Detection of Highly Pathogenic Avian Influenza H5N1 Viral Load by Real-Time RT-PCR in Tissues of Experimentally Infected Chickens

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ABSTRACT

This study reports the highly pathogenic avian influenza (HPAI) H5N1 virus load estimation in different organs of chickens following experimental infection. The TaqMan probe based quantitative real-time reverse transcriptase PCR (qRT-PCR) assay was optimized for quantification of HPAI virus RNA in tissues collected from experimentally infected chickens. Conserved region in the matrix gene of avian influenza virus served as target for the primers and TaqMan probe. A recombinant plasmid containing the matrix protein gene amplicon was constructed for a quantitative estimation of copy numbers of the target gene. Quantification of avian influenza virus RNA was accomplished using a standard curve generated from ten-fold serial dilutions of IVT RNA generated from recombinant plasmid containing matrix gene. High viral RNA load was detected in spleen, brain and lung indicating enormous replication of virus in these tissues. However, spleen showed significantly higher viral RNA load (P<0.03) over other organs.

Keywords: Avian influenza virus, chicken, HPAI, H5N1, Real-Time RT-PCR, TaqMan probe

Introduction

Avian influenza virus belongs to the influenza virus A genus of the Orthomyxoviridae family and are negative-stranded, segmented RNA viruses. In influenza A viruses, 18 haemagglutinin subtypes (Tong et al., 2013) and 11 neuraminidase sub-types are currently known. All the subtypes have been found in water birds, which are a natural reservoir of avian influenza virus, and particularly in those of the Anseriformes and Charadriiformes families (Brown and Stallknecht, 2008). Transmission of avian influenza to domestic birds and their conversion to highly virulent forms often result in great economic losses (Alexander, 2007; Senne, 2007) and also involve a potential risk of transmission to the human population (Wong and Yuen, 2006). The causative agents of highly pathogenic avian influenza (HPAI) in poultry are most often viruses with H5 or H7 haemagglutinin. The pathobiology of H5N1 influenza viruses circulating in domestic poultry in Asia changed dramatically in late 2002; the virus acquired the ability to kill a large number of aquatic bird species (Sturm-Ramirez et al., 2004). In South Asia, H5N1 virus was first detected in domestic poultry in India and Pakistan during February 2006 subsequently confirmed in Bangladesh, Nepal and Bhutan in March 2007, January 2009 and February 2010, respectively (WHO, 2011). India and Bangladesh are experiencing outbreaks of H5N1 virus every year since 2006 and 2007, respectively (WHO, 2011; Dubey et al., 2009). All the H5N1 viruses isolated from poultry and humans in South Asia until 2010 belonged to clade 2.2 (Tosh et al., 2011). The first introduction of clade 2.3.2 H5N1 virus to South Asia was reported in Nepal in February, 2010 (Reid...
et al., 2011). It has been previously shown that real time PCR is a sensitive, specific and rapid test for diagnosis of influenza A viruses (Van Elden et al., 2000; Spackman et al., 2002 and 2003; Ellis et al., 2007). Real Time RT-PCR assay has also been used for quantitation of influenza A viruses (Lee and Suarez, 2004; Wards et al., 2004).

In the present study, TaqMan probe based real time PCR was performed for quantitation of H5N1 virus. The virus (A/Chicken/India/CL03485/2011) belonging to clade 2.3.2 and isolated from cloacal swab of chickens collected during Tripura outbreak in 2011 was used in this study. The aim of study was to determine the amount of avian influenza virus in the various organs of experimentally infected chickens.

Materials and Methods

Virus

The HPAI (H5N1) virus isolate A/Chicken/India/CL03485/2011 obtained from ‘Avian Influenza Virus Repository’ of High Security Animal Disease Laboratory, IVRI, Bhopal (HSADL) was used in this study. Virus was isolated from natural outbreak in chicken from Tripura in 2011. The study was conducted at BSL 3+ containment laboratory, High Security Animal Disease Laboratory, IVRI, Bhopal.

