Comparative Analysis of Molecular Methods for Detection of Influenza Viruses

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Authors' contributions

This work was carried out in collaboration between both authors. Author VS designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript and managed literature searches. Author SK managed the analyses of the study and literature searches. Both authors read and approved the final manuscript.

Original Research Article

ABSTRACT

Aims: Influenza is a serious threat to human population worldwide therefore continuous surveillance is required to update influenza seasonal vaccines. A rapid, sensitive, specific and cost effective diagnostic method will be much helpful for patient management in the present scenario. Present study is conceptualized for detection of influenza viruses by molecular methods and compare with 'gold standard' virus isolation.
Study Design: Standard strains of Influenza virus were used to standardize the molecular diagnostic assays and results were then compared with virus isolation.
Place and Duration of Study: Centre for Biotechnology, Maharshi Dayanand University, Rohtak, Haryana, India, between December 2015 and April 2016.
Methodology: Standard strains of Influenza A and B virus were used for influenza virus isolation using virus culturing in MDCK (Madin-Darby Canine Kidney) cell line by following standard tissue culture procedure. Isolated viruses were detected by Hemagglutination assay (HA) and typed by Hemagglutination inhibition assay (HI). Conventional one step RT-PCR, Taqman real time RT-PCR and RT-LAMP (Reverse transcription loop mediated isothermal amplification) were standardized on RNA extracted from standard strains. Sensitivity and specificity of these molecular methods were

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compared with each other as well as with virus culture (gold standard).

Results: Both influenza A and B virus strains were cultured in MDCK cells and produced cytopathic effect during virus culture. Conventional RT-PCR and real time RT-PCR detected both type of Influenza viruses. RT-LAMP also successfully detected and typed influenza viruses. RT-LAMP proved to be more rapid than other two molecular assays.

Conclusion: Molecular diagnostic methods are useful in detection and typing of Influenza viruses and these methods provide results in short period of time when compared with traditional virus culture methods. RT-LAMP is rapid, sensitive, specific and cost effective method for influenza virus detection and subtyping.

ABBREVIATIONS

PFU: Plaque forming units; TPCK: Tosyl phenylalanyl chloromethyl ketone; PCR: Polymerase chain reaction; DMEM: Dulbecco's Modified Eagle's medium; FBS: Fetal bovine serum; BSA: Bovine serum albumin.

1. INTRODUCTION

Influenza viruses are mainly accountable for respiratory illness and significant loss of human lives worldwide. There are many epidemics and pandemics due to Influenza viruses and causes of high morbidity and mortality in humans and other organisms. Influenza pandemics are very old and at least thirty one times have been recorded since the last 400 years [1]. Influenza viruses (family *Orthomyxoviridae)* are characterized by a segmented, minus-single stranded RNA genome. Influenza viruses are irregular in shape, spherical (80-120 nm diameter) or filamentous structures and their surfaces are marked with rod-shaped hemagglutinin (HA) and neuraminidase (NA) spikes [2]. Influenza viruses can be further typed into A, B and C based on the antigenic differences in the nuclear and matrix proteins. Fourth type of Influenza virus (type D) is proposed recently and is related to bovine infections [3-5]. Influenza A viruses are further sub-typed according to the antigenicity of their surface glycoprotein HA and NA [6]. Eighteen different hemagglutinin subtypes (H1-H18) and eleven different neuraminidase subtypes (N1-N11) are detected so far. H1N1 and H3N2 strains are prevalent among human population on seasonal basis [7]. Almost all sub-types of HA and NA have been isolated from aquatic birds as these are the natural host to the viruses [8-9]. Influenza A viruses show more antigenic variability due to antigenic drift and shift phenomenon which may cause both epidemics and pandemics due to influenza. Influenza B viruses show lesser antigenic variation resulting in epidemics whereas influenza C viruses have limited clinical significance [10-11]. New strains of the Influenza A viruses with human infection potential constantly appear due to this drift and shift, like the new avian influenza A (H7N9) virus [12]. Thus influenza affects a large segment of world population resulting in high mortality, morbidity and economic loss. World health organization (WHO) has estimated that globally, about 3-5 million cases of hospitalization and 250,000 to 500,000 deaths occur due to Influenza every year [13]. Considering the impact of influenza on the health, WHO has established an Influenza surveillance network to collect influenza virus isolates and epidemiological data. At present, five WHO collaborating centres are in Atlanta, Beijing, London, Melbourne, Tokyo and 136 National Influenza Centers (NIC) are located in 106 countries [14]. These centres are working in collaboration with each other on continuous surveillance of Influenza virus and provide the data for effective vaccine development against current circulating strains of influenza viruses. Laboratory diagnosis for influenza virus can be by virus isolation in chicken egg embryo or cell culture, antigenic and serological analysis [15], rapid detection kits as well as molecular detection methods based on nucleic acid amplification like NASBA (nucleic acid sequence based amplification) [16], conventional reverse transcription PCR [17-18]. Latest molecular diagnostic methods include Real-time RT-PCR [19-20], DNA Microarray based tests [21-22] and reverse transcription loop mediated isothermal amplification (RT-LAMP) [23-25]. Rapid, sensitive, specific and cost effective methods are required for surveillance, best patient management and virus outbreaks prevention. The present study is conceptualized to detect

