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Comparison of a nucleoprotein gene based RT-PCR with real time RT-PCR for diagnosis of avian influenza in clinical specimens

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ABSTRACT

A nucleoprotein (NP) gene based reverse transcription polymerase chain reaction (npRT-PCR) assay was developed in our laboratory which could detect 35.09% of the experimental samples negative for virus isolation in first passage but positive by third passage. Reducing the reaction volume to 12.5 μl did not alter the test sensitivity and the results did not vary when duplicate samples were run in a different thermal cycler. The positive and negative agreements of this test in clinical specimens were compared with a matrix gene based real time RT-PCR with virus isolation as standard. A total of 516 clinical specimens including tissues, swabs and feces submitted from various States of India as part of active surveillance for avian influenza were tested by npRT-PCR, RRT-PCR and virus isolation in 9–11 day old embryonated specific pathogen free chicken eggs. The positive and negative agreements of npRT-PCR with virus isolation were found to be 0.909 ± 0.022 and 0.980 ± 0.004 respectively and that of RRT-PCR with virus isolation were 0.902 ± 0.023 and 0.977 ± 0.005 respectively. Since the positive and negative agreements of both npRT-PCR and RRT-PCR tests were similar, we suggest that this test can be used by peripheral veterinary laboratories that do not have real time PCR facility for active surveillance of AIV.

1. Introduction

Influenza viruses are members of the family *Orthomyxoviridae* and are classified as Type A, B and C based on antigenic differences in their nucleoprotein (NP) and matrix (M1) proteins. All avian influenza viruses (AIV) are classified as Type A. Further subtyping is based on antigenicity of the surface glycoproteins viz., haemagglutinin (HA) and neuraminidase (NA). Currently 16 HA and 9 NA subtypes have been identified among influenza A viruses (Fouchier et al., 2005). The NP and M are group specific viral proteins which are conserved across all the subtypes of Type A Influenza virus.

Identification of AIV by conventional methods is achieved through isolation of the virus in specific pathogen free (SPF) or AIV antibody negative embryonated chicken eggs. As virus isolation is time consuming, more rapid, precise and sensitive techniques like enzyme immunoassays (Boon et al., 2001) and reverse transcriptase polymerase chain reaction (RT-PCR) (Herrmann et al., 2001) are being used for the detection and identification of influenza viruses. Of late, TaqMan based Real Time RT-PCR (RRT-PCR) has also been successfully used in screening of AIV suspected field specimens, and reported as a reliable alternative to virus isolation in embryonated chicken eggs (Spackman et al., 2002).

India has experienced outbreaks of H5N1 highly pathogenic avian influenza virus every year since the first detection of H5N1 virus in February, 2006 (Nagarajan et al., 2006, 2009a; Tosh et al., 2007; Murugkar et al., 2008) and H9N2 low pathogenic avian influenza virus (LPAIV) in 2003 (Nagarajan et al., 2009b). During the outbreaks of H5N1 AIV in 2006 and 2007, virus isolation was mandatory for declaration of the outbreak of H5N1 HPAIV as per the Action Plan of Department of Animal Husbandry, Dairying and Fisheries (DADF), Ministry of Agriculture, Government of India (DADF, 2006). However, during the outbreak in West Bengal in January 2008, RRT-PCR and RT PCR were used as confirmatory tests for samples collected within the same geographical area after initial isolation of the virus to enable rapid implementation of control measures. Even though there are literatures available regarding development of RT-PCR for diagnosis of AIV (Fouchier et al., 2000; Lee et al., 2001), those comparing the efficacies of NP gene based RT-PCR and real time RT-PCR in clinical samples collected during active surveillance are not available to the best of our knowledge. In this paper, we report the comparison of the positive and negative agreements of a NP gene based RT-PCR assay (npRT-PCR) developed at our laboratory and a matrix based RRT-PCR for diagnosis of AIV in suspected field specimens.

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2. Materials and methods

2.1. Viruses

A total of 12 H9N2 and 53 H5N1 viruses isolated from Indian poultry were tested during this study. Further, one H7N3 gamma irradiated virus (H7N3 A/TY/OREGON/71) procured from National Veterinary Services Laboratory (NVSL), USA was also used in this study.

2.2. Clinical samples

A total of 516 clinical samples (170 tissues, 66 feces, 212 cloacal swabs and 68 nasal/tracheal/oro-pharyngeal swabs) collected from various States of India as part of active surveillance for avian influenza sent by Regional Disease Diagnostic Laboratories (RDDL) and Disease Investigation Laboratories were used in this study for evaluation of the test. The details of the samples tested are given in Table 1.