Experimental birds

Six week old specific pathogen free (SPF) chickens produced in SPF unit of HSADL were used in this study. The birds were housed in an isolator, ventilated under negative pressure with HEPA-filtered air. All personnel were required to use respiratory protection while working with live viruses or experimentally infected animals.

Experimental infection

Six week old SPF chickens were inoculated via intranasal route with $10^6$ EID$_{50}$ of A/Chicken/India/CL03485/2011. All the birds were monitored on a daily basis for clinical signs and temperatures. Tissues (Lung, brain and spleen) were collected from sacrificed chickens at 2 dpi.

RNA extraction

The total RNA was extracted using PureLink® RNA Mini Kit (Life technologies), as per the manufacturer’s protocol. Briefly, 100 µl of tissue homogenate (10% suspension) was mixed with 2 ml lysis buffer (Kit component) containing 2-mercaptoethanol. Following centrifugation at 12,000 x g for 2 min, the supernatant was collected and transferred to a fresh RNase-free eppendorf tube. One volume of 70% ethanol was added to supernatant. Subsequently entire sample was applied to the PureLink spin cartridge and centrifuged at 12,000 g for 15 sec. The extracted RNA was eluted in 30 µl nuclelease-free water.

Amplification and cloning of matrix gene

A matrix protein gene (1027 bp) was PCR amplified. The amplified PCR product was gel purified using a QIAquick gel extraction kit (Qiagen) and cloned into the pGEM® T Easy vector (Promega, USA) using a pGEM®-T Easy Vector System I cloning kit (Promega, USA). The recombinant plasmid was propagated in and purified from E. coli DH5α competent cells. Plasmid DNA was purified using Qiagen Plasmid Miniprep Kit (Qiagen) following manufacturer’s protocol.

In Vitro RNA (IVT RNA) synthesis, quantitation and standard curve preparation

The recombinant M segment transcribed in pGEM®-T easy vector plasmid was linearized using SalI restriction enzyme. M gene in vitro transcribed RNA (IVT RNA) was produced using T7 polymerase as per the protocol recommended in IVT RNA synthesis Kit (Fermantas, USA). Briefly, reaction was performed in a total volume of 50 µl containing 1x transcription buffer (4 mM Tris-HCl (pH 7.9 at 25°C), 6 mM MgCl$_2$, 10 mM DTT, 10 mM NaCl, 2 mM spermidine), 2 mM ATP/GTP/CTP/UTP mix, 50 U RiboLock™ RNase Inhibitor, 30 U T7 polymerase enzyme and 1 µg of linearized plasmid DNA template at 37°C for 4 h followed by DNAase I treatment (2U) at 37°C for 15 min. The resulting IVT RNA was purified using QIAamp® viral RNA Kit (QIAGEN, Germany) and quantified by Qubit® quantitation platform (Invitrogen, USA). The integrity of the IVT RNA was analyzed in 1% agarose gel after treating
all electrophoresis components with 0.001% DEPC (Diethylpyrocarbonate) overnight.

The copy number was calculated by the standard formula:

$$\text{Transcript copy number} = \left(\text{RNA concentration (g/µl)} \times 6.02 \times 10^{23}\right) \times \frac{\text{Molecules/µl}}{\text{[Length of in vitro RNA x 340]}}$$

A standard curve was generated using ten-fold serial dilutions of the IVT RNA. The standard curve generated was used for the quantification of influenza A virus RNA in tissue samples.

**Matrix gene based quantitative real time reverse transcriptase (qRT-PCR)**

TaqMan Probe based on conserved region of influenza virus 'M' gene reported by Payungporn et al., (2006) was used in the TaqMan one step real time RT-PCR. The sequence of a sense primer was 5'-TGATCTTCTGGAAAAATTTTGAG-3', the sequence of an antisense primer was 5'-CGTAGMGACCTCTTTTCA-3' and probe sequence was 5'-[FAM] TTGTTGATTCTTGTAGC [TAMRA]-3'. The probe was labelled at the 5’end with 6-carboxyfluorescein (FAM) reporter dye and, at the 3’end, with 6-carboxytetramethylrhodamine (TAMRA) quencher dye. Verso 1-step RT-qPCR ROX Mix (Thermo Scientific) was used with a 12.5 µl mixture under the following conditions: 0.125 µl kit-supplied RT-PCR Enzyme Mix, 6.25 µl qRT-PCR ROX Mix (2x), 0.625 µl RT Enhancer, 10 pmol of each primer, 10 µM probe and template. Reverse transcription was performed with 1 µl of total RNA at 48°C for 45 min, followed by 95°C for 15 min.

