spite of this difference, isolating AIVs by embryonating egg inoculation is at times more effective, hence sensitive, than molecular genetic detection.

Conclusion

In conclusion, we here compared the sensitivity of two molecular as well as two cell culture systems for detection of AIV, and found that both molecular tests are adequately sensitive. Both molecular methods can detect 0.1 EID_{50}, while CFE and MDCK cells can detect about 1 EID50 of viruses tested.

Both molecular methods can generate results within few hours, and may provide suitable assays system for routine screening. Besides, comparison of cell cultures to chicken embryos showed that MDCK was more sensitive than CEF and less sensitive than embryonating eggs. Depending on the facilities available in an individual laboratory, any of the methods we utilized in a comparative manner, may expediently be used as routine screening method. Concurrent applications pertaining to virus typing, antigenic subtyping, and pathotyping of the detected AIVs may be regarded as by products or further objectives which could extend the usefulness of the various methods.

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Table 1. Detection of avian influenza viruses in chicken embryo and tissue cultures

Virus	$EID_{50}/100\mu l$	Trypsin[a]	Mean $TCID_{50}/100\mu l$	
			CEF	MDCK
Aomori/395/04 H7N1	$10^{7.9}$	-	$10^{3.5}$	$10^{5.00}$
		+	$10^{6.9}$	$10^{7.00}$
Miyagi/258/05 H6N2	$10^{6.5}$	-	$10^{2.4}$	$10^{4.00}$
		+	$10^{5.3}$	$10^{6.3}$

[a]Supplementation of trypsin was different for the two different cell culture systems because of varying sensitiveness to trypsin. No supplementation (-)

Table 2. Sensitivity and specificity of rRT-PCR and NASBA-MDM in detecting avian influenza viruses

Dilution of virus	rRT-PCR (Ct value)		NASBA-MDM (OD$_{405}$ value)	
	Aomori/395/04 H7N1[a]	Miyagi/258/05 H6N2[b]	Aomori/395/04 H7N1	Miyagi/258/05 H6N2
10^{-2}	18.39±0.28[c]	22.06±0.70	2.93±0.15	2.84±0.18
10^{-3}	21.11±0.86	24.99±0.45	2.83±0.24	2.79±0.17
10^{-4}	24.88±0.23	26.45±0.96	2.80±0.21	2.80±0.31
10^{-5}	NT	NT	2.84±0.13	2.67±0.14
10^{-6}	32.49±1.68	31.46±1.45	2.79±0.21	2.12±0.18
10^{-7}	32.98±0.43	40.26±0.57	2.79±0.19	0.31±0.11
10^{-8}	32.29±0.86	43.84±1.12	2.76±0.16	0.36±0.10
10^{-9}	38.77±0.61	45.00±0.41	0.28±0.11	0.12±0.18
10^{-10}	42.99±0.30	45.00±00	0.30±0.09	-[d]
NTC	45.00±0		0.37±0.09	
NDV	45.00±00		0.36±0.12	

[a]EID$_{50}$ is $10^{7.9}$/100μl, [b]EID$_{50}$ is $10^{6.5}$/100μl; [c]Mean± standard deviation; [d]could not detected; NT, no tested, NTC: no template control, NDV: Newcastle disease virus

Table 3. Comparative sensitivity of rRT-PCR, NSABA-MDM and two cell culture methods in detecting avian influenza viruses.

Dilution (Log10)	Aomori/395/04 (H7N1)[a]				Miyagi/258/05 (H6N2)[b]			
	rRT-PCR	NASBA-MDM	CEF cell	MDCK cell	rRT-PCR	NASBA-MDM	CEF cell	MDCK cell
10^{-2}	+	+	+	+	+	+	+	+
10^{-3}	+	+	+	+	+	+	+	+
10^{-4}	+	+	+	+	+	+	+	+
10^{-5}	NT	NT	+	+	+	+	+	+
10^{-6}	+	+	+	+	+	+	-	+
10^{-7}	+	+	+	+	-[c]	-	-	-
10^{-8}	+	+	-	-	-	-	-	-
10^{-9}	-	-	-	-	-	-	-	-
10^{-10}	-	-	-	-	-	-	-	-

[a]EID$_{50}$ is $10^{7.9}$/100μl, [b]EID$_{50}$ is $10^{6.5}$/100μl, [c]could not detected; NT: Not tested; Data for CEF and MDCK cells based on the results obtained with supplementary trypsin.