influenza viruses by nucleic acid based methods (conventional RT-PCR, real time RT-PCR, RT-LAMP), in comparison with gold standard i.e. virus culture.

2. METHODOLOGY

2.1 Standard Strains of Influenza Virus and MDCK Cell Line

The standard strains of Influenza A (A/New Caledonia/20/99 (H1N1-like) and Influenza B (B/Hong-Kong/330/2001) viruses and stocks of Madin-Darby Canine Kidney (MDCK) cell lines were kindly provided by Department of Microbiology, AIIMS, New Delhi, India.

2.2 Virus Culture

Virus isolation can be done in both chicken egg embryo and cell culture; however egg culture has certain disadvantages like availability of large number of embryonated eggs, presence of other infective agents/pathogens and highly labour intensive. Therefore, we have used MDCK cell line of low passage level (up to #20) for virus culture. Tissue culture flasks having confluent monolayer of MDCK cells were used for growth of influenza viruses. Before inoculation, growth medium (DMEM with 10% FBS) was removed and cells were washed once with phosphate buffer saline (PBS) and twice with virus growth medium (VGM) (serum free DMEM with 2 µg/ml TPCK treated trypsin and BSA 150 mg/ml) [26].HA (Hemagglutination assay) was done to know the virus titre by following WHO standard protocol [26,27]. Two folds serial dilutions were made for both of Influenza viruses A and B to get the HA titre 32. Five hundred micro liters of each dilution (with HA titre 32) was inoculated into respective culture flask. Inoculums was allowed to adsorb for 30 minutes at 37°C, followed by addition of 5-6 ml of serum free, virus growth medium to each flasks. Flasks were incubated at 37°C and observed daily for cytopathic effect (CPE). Flasks were harvested either when 3+ or 4+ CPE (more than one large patch per field of observation) was observed or on seventh day even if no CPE was observed.

2.3 RNA Extraction and Aliquots

Viral RNA was extracted from an aliquot of standard strains of influenza virus using commercial GeneJET Viral DNA/RNA Purification Kit (Thermo Fisher Scientific, USA) following the manufacturer's instructions. RNA was eluted in a final volume of 50 µl in elution buffer (provided with kit) and divided into aliquots of 5 µl for storage at -80°C until use.

2.4 Standardization of Conventional One-step Reverse Transcription (RT-PCR)

One step RT-PCR was standardized with extracted RNA using Verso 1-Step RT-PCR Kit (Thermo Fisher Scientific, USA) using manufacturer's instructions. Separate reactions were standardized for detection of Influenza A and Influenza B virus. For Influenza A virus, primers were taken from the Matrix gene (M gene) as given in the WHO update for molecular diagnosis of Influenza virus [28]. The sequence of primer pairs for influenza A were Influenza A forward (5′-AGTCTTCTAACCGAGGTCGAAACG-3′ and Influenza A reverse (5′-TGGACAAAGCGTCTACGCTGCA-3′). External primers (F3 and B3) used in RT-LAMP were taken as forward and reverse primers for detection of Influenza B virus, which were targeted against the Nucleoprotein gene [29]. The sequence of primer pairs for influenza B were Influenza B forward (5′-ACGGTATCAACACTGGGACA-3′) and Influenza B reverse (5′-TCGGGGTCTGTTTCTTTTGG-3′). The expected product size was 239 bp and 198 bp for Influenza A and B virus, respectively. Reaction mixture was comprised of 12.5 µl of 2× RT-PCR buffers, 1 µl of RT enhancer (provided with kit), 0.2 µmol/L of both forward and reverse primers, 0.5 µl of enzyme mix (Reverse Transcriptase (RT) and Taq polymerase) and RNase free water to make a final volume of 20 µl. Then, 5µl of viral RNA were added to the test tube to make a final volume of 25 µl. Negative controls were also included which contain 5 µl of RNase free water in place of viral RNA. Reaction mixtures were incubated at 50°C for 50 min (reverse transcription), 94°C for 15 min (RT inactivation), then 40 cycles of PCR: denaturation at 94°C for 30 sec, annealing at 55°C for 45 sec and extension at 72°C for 1 min, followed by a final extension at 72°C for 7 min. The RT-PCR amplicons were visualized by electrophoresis in 2.5% (w/v) agarose gel using standard gel electrophoresis procedures.

