International Scholars Journals

International Journal of Urology and Nephrology ISSN 2091-1254 Vol. 7 (9), pp. 001-010, September, 2019. Available online at www.internationalscholarsjournals.org © International Scholars Journals



*Full Length Research Paper*

# Quantitative analysis of the 2009 pandemic A (H1N1) influenza virus genome at different time course of infection in virion and in Madin-Darby Canine Kidney (MDCK) cells

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Accepted 28 March, 2019

**The 2009 pandemic A (H1N1) influenza virus was first identified in Mexico in April 2009 and spread world wide over a short period of time. Well validated diagnostic methods that are rapid and sensitive for detection and tracking of this virus are urgently needed. In this study, time course kinetic characterizations of the abundances of all ten genes of the 2009 pandemic A (H1N1) influenza virus in standard virions and infected Madin- Darby Canine Kidney (MDCK) cells were monitored. Results showed that the amounts of each gene in infected cells were significantly higher than those in virions, so that cell lysates were more recommended to be the nucleotide materials detection object than virions. Meanwhile, all genes were present in virions in approximately equimolar amounts, whereas the copy numbers of each gene in cell lysates were distinguishing. The abundances of M1 and NP genes were highest and may be the optimized choice for nucleotide detection in infected cells. Furthermore, the most sensitive time point for viral nucleotide detection in cells was 48 to 56 h post infection. In infected MDCK cells, the total RNAs amounts of NP and NS1 genes began to rise at 3 h post infection, whereas other eight genes escalated from 8 h post infection just as the situation of all genes in standard virions. All these data may be useful for more sensitive diagnosis and surveillance of the novel A (H1N1) virus, and might further limit the transmission of this pandemic disease in the future.**

**Key words:** The 2009 pandemic (H1N1) influenza virus, real-time PCR, virions, MDCK cells, abundance, sensitivity.

## INTRODUCTION

On June 11, 2009, the World Health Organization raised the global pandemic alert level to phase 6, the pandemic phase, in response to the emergence and global spread of a novel influenza A (H1N1) virus, which emerged in Mexico in early 2009 (Garten et al. 2009). The transmissibility of this virus was estimated to be higher than that of seasonal influenza viruses (Fraser et al. 2009). The 2009 pandemic A (H1N1) influenza viruses infections have been primarily seen among young and previously healthy adults, which suggesting that they are most vulnerable to infection. To limit community or hospital transmission, as well as to initiate antiviral therapy in time as recommended by the WHO, accurate and rapid diagnosis for confirming infection with the novel A (H1N1) virus is urgently needed and critical.

Polymerase chain reaction (PCR), especially real-time PCR, remains the best choice for early clinical diagnosis method of this virus (Bolotin, 2009; Robertson et al., 2009; Carr et al., 2009; Chan et al., 2009; Ellis et al., 2009; Gunson et al., 2009; Jiang et al., 2009; Liu et al., 2009; Pabbaraju et al., 2009; Panning et al., 2009; Poon et al., 2009; Wang et al., 2009; Whiley et al., 2009; WHO, 2009; Wu et al., 2009). To date, most research and commercial detection kits for PCR diagnosis of the pandemic A (H1N1) influenza virus are targeted on

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hemagglutinin (HA) gene to distinguish the novel virus from other subtype influenza viruses (Jiang et al., 2009; Liu et al., 2009; Panning et al., 2009; Poon et al., 2009; Wang et al., 2009). Meanwhile, there is also a large number of PCR assays in use that target on matrix (M) gene for its high conservation between various strains (Carr et al., 2009; Chan et al., 2009). However, the efficiency and sensitivity of detection based on other genes of this novel virus have never been reported and compared.

In the present study, we compared the abundance of all ten genes of the pandemic A (H1N1) influenza virus in supernatant virions and infected MDCK cell lysates by using real-time quantitative PCR based on SYBR green dye. Twenty pairs of primers were designed. Their amplified products were overlapped, and covered full genome. Meanwhile, cell supernatants and lysates were collected continuously from 0 to 96 h post infection (h.p.i) to monitor the time course and kinetic characteri-zation of viral nucleotide material amounts. We hope the results may close two gaps in knowledge about the detection for the pandemic A (H1N1) influenza virus in laboratory: (1) which time point post infection is most sensitive for virus detection? (2) which gene possesses the highest abundance in virions and infected cells? New insights into these two problems may lead to new strategies for inhibiting future extensive transmission of the the pandemic A (H1N1) influenza viruses.

## MATERIALS AND METHODS

### Viruses and cells

The 2009 pandemic A (H1N1) influenza virus strain A/California/07/2009 was used in this study. Virus was cultured in Mardin Darby Canine Kidney (MDCK) cells and aliquots were frozen at -80°C. The 50% tissue culture infectious dose ($TCID_{50}$) was determined by serial titration of viruses in MDCK cells respectively, and the titers were calculated according to the Reed-Muench method (Reed and Muench, 1938). All experiments involving the 2009 pandemic A (H1N1) influenza viruses were conducted under biosafety level 3 (BSL-3) conditions, in associated with guidelines of the World Health Organization (http://www.who.int/entity/csr/resources/publications/swineflu/Laboratorybioriskmanagement.pdf ).