**Table 1. Amount of viral RNA (45-cycle threshold [Ct]) detected in real time PCR in the lung, spleen and brain at 2 dpi of HPAI H5N1 in chickens**

<table>
<thead>
<tr>
<th>Chicken No.</th>
<th>Tissues</th>
<th>Result of RT-PCR 45-cycle threshold (Ct)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-1</td>
<td>Lung</td>
<td>31.40*</td>
</tr>
<tr>
<td></td>
<td>Brain</td>
<td>33.23*</td>
</tr>
<tr>
<td></td>
<td>Spleen</td>
<td>33.15*</td>
</tr>
<tr>
<td>C-2</td>
<td>Lung</td>
<td>31.84*</td>
</tr>
<tr>
<td></td>
<td>Brain</td>
<td>32.11*</td>
</tr>
<tr>
<td></td>
<td>Spleen</td>
<td>35.03*</td>
</tr>
<tr>
<td>C-3</td>
<td>Lung</td>
<td>31.01*</td>
</tr>
<tr>
<td></td>
<td>Brain</td>
<td>32.92*</td>
</tr>
<tr>
<td></td>
<td>Spleen</td>
<td>33.23*</td>
</tr>
</tbody>
</table>

a: Virus amount represented as RT-PCR Ct values; *: Presence of infectious virus confirmed by virus isolation in embryonated eggs.

**Table 2. Viral RNA amount (log copies) in tissues collected at 2 dpi from chickens challenged with A/Chicken/India/CL03485/2011 virus**

<table>
<thead>
<tr>
<th>organs</th>
<th>Viral RNA amount(Log copies)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A/chicken/India/CL03485/2011</td>
</tr>
<tr>
<td>Lung</td>
<td>9.15</td>
</tr>
<tr>
<td>Brain</td>
<td>9.63</td>
</tr>
<tr>
<td>Spleen</td>
<td>9.61</td>
</tr>
</tbody>
</table>

Viral RNA amount in tissues from intranasally inoculated chickens with $10^6$ EID50 A/chicken/India/CL03485/2011. The tissues were collected after euthanasia. Spleen showed significantly higher viral RNA load (P<0.03, using the student’s t-test.)
initial denaturation. The PCR amplification programme consisted of 40 cycles of 95°C for 15 sec, followed by 55°C for 30 sec. Fluorescence data was acquired at the end of each annealing step. The reactions were carried out in LightCycler®480 System (Roche Applied Science, USA).

Results

Standard curve

The concentration of IVT RNA produced was 47.1 µg/ml. The viral RNA copy number calculated using formula mentioned in materials and methods was $2.06 \times 10^{10}$ copies/µl. Serial tenfold dilutions of IVT RNA were used to produce a standard curve for quantitative analysis. The standard curve was generated by amplification of the diluted IVT RNA in the range of $10^1$ to $10^9$ copies per 1 µl (Fig. 1). The log concentration of IVT RNA copies was plotted against the measured crossing point ($C_p$) values. Fig. 2 presents a linear correlation between the logarithmic number of IVT RNA copies and the ($C_p$).

Quantitative real time reverse transcriptase (qRT-PCR)

All tissue samples were tested by the TaqMan qRT-PCR system. Positive amplification by qRT-PCR was observed in all tissues tested. RT-PCR analysis does not differentiate between viable, infectious virus and partial, inactivated or defective virus in the samples, therefore virus isolation from tissues was performed. Virus isolation in embryonated chicken eggs (ECEs) showed that all tissues with measurable viral RNA had infectious virus. Viral RNA amount represented as RT-PCR values is showed in Table 1. The RT-PCR Ct values representing viral RNA amount was converted into viral RNA copy number by importing standard curve in the experiment. Viral RNA amount represented as copy number is summarized in Table 2 and Fig. 3.