2.5 Development of Taqman One-Step Real-Time RT-PCR Assay

Viral RNA extracted from the standard strains of influenza was used to standardize the Taqman

One-Step Real-Time RT-PCR Assay. The Influenza A primer/probe set is for universal detection of type A Influenza virus and it amplified a product of 105 bp of Matrix gene M1 of Influenza A viruses; while the Influenza B primer/probe set amplified a product of 102 bp of Nonstructural protein (NS) gene of Influenza B virus. The details of primers and probes used are given in the Table 1.

A single-step Real-Time RT-PCR was carried out using SuperScipt III Platinum One-Step RT-PCR system (AgPath-ID^{TM} One-Step RT-PCR Kit, Applied Biosystems, ThermoFischer Scientific, USA). Two sets of primer/probe (Influenza A and B) in separate tubes/wells were used in RT-PCR. Each primer and probe was used at a working concentration of 40 µmol/L and 10 µmol/L, respectively. The 25 µl reaction comprised a combination 12.5 µl of 2× master mixture, 1.5 µl of primer/probe mixtures (0.5 µl each), 1 µl of 25× enzyme mixture, 5 µl of RNase free water, 5 µl of viral RNA or 5 µl of RNase free water for the negative control. The cycling parameters for Real-Time RT-PCR were: reverse transcription at 50°C for 30 min; Enzyme activation at 95°C for 15 min; 45 cycles of two step PCR amplification of, 95°C for 15 sec and 55°C for 1 min. Fluorescence data was collected during the 55°C (annealing) step [30]. The threshold cycle (Ct) is calculated from the curve as the minimum number of cycles at which florescent signal/normalized reporter signal (ΔRn) crosses the baseline or threshold value.

2.6 Reverse Transcription Loop-mediated Isothermal Amplification (RT-LAMP)

RT-LAMP is standardized for the detection of influenza viruses using viral RNA extracted from the standard strains of Influenza A and B viruses. Four primers are used in RT-LAMP which corresponds to six regions on the template RNA. Two primers are outer (F3 and B3) while another two are inner (FIP and BIP). The details of primers are given in Table 1. The primers used for influenza A virus were taken from the matrix gene [23] and for influenza B virus primers were

Table 1. Primers and probes used in standardization of real time RT-PCR and RT-LAMP for Influenza A and B viruses. K, R and Y represent mixture of (G,T), (A,G) and (C,T) respectively

Assay	Primers and probes	Sequence (5'→ 3')	Working Concentration (µmol/L)	Reference
Real time RT-PCR	Influenza A forward	GACCRATCCTGTCACCTCTGAC	40	
	Influenza A reverse	AGGGCATTYTGGACAAAKCGTCTA	40	[30]
	Influenza A probe*	TGCAGTCCTCGCTCACTGGGCACG	10	
	Influenza B forward	TCCTCAAYTCACTCTTCGAGCG	40	
	Influenza B reverse	CGGTGCTCTTGACCAAATTGG	40	[31]
	Influenza B probe*	CCAATTCGAGCAGCTGAAACTGCGGTG	10	
RT-LAMP	**Influenza A virus**			
	Inf A-F3	TGGTGCACTTGCCAGTTG	5	
	Inf A-B3	CCAGCCATCTGTTCCATAGC	5	
	Inf A-FIP	TGCTGTGAATCAGCAATCTGTTACAGGATGGGAACAGTGACC	40	[23]
	Inf A-BIP	AGACAAATGGCTACTACCACCCGTAGTGCTAGCCAGCACC	40	
	Influenza B virus			
	Inf B-F3	ACGGTATCAACACTGGGACA	5	
	Inf B-B3	TCGGGGTCTGTTTCTTTTGG	5	
	Inf B-FIP	GCAAGGGTTGCTGGTCTAATGAATTGACAAAACACCGGAAGA	40	[29]
	Inf B-BIP	CCCACCAAGCAACAAACGAACCCGACATCATCTTCACTGC	40	