### Preparation of total RNAs from infected cells and supernatants at each time point

$10^2$ $TCID_{50}$ viruses were added to MDCK monolayers in 35 mm dishes (Corning, USA) . After a 60 min adsorption at 37°C, cells were fed with 3 ml serum-free minimum essential medium containing tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK) - treated trypsin (0.5 g/ml) (Sigma, USA) and antibiotics (Sigma, USA). This was designated as 0 h.p.i. Other dishes were then incubated at 37°C. At each time point post infection, 100 l viral supernatants were harvested and clarified from cell debris by centrifugation at 3,000 g for 10 min. Cells were also harvested, and washed twice with PBS, followed by resuspending in 100 l PBS. Total RNAs in virion and cell lysates at each time point were extracted by using the instructions. RNAs were dissolved in 30 l diethyl pyrocarbonate-treated water and stored at -80°C. First-strand cDNA was produced by using random primers with 8 l RNA in a 20 l reaction mixture containing 200 U Superscript reverse transcriptase (Invitrogen, USA).

### Calibration sample preparation

Eight full-length segments of the 2009 pandemic A (H1N1) influenza virus (PB2, PB1, PA, HA, NP, NA, M2 and M1, NEP and NS1) were amplified from A/California/07/2009 strain, and primers were designed by using Primer Premier 5.0 software based on the genomic sequences (Genbank accession number FJ966976 (PB2), FJ966978 (PB1), FJ966977 (PA), FJ966974 (HA), GQ338390 (NP), GQ377078 (NA) , FJ966975 (M2 and M1), FJ969538 (NEP and NS1) respectively (Table 1). PCR products were cloned into the pGEM- T easy vector (Promega, USA) separately and positive clones were selected. The calibration DNAs were extracted and purified by QIAprep Spin Miniprep Kit (Qiagen, Germany) and the concentrations were determined with a Lambda 25 UV spectrometer and converted to copy numbers. Each DNA was serially diluted 10-fold from $10^{10}$ copies to $10^1$ copies per microliter and stored at -20°C.

### Real-time PCR assays

The real-time quantitative PCR assays based on SYBR Green dye were performed on Step one PCR system (ABI) with 2 l cDNA in a 20 l reaction mixture which also containing 10 l of 2 x SYBR Green PCR Master Mix (ABI), 1 l each of 10 M forward and reverse primers (Table 1), and 6 l nuclease-free water. Thermal cycling was done under the following conditions: 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 51-59°C for 30 s, and 72°C for 45 s. Fluorescence measurements were taken after each cycle. For M2, M1, NEP, and NS1, the PCR products were the full length genes, and for other six genes, there were two to three pairs of primers each which amplified products were overlapped with each other and covered the full length genes respectively (Table 1).

### Statistical analysis

One-way ANOVA with DUNCAN and LSD methods were used for comparing mean viral load by using different primers at different time points (SPSS 11.5 for Windows).

## RESULTS

### Assessment of real-time PCR assays for each pair of primers

Each calibration DNA was serially diluted 10-fold from $10^{10}$ copies to $10^1$ copies per microlitre. For each real-time PCR assay, the analytical sensitivity could reach $10^2$ copies per microliter. Calibration curves showed that there were strong linear dynamic range ($r^2$>0.99) between the logarithms of the copy numbers and the mean $C_q$ values. The slopes of the log- linear portion of the calibration curves were between -3.5 to -3.0, and the amplification efficiencies were ranged from 87 to 110%. Based on three intraassay repeats of each calibration sample, and three interassay repeats of each real-time PCR, the repeatability and reproducibility of all assays

