CHAPTER 1

INTRODUCTION

Influenza is a serious respiratory sickness which poses a major concern for public health and economy around the world. Influenza viruses are accountable for this highly infectious disease which infects the mucosal lining of respiratory tract. Seasonal outbreaks, epidemics and pandemics have been witnessed for influenza virus and have resulted in immense loss to public health and economy (Kamp and Reyes-Terán, 2006; Wright et al., 2007; Shaw and Palese, 2013; WHO, 2016). Seasonal influenza viruses are the cause of mild illness but may also result in severe illness or even death, particularly in high-risk individuals including pregnant women, children, aged people, immunocompromised persons and the people having chronic medical conditions. Seasonal influenza is concentrated during winter in temperate zones and in rainy seasons in tropical zones, but influenza pandemics can appear at any time of the year (Finkelman et al., 2007; WHO, 2016). Influenza pandemics are the rare events, caused by those virus subtypes against which none or very less individuals have immunity and thus rapidly spread across the globe. In the last century, three major pandemics were witnessed; 1918 (Spanish flu/H1N1), 1957 (Asian flu/H2N2) and 1968 (Hong Kong flu/H3N2) (Lazzari and Stohr, 2004; Kilbourne, 2006). Mortality of 1918 pandemic is estimated to be 50-100 million whereas in 1957 and 1968 approximately one million people died during each pandemic (Johnson and Mueller, 2002). In current century, we have already faced influenza pandemic due to a novel Influenza A(H1N1) virus strain which emerged in April 2009 in California and Mexico and spread across the globe in a short span of time (CDC, 2009). This strain was spread across 214 countries across the world and was responsible for about 284,500 deaths (Dawood et al., 2012).

Influenza viruses are grouped in the family Orthomyxoviridae which is characterized by segmented, negative-sense, single stranded RNA genome (Shaw and Palese, 2013). This family contains seven genera viz., Influenzavirus A, Influenzavirus B, Influenzavirus C, Influenzavirus D, Isavirus, Quaranjavirus and Thogotovirus (ICTV, 2016). Out of the first four genera, Influenzavirus A and Influenzavirus B are responsible for seasonal epidemics while Influenzavirus C causes mild reparational illness and Influenzavirus D affects cattle and not known to cause any infection in humans. The other
three genera including *Isavirus*, *Quaranjavirus* and *Thogotovirus* are of not known to cause any significance infection in humans (Hause et al., 2014; CDC, 2016; Ferguson et al., 2016). Influenza viruses can be spherical (80-100 nm), irregular or filamentous (100-300 nm) in shape and have two main surface glycoproteins i.e., Hemagglutinin (HA) and Neuraminidase (NA) (Shaw and Palese, 2013). These proteins give the appearance of little spikes on the virus surface under electron microscope and are responsible for the virus entry inside of the host cells. Influenza viruses are classified into four genera on the basis of differences in the nuclear and matrix proteins of the virus. Influenza A viruses are further divided into subtypes according to their surface antigenic proteins, HA and NA (Cox and Subbarao, 1999, 2000). Till now, there are 18 different HA (H1-H18) subtypes and 11 different NA (N1-N11) subtypes (CDC, 2016). This genetic diversity of influenza A viruses makes them very successful pathogens and helps them to evade the host immunity (Shaw and Palese, 2013).

For control and prevention of influenza, use of antivirals and vaccines has been recommended. Vaccination is considered the best approach for disease control and to prevent its transmission, but there is no universal influenza vaccine yet available due to highly changeable nature of influenza viruses. Each year, influenza vaccines are updated according to the circulating strains and this information is provided by the surveillance system of WHO’s Global Influenza Surveillance and Response System (GISRS) for both the hemispheres (WHO, 2012; Webster and Govorkova, 2014). Surveillance provides the data from various regions to WHO network of influenza laboratories for appropriate vaccine strains selection. Antivirals treatment proved effective against influenza if given within 24-48 h of the onset of disease (Moscona, 2005). Hence, accurate and rapid diagnosis of influenza is crucial for timely intervention of therapy, clinical management and thus controlling the spread of disease.

Clinical symptoms of influenza and other respiratory infections can be very similar, thus making it hard to differentiate. Only laboratory diagnosis can provide an accurate influenza diagnosis. Presently, influenza diagnosis is done by virus isolation, antigen detection, serological analysis and molecular methods. However, each of these methods has its own advantages and limitations. Virus isolation is considered as ‘gold standard’ and it is a robust technique for the detection of influenza but it is labour-
intensive and time consuming. Virus isolation is performed in conventional chicken egg embryo culture or using cell culture. Both of these methods require high level of biosafety, trained professionals and the results are obtained in several days. Improvements of conventional virus culture like shell vial culture has also been utilized but these methods compromise with sensitivity of the assay (Espy et al., 1986; Matthey et al., 1992; George et al., 2002). Rapid detection kits work on the principle of antigen detection and are rapid, easy to perform, do not require sophisticated instruments but lacks in specificity and sensitivity (Louie et al., 2010). Serological assays focus on detection of influenza specific antibodies e.g., enzyme linked immunosorbent assay (ELISA), hemagglutination inhibition assay (HI), immunofluorescence assays, virus neutralization assays etc. The sensitivity of these assays is low when compared with virus culture and molecular assays (Stephenson et al., 2009).