TaqMan® probes were labeled at the 5'-end with the reporter molecule 6-carboxyfluorescein (FAM) and with the quencher, Blackhole Quencher 1 (BHQ1) (Biosearch Technologies, Inc., Novato, CA) at the 3'-end

taken from the nucleoprotein gene [29]. The reaction was standardized using Loopamp RNA amplification kit (Eiken Chemical Co. Ltd, Japan) following the manufacturer's instruction. Reaction mixture was prepared in two different tubes for influenza A and B virus respectively. Each reaction cocktail was made of 12.5 µl of 2× reaction buffer, 0.5 µl of enzyme mix, 1 µl of each F3 and B3 primers (5 µmol/L), 1 µl each FIP and BIP primers (40 µmol/L) (Table 1) and RNase free water was added to make final volume 20 µl. Then 5µl of viral RNA was added to make a final reaction volume of 25 µl. Negative controls were also included. The reaction is incubated at 63°C for 1 hour (amplification) and 80°C for 15 min (enzyme inactivation). The amplicons were visualized on 2.5% (w/v) agarose gel using standard gel electrophoresis procedures.

2.7 Sensitivity and Specificity of Molecular Assays

Standard strain of Influenza A virus (A/New Caledonia/20/99 (H1N1-like) with known viral titre (3×10^3 PFU/µl) was used to compare the sensitivity of three molecular assays i.e. conventional RT-PCR, real time RT-PCR and RT-LAMP. The RNA was extracted from 100 µl of standard strain of influenza A virus using GeneJET Viral DNA/RNA Purification Kit (Thermo Fisher Scientific, USA) following the manufacturer's instructions and eluted in final volume of 50 µl. Final eluted viral RNA is supposed to have viral titre of 10^3 PFU/µl. Decimal dilutions of viral RNA were prepared in RNase free water from 10^{-1} to 10^{-5} PFU/reaction and were used as sample RNA in all three assays as described earlier. When dealing with RNA, care was taken to minimize freeze/thaw cycles and all the three molecular assays were

setup on the same day to minimize viral RNA degradation.

Specificity of the molecular assays was checked against other viruses causing influenza like illness such as parainfluenza viruses, adenoviruses and Respiratory syncytial virus (RSV).

3. RESULTS AND DISCUSSION

A rapid, specific and sensitive method to detect influenza virus is much important for effective patient management and to early start of antiviral therapy. It also helps in surveillance of newly emerging influenza strains, vaccine development and to check the spread of virus in early stages, hence preventing epidemics or pandemics of influenza. Conventional methods like virus isolation using egg embryo culture, cell culturing, shell vial culturing, antigen detection and serological analysis are used for the laboratory diagnosis of influenza viruses [32]. In our study virus culture in MDCK cell line both influenza A and B viruses showed cytopathic effect (CPE) in MDCK cells on 4th days of post inoculation. CPE due to influenza virus in MDCK cells was seen as rounding and lacy appearance of cells (Fig. 1). HA titers of both isolates were found to be 1:256 for influenza A and 1:64 for influenza B respectively.

Although virus isolation using cell culture is considered as 'gold standard', it is labour-intensive and the results depend on the optimal sample transport. Moreover, the virus can become undetectable in the later course of the infection due to decline in viable virus count with time [33-35]. Other diagnostic techniques, such as viral antigen detection and shell vial culture provide results faster but generally
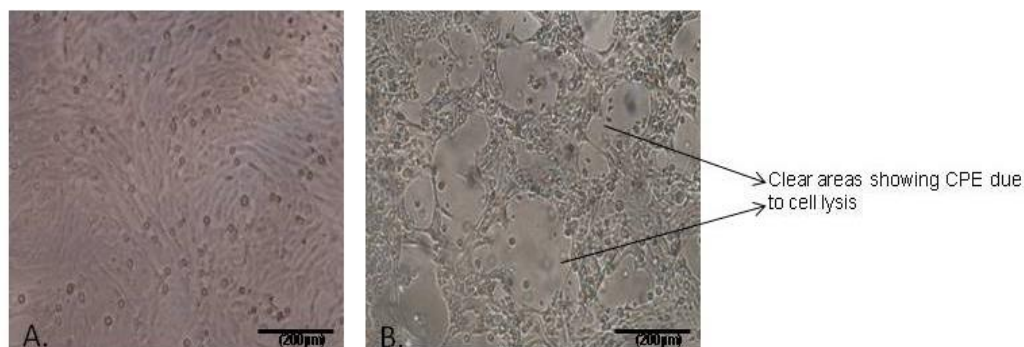

Fig. 1. Influenza virus culture in MDCK cell line A. Normal cell line B. Cell line showing cytopathic effect (CPE)

are less sensitive than conventional cell culturing [36-39]. Rapid detection kits are also available to detect influenza viruses and most of these tests distinguish either influenza A or influenza B but are unable to further subtype influenza A viruses. Although these rapid tests give results in less than 30 min, but the performance and reliability are the issues faced by such tests [40-43].