**Table 1.** Primers used for calibration samples preparation and real-time PCR assays.

| Target genes | Fragment | Primer | Sequence (5' 3') | Annealing temperature (°C) | Product length (bp) |
|---|---|---|---|---|---|
| PB2 | PB2-1 | PB2-F1# | atggagagaataaaagaactg | 51 | 800 |
| | | PB2-R1 | actatgtttctagcagcgata | | |
| | PB2-2 | PB2-F2 | tacactccaggaggagaagtg | 57 | 831 |
| | | PB2-R2 | ttcactgacttcttcgggaga | | |
| | PB2-3 | PB2-F3 | gtggtagtgagtattgaccga | 55 | 801 |
| | | PB2-R3# | ctaattgatggccatccgaat | | |
| PB1 | PB1-1 | PB1-F1# | atggatgtcaatccgactcta | 55 | 800 |
| | | PB1-R1 | tcaagcttttcgcaaatgctc | | |
| | PB1-2 | PB1-F2 | ggcaagttaaaaagaagggct | 55 | 851 |
| | | PB1-R2 | actccagacactccaaagctg | | |
| | PB1-3 | PB1-F3 | agctttttttatcgctatgga | 51 | 795 |
| | | PB1-R3# | ttatttttgccgtctgagttc | | |
| PA | PA-1 | PA-F1# | atggaagactttgtgcgacaa | 55 | 751 |
| | | PA-R1 | ttgacatttgggaaagcttgc | | |
| | PA-2 | PA-F2 | aaaactttagagcctatgtag | 51 | 791 |
| | | PA-R2 | cctacatttgcttatcattgg | | |
| | PA-3 | PA-F3 | tacataaatacggccttgctc | 53 | 762 |
| | | PA-R3# | ctacttcagtgcatgtgtgag | | |
| HA | HA-1 | HA-F1# | atgaaggcaatactagtagtt | 51 | 600 |
| | | HA-R1 | agatggatggtgaatgcccca | | |
| | HA-2 | HA-F2 | gataaagggaaagaagtcctcg | 57 | 599 |
| | | HA-R2 | ctcttcaggtcggctgcatat | | |
| | HA-3 | HA-F3 | gttatcaccatcaaaatgagca | 53 | 602 |
| | | HA-R3# | ttaaatacatattctacactgtag | | |
| NP | NP-1 | NP-F1# | atggcgtctcaaggcaccaaa | 57 | 780 |
| | | NP-R1 | tgccaggaaaatgaggtcttc | | |
| | NP-2 | NP-F2 | caagtaagagaaagtcgaaac | 53 | 777 |
| | | NP-R2# | tcaactgtcatactcctctgc | | |
| NA | NA-1 | NA-F1# | atgaatccaaaccaaaagataa | 51 | 731 |
| | | NA-R1 | tcggtcattacagtaaagcaag | | |
| | NA-2 | NA-F2 | aagagtctgaatgtgcatgtg | 51 | 731 |
| | | NA-R2# | ttacttgtcaatggtaaatgg | | |
| M2&M1 | M2 | M2-F# | atgagtcttctaaccgaggtc | 53 | 982 |
| | | M2-R# | ttactctagctctatgttgac | | |
| | M1 | M1-F | atgagtcttctaaccgaggtc | 57 | 759 |
| | | M1-R | tcacttgaatcgctgcatctg | | |
| NEP&NS1 | NEP | NEP-F# | atggactccaacaccatgtca | 57 | 838 |
| | | NEP-R | ttaaataagctgaaacgagaaagc | | |
| | NS1 | NS1-F | atggactccaacaccatgtca | 57 | 660 |
| | | NS1-R | tcatttctgctctggaggtag | | |
| | | NEP&NS1-R# | gtagaaacaagggtgtttttt | 51 | 863 |

# Primers used for full length genes amplification for calibration DNAs preparation.

were verified to be excellent, since the CV values were less than 1% (Data not shown).

### Time course of viral RNAs amounts in virion

To determine the time course of viral RNAs amounts in virion, MDCK cells were infected with the 2009 pandemic A (H1N1) influenza virus strain A/California/07/2009, and 100 l viral supernatants were harvested and clarified at 0, 0.5, 1, 1,5, 2, 2.5, 3, 4, 6, 8, 12, 24, 32, 48, 56, 72 and 96 h.p.i separately. RNAs were extracted and reverse transcribed to cDNAs which were quantified by real-time PCR with twenty primer pairs based on ten viral genes with SYBR green dye. Quantification results showed that for PB2, PB1, PA, HA, NP and NA genes, which used two or three primer pairs for quantification, the results of each gene were not totally identical when using diverse primers (Figures 1a~f), however, statistical analysis by SPSS software indicated that there were no difference among the quantification results of each gene by different primers ($P>0.05$) . The discrepancy was probably caused by the different match abilities of each primer set in annealing with the templates. For all ten genes, the total RNAs presented in standard virions in approximately equimolar amounts at each time point (Figures 1a~h), since statistical analysis indicated that there were no difference among them ($P>0.05$). Time course kinetic curve of the RNAs amounts of each gene in virions began to rise at 8 h.p.i, and quantification results by using PB2-1, PB2-2, PB1-3, NP2, NA1, NA2, M1, M2 and NS1 primers reached their peak copy numbers at 48 h.p.i, whereas PB2-3, PB1-1, PB1-2, PA-1, PA-2, PA-3, HA-1, HA-2, HA-3, NP-1 and NEP primers detected the climax copies at 56 h.p.i. However, the differences of amounts between these two time points were extremely indistinctive (Figure 1), which suggested that when clinical suspected positive specimens were cultured in MDCK cells, the most sensitive time course for nucleotide materials detection from the viral particles was 48 to 56 h.p.i.