Discussion

The number of RNA copies can be assessed by the quantitative real-time reverse transcriptase PCR

Fig. 1. Amplification curves of 10-fold serial dilution of AIV-M gene IVT RNA ranging from copy no. $2.06 \times 10^3$ to $2.06 \times 10^9$ using TaqMan probe based one step real time RT-PCR. X-axis represents the cycle no. and Y-axis represents the fluorescence acquired at 465-510 nm. The curve shows logarithmic amplification of IVT RNA dilutions.
Fig. 2. Standard curve for TaqMan probe based one step Real time RT-PCR. X-axis represents the AIV-M gene IVT RNA copy no. (log concentration) and Y-axis represents the crossing point (Cp). The assay was linear from $2.06 \times 10^9$ to $2.06 \times 10^2$. The error and efficiency is 0.0107 and 1.975, respectively, indicate high sensitivity of the assay.

Fig. 3. Viral RNA load (Log copies) in lung, brain and spleen collected at 2 dpi from chickens experimentally infected with A/chicken/India/CL03485/2011. The Viral RNA load is shown as mean level ± standard deviation.
assay (qRT-PCR). This method has previously been used to determine the amount of avian influenza virus (Lee and Suarez, 2004), as well as other avian viral pathogens (Markowski-Grimsrud et al., 2002; Peters et al., 2005; Islam et al., 2006; Callison et al., 2007). A comparison of the number of viral RNA copies detected by qRT-PCR with that of infectious virions in a Vero cell line, gave a ratio of one RNA copy to 0.00157 TCID$_{50}$ (Rosenbergova et al., 2009). Similarly Di Trani et al. (2006), using the qRT-PCR assay, could detect up to 0.001 TCID$_{50}$ of the reference virus - an equivalent to 0.08 EID$_{50}$. To compare the virus concentration in swan tissues with those in the organs of other avian species, TCID$_{50}$ values were converted into EID$_{50}$ values (Rosenbergova et al., 2009). We could compare our results with that of experimentally infected chickens, ducks and geese, where concentration of the virus was estimated by inoculating chicken embryo and EID$_{50}$. To compare our values with that of ducks, chickens and other water and gallinaceous avian species reported, we converted the RNA copy no to EID$_{50}$ values.

In the experiments with a South-Korean H5N1, the virus concentration in lung tissue was $10^{9.5}$-$10^{9.5}$ EID$_{50}$ in chickens infected intranasally, resulting in 100% mortality. In the experiments with Korea chicken H5N1 virus, the virus concentration in lung tissue was $6.4$ TCID$_{50}$/g in chickens, ducks and quails respectively (Jeong et al., 2009). In our study, the virus concentration estimated in lung tissues was between $10^{7.9}$-$10^{8.1}$ EID$_{50}$. The virus concentration in the examined spleen tissues of the chickens was $10^{8.5}$-$10^{8.7}$ EID$_{50}$. In the brain tissue of experimentally infected chicks, the virus concentration was found to be $10^{8.9}$ to $10^{8.1}$ EID$_{50}$ (Zhou et al., 2006). In the experiments with Korea chicken H5N1 virus, the virus concentration in brain tissue was $4.9$ TCID$_{50}$/g (Jeong et al., 2009). In this study, the virus concentration in the examined brain tissue of chickens was $10^{8.2}$-$10^{8.5}$ EID$_{50}$.

In present study high viral RNA load was detected in spleen, brain and lung indicating enormous replication of virus in these tissues (Fig. 3). However spleen has significantly higher titer (P<0.03 using the student’s t-test) followed by brain and lung. Similar findings have also been reported by Schat et al. (2012). No significant difference in virus titer was found between brain and lung.

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References