Molecular detection methods include conventional PCR based tests, NASBA, Real time PCR, RT-LAMP and microarray based assays. Conventional RT-PCR is successfully applied in the detection and typing of influenza viruses. In most of the developing countries conventional RT-PCR is preferred over real time PCR due to high instrument and reagent costs of the latter [44,45]. In our study, standardized conventional RT-PCR detected both influenza A and B virus while no amplification was seen in negative controls. Influenza A virus showed a strong band of 239 bp while influenza B virus showed the product band of 198 bp (Fig. 2).

Real time RT-PCR is rapid, sensitive and specific method for diagnosis of influenza viruses [19,20,46] and the results obtained are same with that of virus isolation but in shorter time. Real time RT-PCR using Taqman probes is more rapid, sensitive and specific than conventional RT-PCR [46]. As Taqman probes are costly so SYBR Green based Real time RT-PCR can also be used to reduce cost, but it also compromise with the specificity of the assay. In our present study, both Influenza A and B virus showed Ct value of 17 (Fig. 3) in Taqman Real-Time RT-PCR assay.
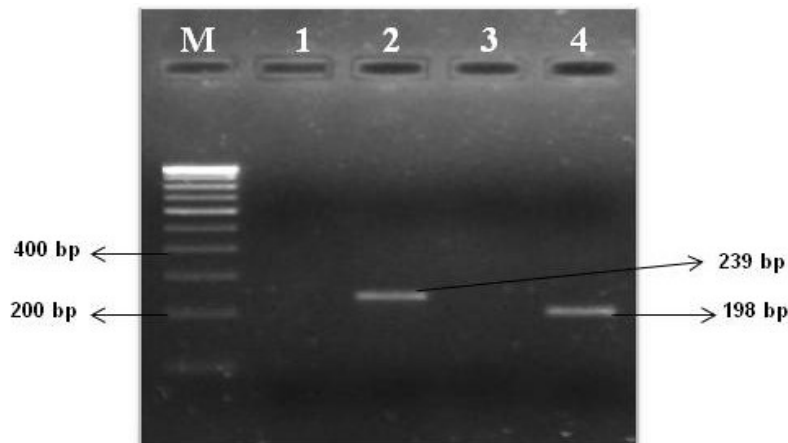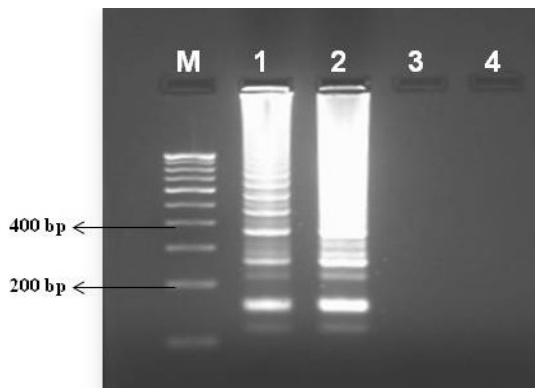

Fig. 2. Agarose electrophoresis results of conventional one step RT-PCR products. Lane M, 100 bp DNA ladder; lane 1 and 3, negative control for Influenza A and B virus respectively; lane 2 and 4, positive control for influenza A and B virus respectively
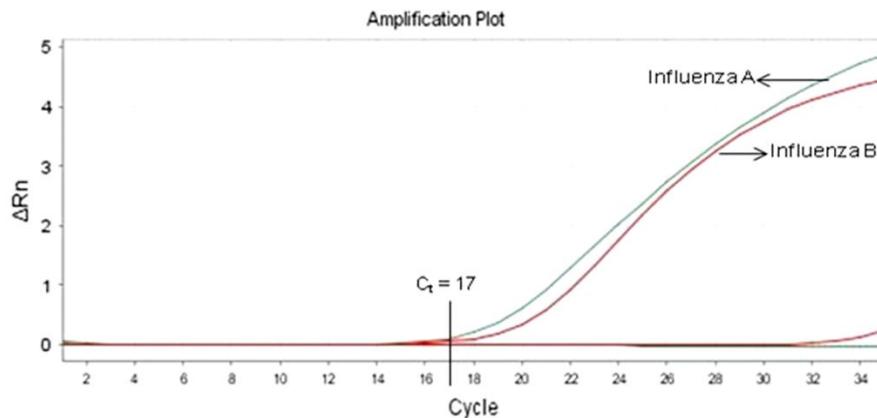
Fig. 3. Amplification curve of Taqman real time RT-PCR for Influenza A and B virus, both viruses showing Ct value 17