### Time course of viral total RNAs amounts in infected cells

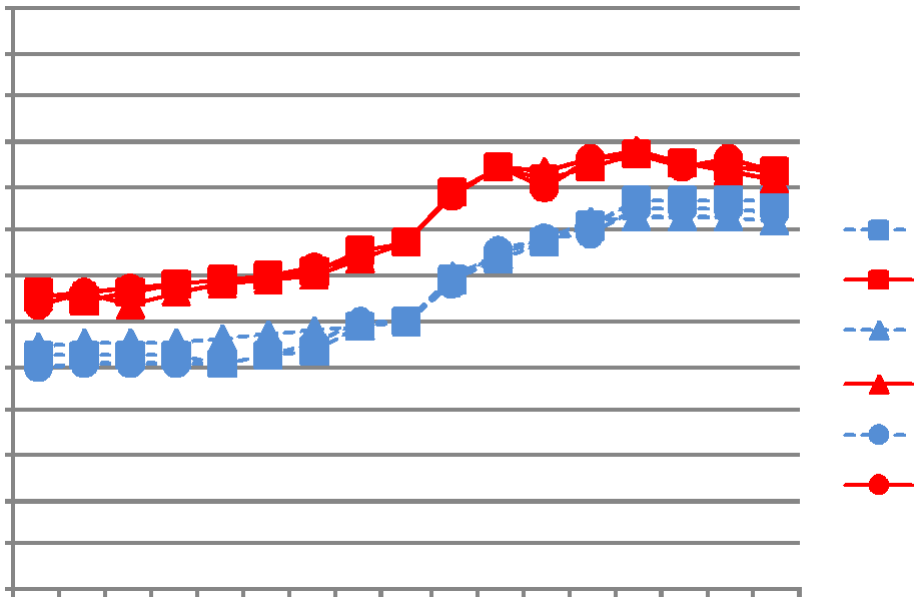
Similarly, infected MDCK cell were harvested, washed twice, and resuspended in 100 l PBS at 0, 0.5, 1, 1,5, 2, 2.5, 3, 4, 6, 8, 12, 24, 32, 48, 56, 72 and 96 h.p.i separately. Real-time PCR results showed that although there were no differences among the quantification results of PB2, PB1, PA, HA, NP, and NA genes respectively by two or three primer pairs each ($P>0.05$), the abundance of each gene in cell lysates was distinguishing (Figure 2). Statistical analysis indicated that the amounts of ten genes in cell lysates could be divided into three grades. The first grade included M1 and NP genes. The highest copies at all time points originated from M1 gene, followed by NP gene, with lower abundance than M1 but higher than other eight genes. Amounts of PB2, PB1, PA, HA, NA, M2 and NS1 genes in cell lysates could be classified into the second grade, and there were no significant differences among them ($P>0.05$). The abundance of NEP gene was the last grade, which was significant lower than other nine genes in infected cells ($P<0.01$). Meanwhile, time course kinetic curves of all ten genes in infected cells were not uniform either. The total RNAs amounts of NP and NS1 genes were observed to begin to rise at 3 h.p.i, and were significantly predated compared with other eight genes which escalated from 8 h.p.i (Figures 1 and 2). Quantification results by using PB2-1, PB2-2, PB2-3, PB1-1, PB1-2, PB1-3, PA3, HA1, NP1, NP2, NA1, NA2, M1, M2 and NS1 primers reached their peak copy numbers at 48 h.p.i, whereas PA-1, PA-2, PA-3, HA-2, HA-3, and NEP primers detected the climax copies at 56 h.p.i (Figure 1). Similar to the situation in virions, the differences of copy numbers between these two time points were extremely indistinctive too, which illustrated that the most sensitive time course for viral nucleotide materials detection in infected MDCK cells was also 48 to 56 h.p.i.

## DISCUSSION

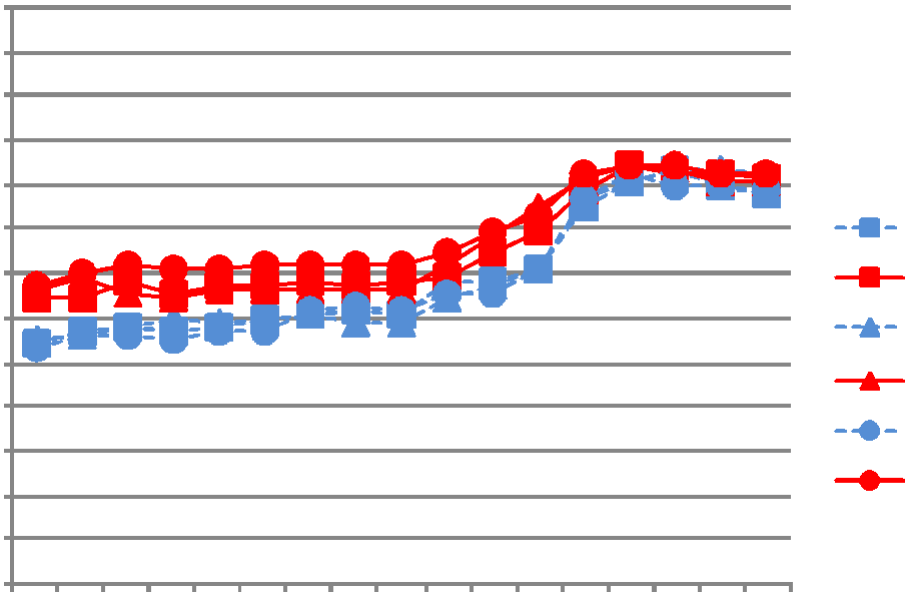
In the 2009 evolving influenza pandemic, rapid and reliable diagnostic methods remains crucial to limit extensive transmission and to initiate therapy. The WHO defines a probable clinical case as one that is confirmed by (1) specific real-time PCR based detection methods, (2) isolation of the pandemic A (H1N1) influenza virus, or (3) detection of 4-fold rise of neutralization antibodies to this virus (WHO, 2009). BSL-2 laboratories with BSL-3 practices are recommended for virus isolation and serology, whereas PCR detection requires only a BSL-2 environment. Of the above diagnostic tests, only real-time PCR based detection methods allow rapid detection of this virus within a few hours.