Molecular diagnostic methods are based on detection of viral nucleic acids thus are highly specific and sensitive. Molecular diagnosis of influenza infections include conventional reverse transcriptase polymerase chain reaction (RT-PCR), real-time RT-PCR (rRT-PCR), simple amplification-based assay (SAMBA), nucleic acid sequencing based amplification (NASBA), reverse transcriptase loop-mediated isothermal amplification (RT-LAMP), DNA microarray and sequencing based tests. Introduction of conventional RT-PCR and rRT-PCR as diagnostic tools has revolutionised the laboratory diagnosis approach. Using molecular diagnosis turnaround time is reduced to 4-6 hr as compared to two weeks by virus isolation. Conventional RT-PCR is a rapid, specific and sensitive diagnostic method for detection and typing of RNA virus, like influenza virus (Zhang and Evans, 1991; Pregliasco et al., 1998; Herrmann et al., 2001). Post-PCR analysis makes conventional RT-PCR, time-consuming and prone to laboratory contamination. Real-time RT-PCR solves this problem by using fluorescent dye (such as SYBR green) or gene specific fluorescent probes (TaqMan assay) during the amplification and enables the real time monitoring of the amplified product. It is faster, sensitive and specific than conventional RT-PCR. (van Elden et al., 2001; Spackman et al., 2002; Mackay et al., 2002; Spackman and Suarez, 2008; Carr et al., 2009). But cost is the major limitation of rRT-PCR, as it requires a thermal cycler, computer/optics for fluorescent data generation and collection and trained personnel, making it hard to use in
laboratories of developing countries. Both conventional and real time RT-PCR can be transformed into multiplex PCR by using the many sets of primers in a single reaction tube thereby detecting more than one pathogen in a single reaction. Multiplex RT-PCRs have been applied successfully in detecting influenza viruses (Spackman et al., 2003; Wu et al., 2008; Chaharaein et al., 2009).

Sequencing based methods like DNA microarray, pyrosequencing etc. are also used for influenza diagnosis (Dawson et al., 2007; Townsend et al., 2006; Gall et al., 2009). But these assays are complex, labour intensive, prone to cross-contamination and expensive to be used at resource-limited laboratory conditions of the developing countries. NASBA, SAMBA and RT-LAMP are nucleic acid detection methods based on isothermal amplification of viral RNA in the clinical sample. Both NASBA and SAMBA has been applied successfully for the detection of Influenza A and B viruses (Lau et al., 2004; Wu et al., 2010; Ge et al., 2010; Wang et al., 2013).

Loop-mediated isothermal amplification (LAMP) is a specific, efficient and rapid technique that is similar to PCR amplification but the target DNA amplification is under isothermal conditions. This method uses a special DNA polymerase having strand displacement activity and two pairs of specially designed primers which identify six distinct sequences on the target DNA (Notomi et al., 2000). The method is applicable to amplification of RNA templates by combination with a reverse transcription reaction (RT-LAMP). Due to the use of four primers (or six, including two loop primers), RT-LAMP is highly specific and faster than PCR because amplification is carried out under isothermal conditions. LAMP has been effectively applied for the detection of pathogens such as *Mycobacterium tuberculosis* (Iwamoto et al., 2003) and DNA viruses such as herpes viruses (Ihira et al., 2004; Okamoto et al., 2004). RT-LAMP assay has successfully detected RNA viruses, including SARS virus (Thai et al., 2004; Fujino et al., 2005), RSV (respiratory syncytial virus) (Valette and Aymard 2002), influenza viruses (Poon et al., 2005; Ito et al., 2006; Gu et al., 2010; Parida et al., 2011; Nakauchi et al., 2011; Luo et al., 2015), dengue virus, West Nile virus and Japanese encephalitis virus (Li et al., 2011).

A rapid, accurate and sensitive diagnostic assay plays an important role in early patient management, timely intervention of antiviral therapy and in preventing spreading
of infection at community level. Thus, this study entitled “Development of Reverse Transcriptase-Loop-Mediated Isothermal Amplification (RT-LAMP) for Rapid Detection and Typing of Influenza Viruses” was conceptualized for cost-effective, rapid, sensitive, and specific diagnosis of influenza viruses. The present study is focused on applicability of RT-LAMP assay as a diagnostic tool for influenza viruses and assessing its sensitivity and specificity in comparison with other methods including virus isolation, conventional RT-PCR and real-time RT-PCR. In the present study, RT-LAMP was standardized on the reference strains of influenza A and B viruses. The clinical applicability of standardized RT-LAMP assay was checked on the patients’ samples showing influenza like illness, collected during September 2014 to August 2017 from PGIMS Rohtak, Haryana, India. The results of RT-LAMP were compared with conventional RT-PCR and real time RT-PCR. The sensitivity and specificity of RT-LAMP was compared with virus culture (gold standard) using MDCK (Madin-Darby canine kidney) cell line. Sequence analyses and phylogenetic analyses were also performed with some of the influenza positive isolates obtained during the study.