Loop mediated isothermal amplification (LAMP) is an efficient method of nucleic acid amplification like PCR based methods but it amplify the target sequence under isothermal conditions. It is highly specific as four primers corresponding to six target sequences were used for amplification [47]. LAMP reaction when combined with reverse transcription i.e. RT-LAMP, has been used to detect various RNA viruses like Japanese Encephalitis, Chikungunya, Dengue, Zika virus, Norovirus, human metapneumovirus, Respiratory Syncytial virus and West Nile virus [48,49]. RT-LAMP can be customized for real time detection using dyes such as SYBR green or Calcein like Real Time RT-PCR, which eliminate the need of electrophoresis for analysing the results [50]. RT-LAMP assay has been applied successfully for Influenza virus detection and sub-typing [23-25,29,48-53].

In our study, RT-LAMP detected Influenza A and B virus presence in the respective tubes while no amplification was observed in negative controls, as shown in Fig. 4.

Fig. 4. Gel electrophoresis analysis of RT-LAMP products. Lane M, 100 bp DNA ladder; lane 1 and 2, positive controls for influenza A and B virus respectively; lane 3 and 4, negative controls for influenza A and B virus respectively

In our study, we found that all three molecular detection assays i.e. Conventional RT-PCR, Real time RT-PCR and RT-LAMP showed similar results when compared with the virus culture. All the three molecular methods are highly specific as none of these methods showed positive results with Adenoviruses, Parainfluenza viruses and RSV. Sensitivity of molecular assays was also compared and we found that RT-LAMP assay showed similar results as that of Real time

RT-PCR but in less time. RT-LAMP was found to be ten times more sensitive than conventional one step RT-PCR (Fig. 5). In the study of Poon et al. [23], sensitivity of RT-LAMP was found ten times greater than that of conventional RT-PCR. Imai et al. [51] have reported 100 times sensitivity of RT-LAMP in comparison to RT-PCR for detecting avian influenza virus. Previous studies has shown that RT-LAMP have comparable sensitivity with that of real time RT-PCR for influenza viruses [24,53].
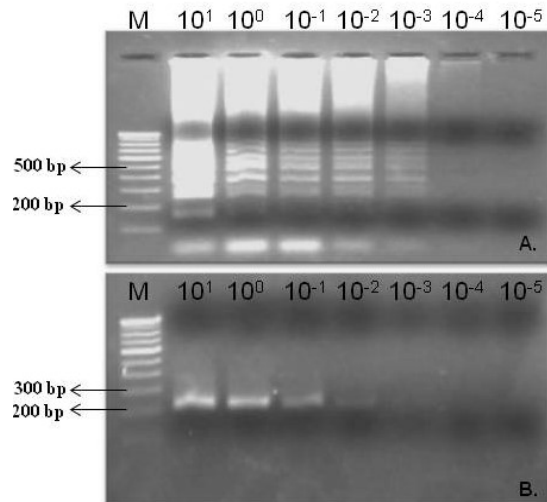
Fig. 5. Sensitivity of RT-LAMP in comparison to conventional RT-PCR. Influenza A virus strain (A/New Caledonia/20/99 (H1N1-like), with known titre of $3X10^3$ PFU/µl, was diluted ten-fold serially and detected with RT-LAMP (gel A) and conventional RT-PCR (gel B)

4. CONCLUSION

The current study showed that the molecular detection methods are fast and provide similar results as that of traditional virus culture methods; therefore these methods provide rapid means of Influenza virus diagnosis. Sensitivity and specificity of RT-LAMP were comparable to that of real time RT-PCR and it was proved to be ten times more sensitive, than conventional RT-PCR. RT-LAMP was rapid and cost effective method for influenza A and B virus detection than other two methods. Development of such rapid sensitive specific and cost effective assays will be useful for influenza diagnosis, early patient management, surveillance and controlling the spread of the disease.

ACKNOWLEDGEMENTS

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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