The genome of the 2009 pandemic A (H1N1) influenza virus comprises eight RNA segments of negative polarity (Smith and Hay, 1982). Viral mRNAs from segments 1 to 6 are monocistronic and encode proteins PB2, PB1, PA, HA, NP and NA separately, whereas viral mRNAs derived from segments 7 and 8 are bicistronic and could undergo alternative splicing for protein expressions, and each encodes two proteins, M2 and M1, NEP and NS1, respectively. In the infected cells, virion RNAs (vRNAs) are transcribed into two different types of transcripts (Hay et al., 1977). The predominant transcripts are the viral messenger RNAs (mRNAs), which contain a 5’ cap structure and a 3’ poly (A) tail. The viral mRNAs are incomplete copies of the vRNAs in that they lack a copy of the last 17-22 nucleotides at the 5’ end of the vRNAs. Another type of viral transcripts, which are complete

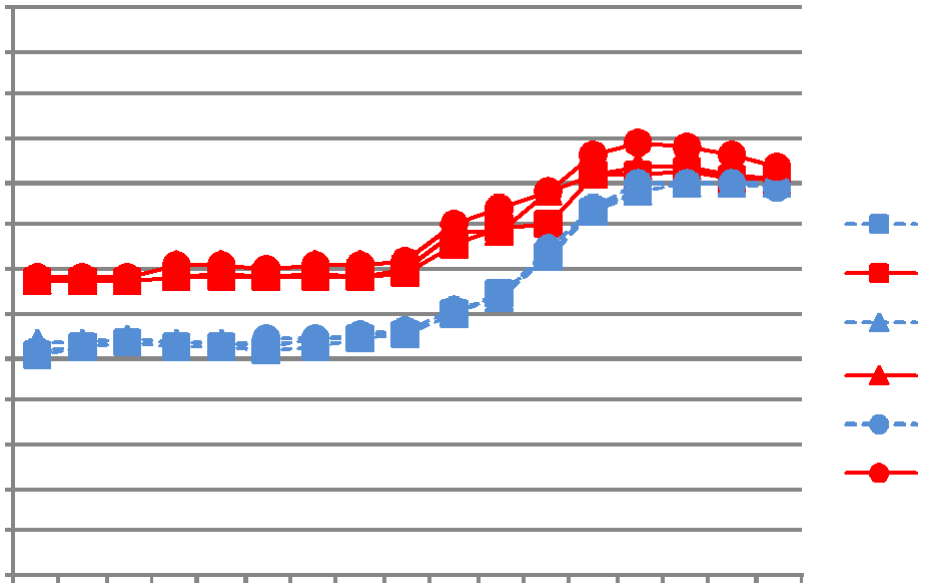
(a)



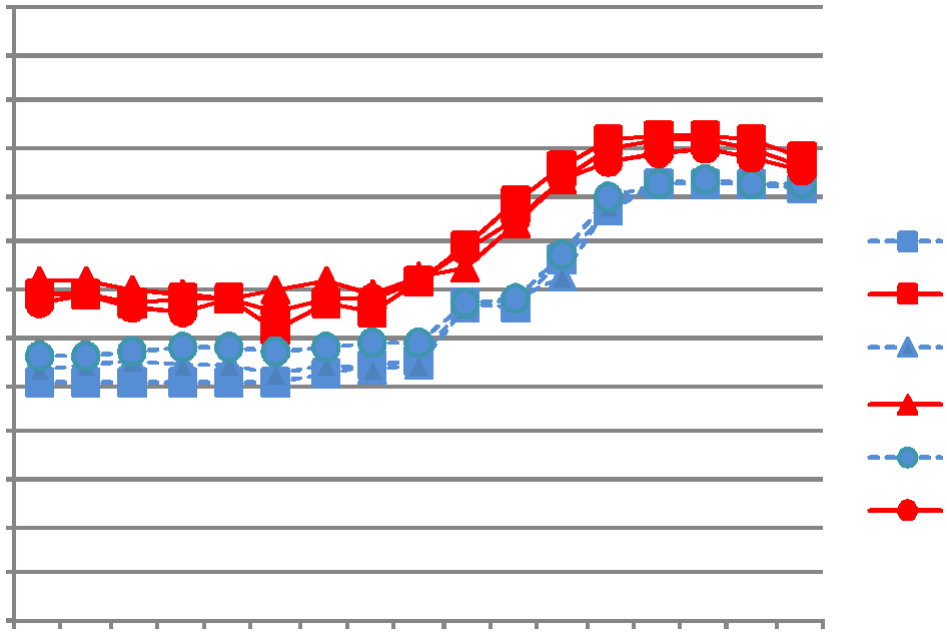
(b)