2.3. Experimental samples

A total of 57 tissue samples collected on 3 DPI from chicken experimentally infected with A/chicken/Uttar Pradesh/India/2543/2003 (H9N2) virus were simultaneously processed for virus isolation and npRT-PCR. The amnio-allantoic fluids (AAFs) harvested from the infected specific pathogen free (SPF) embryonated chicken eggs were subjected to HA test and npRT-PCR and the results were compared. All the samples which gave nil or 1:2 HA titer were subsequently passaged in eggs twice and the HA titer was calculated. The samples which gave no HA titer after third passage were declared negative for virus isolation.

2.4. Virus isolation and subtyping

Virus isolation in 9–11 old embryonated SPF chicken eggs and subtyping were carried out as described previously (Nagarajan et al., 2009b).

2.5. Designing of primers

A total of 205 out of 1303 full length sequences of NP gene of H1 to H13 and H16 subtypes (H14 and H15 subtype NP gene sequences were not available) isolated from various countries of Asia were downloaded from NCBI influenza virus data base (http://www.ncbi.nlm.nih.gov/genomes/FLU/Database/nph-select.cgi accessed on 25.10.2010) and aligned with CLUSTAL W program version 1.6 (Thompson et al., 1994) with a gap penalty of 15. The regions showing consecutive consensus sequences were evaluated for primer characteristics using primer design software of LASERGENE software (DNASTAR Inc., USA) and degenerate primers were designed to bind with NP genes of all the 14 subtypes. The sequences of the primers are HSADLNP1F (*181* 5′AAACTCAGCGAC YAWGAAGG3′ *200*) and HSADLNP1R (*623* 5′ CGAATTAGTTCCAT YACCAT 3′ *604*).

2.6. RNA standard for test sensitivity

Recombinant plasmid DNA were constructed by ligating the full length NP gene (nt 1–1565) of A/chicken/Navapur/India/7972/2006 (H5N1) virus into pGEM-T Easy Vector (Promega, USA). The plasmid was linearized by digesting overnight at 37 °C with *Xba* I restriction enzyme (Fermentas, USA). *In vitro* transcription was performed with RiboMAX™ Large Scale RNA Production System-T7 (Promega, USA) as per manufacturer's protocol. The concentration of the transcribed RNAs was determined using Qubit® (Invitrogen, USA) as per manufacturer's protocol. The copy number of RNA was determined by the formula (concentration of RNA in grams/μl/[length of amplicon × 340]) × 6.022×10^{23} = Number of molecules/μl (http://www.qiagen.com/resources/info/guidelines_rtpcr/quantifying.aspx).

2.7. RT-PCR

RNA was extracted from the clinical samples/allantoic fluids using QIAamp viral RNA mini kit (Qiagen Inc., USA) following manufacturer's instructions. The RT-PCR was done using Access RT-PCR kit (Promega, USA). Two types of reactions were carried out for standardization using ten fold serial dilutions of *in vitro* transcribed RNA (ranging from 3.49×10^9 to 3.49 copies). The master mix for both the reactions included 1X buffer, 1.5 mM of $MgSO_4$, 200 μM of dNTPs. One set of reaction was carried out with 4 μl of RNA, 20 pmols of forward (HSADLNP1F) and reverse (HSADLNP1R) primers, 2.5 units each of *Tfl* polymerase and AMV RT enzymes and RNase free water to a final volume of 25 μl. Another set of reaction was carried out with 2.0 μl of the same set of RNA, 10 pmols of forward and reverse primers, 1.25 units each of *Tfl* polymerase and AMV RT enzymes and RNase free water to a final volume of 12.5 μl. Positive and negative (nuclease free water) controls were included in each run. The master mix was prepared and dis-

Table 1
Details of clinical samples tested.

State	Type of samples[a]				Total
	Dead bird/tissues	Feces	Cloacal swab	Nasal/Tracheal/ Oro-pharyngeal swabs	
Assam	28 (Duck – 1, Crow – 1)	14	30 (Duck – 1)	8	80
Jharkhand	0	0	4	0	04
Kerala	1	0	1	0	02
Madhya Pradesh	0	0	0	1	01
Maharashtra	1	1	4	1	07
Orissa	1 (Migratory bird)	7	10 (Migratory bird)	12 (Migratory bird)	30
Punjab	0	0	1	2	03
Rajasthan	0	0	0	1	01
Sikkim	4	0	7	7	18
Tamil Nadu	6	0	2	3	11
Tripura	2 (Goose – 1, Duck – 1)	0	0	0	02
Uttar Pradesh	0	0	19	0	19
West Bengal	127 (Duck – 1, Parakeet – 2, Crow – 1, Pigeon – 1, Myna – 1, Migratory bird – 1)	44	134	33	338
Total	170	66	212	68	516

[a] All the samples except those in parenthesis were collected from chickens.