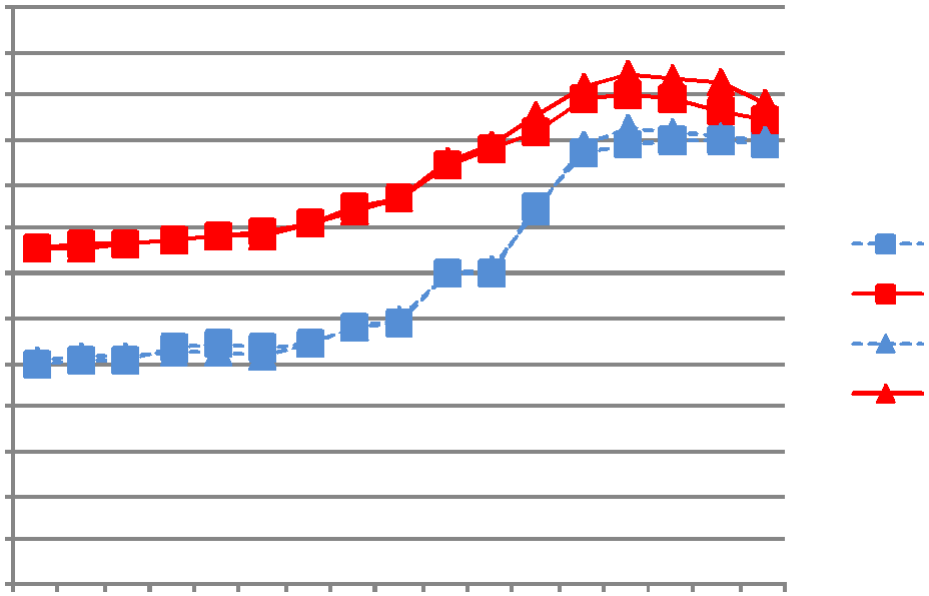
(c)



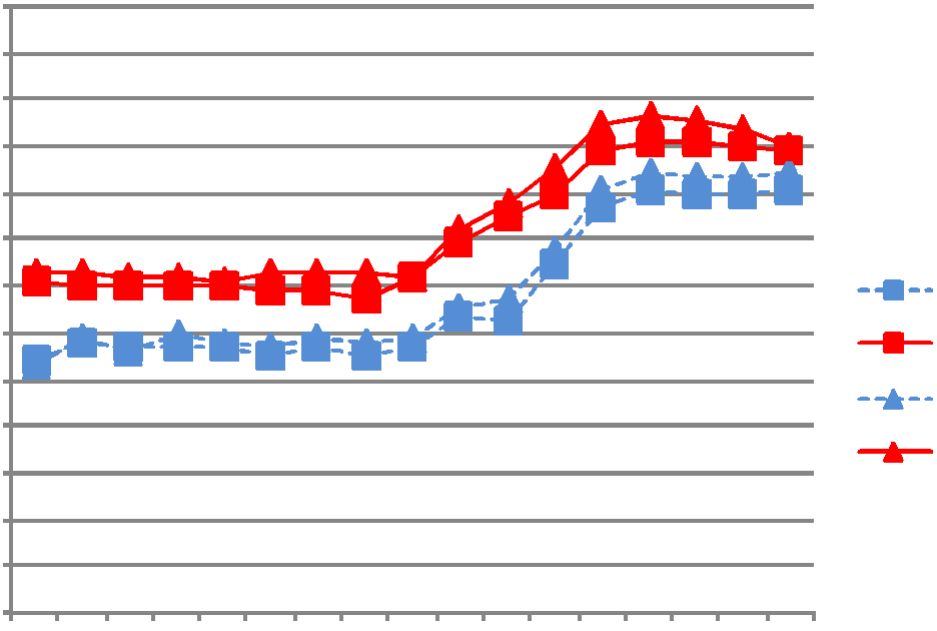
(d)



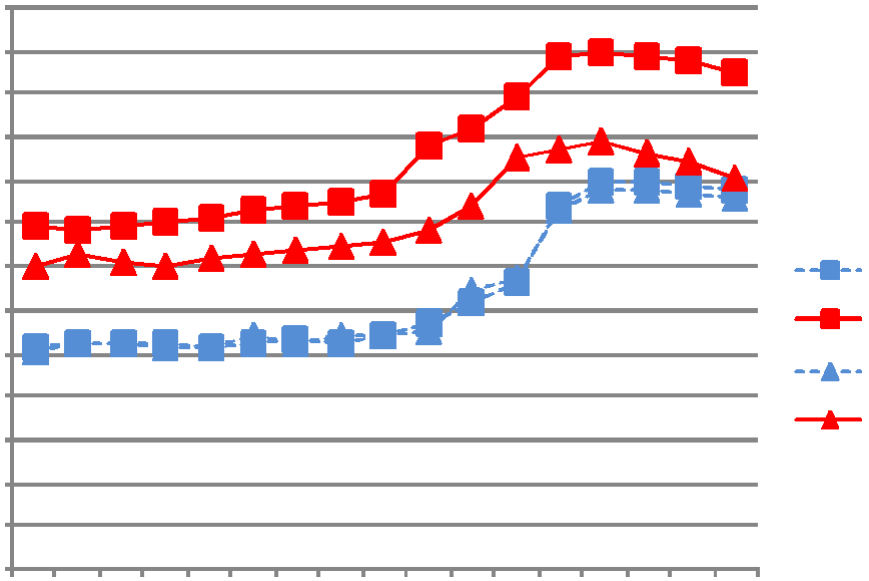
(e)



(f)



(g)



(h)

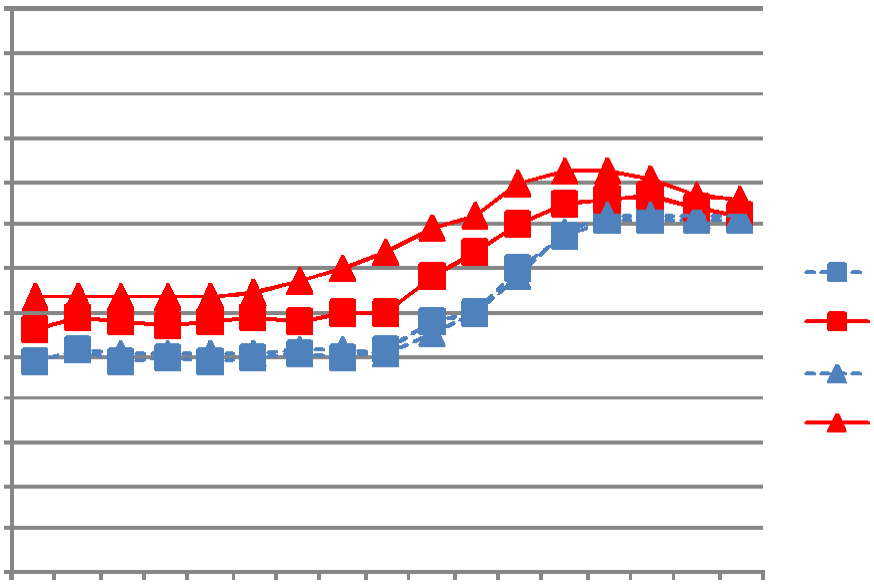


**Figure 1.** Time course kinetic curves of the abundance of each gene from the 2009 pandemic A (H1N1) influenza virus in virions and infected MDCK cells. (a) PB2; (b) PB1; (c) PA; (d) HA; (e) NP; (f) NA; (g) M2 and M1; (h) NEP and NS1.

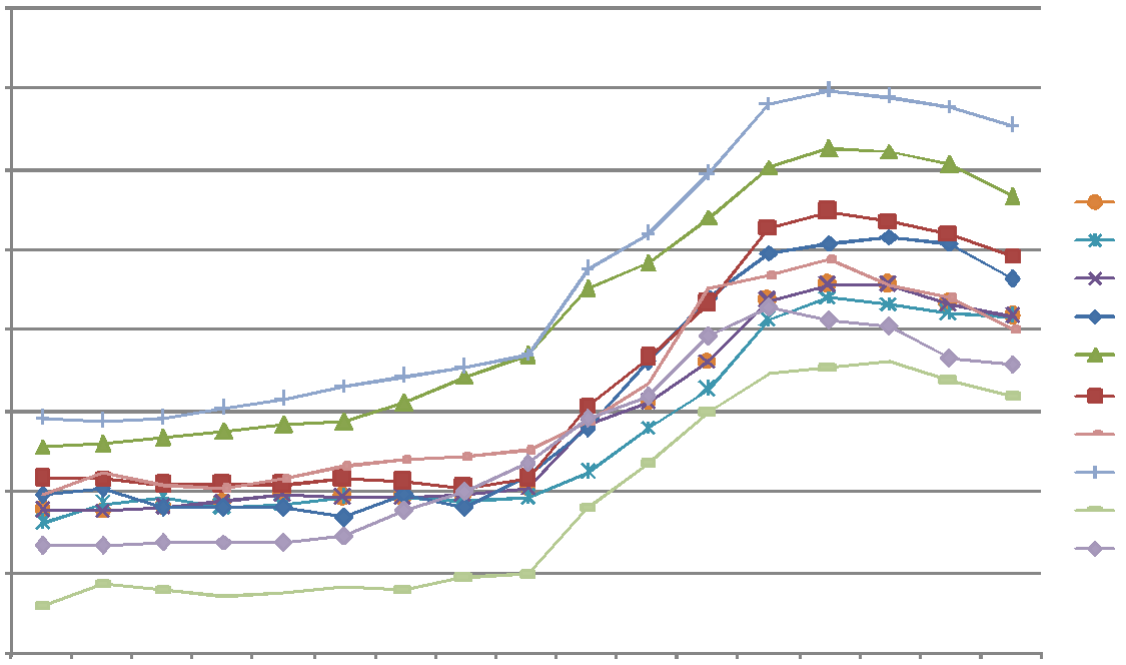


**Figure 2.** Comparison of the time course of the abundance of the 2009 pandemic A (H1N1) influenza virus genome in infected MDCK cells.

copies of the vRNAs, are the templates for progeny vRNA synthesis and represent an intermediate in the replication of the viral genome (complementary RNAs, cRNAs). This may explain why the amounts of total RNAs in infected cells were significantly higher than those in virions, and suggested that after clinical suspected positive specimens were cultured in cells, cell lysates were more recommended to be the nucleotide materials detection object than virions in supernatants.

Our results showed that all eight virion RNA segments were presented in standard virions in approximately equimolar amounts, which demonstrated that this influenza virion packaging is an ordered and selective process that each viral particle must and only have one copy of each eight RNA segments to be incorporated. This phenomenon coincided with other influenza viruses, and also suggested that all primers used in this study exhibited equivalent amplification efficiency, which supplied a firm foundation for further investigation in cell lysates. However, the copy numbers of the genome in cell lysates were distinguishing. The highest amounts at all time points post infection originated from M1 gene, and that is the reason why the conventional PCR detection targeted on M1 gene have been widely used for the first-line screening, since they could identify this virus with high sensitivity. In addition, high conservation between various strains was another reason for the popular applying of M1 gene based PCR detection.

Meanwhile, it was shown by nucleotide materials detection in infected cells that NP was another excellent target gene beside M1 gene, because its abundance in infected cells was only a little lower than M1 gene, but much higher than other genes of influenza virus. High conservation also was the merit of the NP gene targeted detection method to be widely used. However, assays targeted on conserved genes such as M or NP were useful to screen patient specimens for influenza, but could not easily differentiate the 2009 pandemic A (H1N1) virus with other subtype influenza viruses. Assays targeted on surface genes, such as HA, still are extremely critical for sub-typing and antigenic characterization. Another phenomenon in the time course kinetic curve of each gene in virions and infected cells is that the most sensitive time point for viral nucleotide materials detection was 48 to 56 h.p.i. In virions, RNAs amounts of each gene simultaneously began to rise at 8 h.p.i. However, in infected cells, the total RNAs amounts of NP and NS1 genes were observed to begin to rise at 3 h.p.i, whereas other eight genes escalated from 8 h.p.i just as the situation in the virions. We supposed this may be the outcome of the transcriptional characterization of the influenza virus, with which the transcription stage could be divided into different phases. Immediately after infection, primary transcription occurs (Hay et al., 1977).

In this phase, all eight mRNAs are synthesized in equivalent amounts, and this may be 0 to 2.5 h.p.i which

reflected in our results. The following is the second transcription phase, which can be further subdivided into early and late phases. In the early phase of the secondary transcription, NS1 and NP RNAs are preferentially synthesized (Hay et al., 1977; Smith and Hay, 1982; Shapiro et al., 1987). The reason, however, for the preferential early expression of the NS1 and NP proteins is still unknown. It is possible that NP is required for the replication and transcription of viral RNA. NS1 might be required for the regulation of cellular gene expression. During the late phase, RNAs of each gene are synthesized in equivalent amounts, as required for progeny virus genome, and this may be 8 h afterwards post infection demonstrated by our data.

In conclusion, time course kinetic characterizations of copy numbers of the novel A (H1N1) influenza viral genome in standard virions and infected MDCK cells were monitored. The data suggested that cell lysates were more recommended to be the nucleotide materials detection object than virions. Meanwhile, the amounts of each gene in cell lysates was distinguishing. M1 and NP genes might be the optimized choice for viral nucleotide detection in infected cells. Furthermore, the most sensitive time point for nucleotide materials detection in cells was 48 to 56 h.p.i. All these results may provide useful data for rapid diagnosis in the early phase of disease course, and to initiate proper therapy for patients, and further controlling of the pandemic disease.

## ACKNOWLEDGEMENT

This work was supported by the Industry Foundation of Ministry of Health (200802036) and the National Science and Technology Major Projects of Infectious Disease (2009ZX10004-402,2009ZX10004-016).

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