Influenza viruses are notorious pathogens associated with recurring acute respiratory infections and are responsible for considerable morbidity and mortality among human population around the world (Cox and Subbarao, 1999; WHO, 2016). Despite the availability of antivirals and effective vaccines, influenza viruses still remain a cause for annual epidemics and rare pandemics. Individuals such as young children, elderly people, pregnant women, persons having cardiovascular ailments and immunocompromised people are at high risk of developing severe complications due to influenza (Harper et al., 2005). Antiviral therapy is effective if started within 36-48 hours after the onset of infection (Aoki et al., 2003). Thus, accurate and rapid diagnosis of influenza not only saves the lives of high-risk individuals but also helps in its containment, prevention and surveillance of emerging new strains. Influenza like illness (ILI) is characterized by high grade fever, dry cough, sore throat, muscle ache and discomfort. Clinical diagnosis, solely based on symptoms of influenza infections, is difficult as different respiratory viruses produce almost similar symptoms and also co-infections with other pathogens may not become evident (Cox and Subbarao, 1999; Peltola et al., 2005).

Laboratory diagnosis can provide confirmation of influenza infection and information about virus strain. Different laboratory diagnostic techniques have been utilized for identification and characterization of influenza viruses. These include traditional virus culture, serological methods, immunofluorescence, rapid tests and molecular methods. Virus culture is the “gold standard” for influenza diagnosis and can be done by either embryonated egg culture or cell culture (Hsiung, 1984; Leland and Ginocchio, 2007). The disadvantages of virus culture are that it takes time to grow viruses and results are obtained after 10-14 days. Also, the need of well equipped laboratory with biosafety facilities and labour-intensive procedures make it difficult to use virus culture for routine diagnosis of influenza viruses as needed during influenza outbreaks (Stokes et al., 1988). Modern virus culture methods like shell vial culture can provide results in 24-48 hours, but these are laborious, require well-trained professionals and are not as sensitive as traditional culture methods (Kamp and Reyes-Teran, 2006). Serological methods on the other hand, need paired serum samples from acute and convalescent phase and take time (3-4 weeks) to produce results (Zambon et al., 2001). Immunofluorescence-
based tests as direct fluorescent antibody (DFA) and indirect fluorescent antibody (IFA) tests need adequate amount of clinical sample and their results may vary for different samples from same person. Immunofluorescence tests can detect influenza A and B viruses successfully but are not useful to differentiate between subtypes of influenza A viruses (Kim and Poudel, 2013). Rapid influenza diagnostic tests (RIDTs) can provide results within 15-30 min but are not sensitive enough and also not reliable for subtyping of influenza A viruses (Koul et al., 2015). RIDTs provide better results in children than in adults due to high viral loads in children samples (Cruz et al., 2010).

Molecular methods have emerged as better diagnosis approach due to their accuracy, speed, specificity and sensitivity (Ellis and Zambon, 2002; Wang and Taubenberger, 2010; Landry, 2011). Influenza viruses can be detected by using different molecular diagnostic techniques like conventional RT-PCR and real-time RT-PCR, NASBA, DNA microarray, SAMBA, sequencing-based tests and RT-LAMP. Although PCR-based methods are rapid, sensitive and highly specific, however these methods require high cost equipments and technical expertise (Wang and Taubenberger, 2010). NASBA and SAMBA are two non-PCR-based approaches which have been evaluated for the diagnosis of influenza viruses (Moore et al., 2008; Wu et al., 2013). Presence of RNA secondary structures, lengthy primer target sites and poor hybridization of primers are some limitations of NASBA (Luebke et al., 2003). DNA microarray and sequencing-based tests have also been used successfully for detection of influenza viruses but are labour-intensive, require technical expertise and costly instrumentation (Vemula et al., 2016). RT-LAMP (reverse transcriptase-loop-mediated isothermal amplification) is a rapid, simple, non-PCR-based amplification technique, which has been used for diagnosis of viruses based on viral nucleic acids detection (Notomi et al., 2000; Parida et al., 2008).

The aim of the present study was to develop RT-LAMP assays for rapid, sensitive and specific detection, typing and subtyping of influenza viruses and their evaluation on clinical samples. Among influenza viruses, only influenza A and B viruses are responsible for majority of human infections while, influenza C viruses cause mild illness in humans. Influenza A and B viruses are accountable for annual influenza epidemics whereas influenza A viruses are associated with rare pandemics (Shaw and Palese, 2013). Therefore, the present study was focussed on rapid, sensitive, specific and cost-effective
diagnosis of influenza A and B viruses and subtypes of influenza A viruses viz., seasonal H1N1, H3N2 and A(H1N1)pdm09.

Separate RT-LAMP reactions were optimized for typing and subtyping of influenza viruses. The optimization of RT-LAMP reactions was done on reference strains of influenza A and B viruses. Typing RT-LAMP reactions were optimized separately for detection of influenza A and B viruses. For detection of influenza A viruses, we have used published primers targeted against matrix gene (M gene) (Poon et al., 2005). Viral RNAs were extracted from three reference strains of influenza A viruses (A/California/7/2009 (H1N1) pdm09-like virus; A/Malaysia/2089302/2009 (H1N1)-like virus and A/Texas/50/2012 (H3N2)-like virus) and were used for optimizing separate RT-LAMP reactions. The limit of detection (LOD) was assessed by applying RT-LAMP in triplicate reactions on ten-fold serial dilutions of the respective reference strains with known virus titre (expressed as PFU/ml). The assay was successful in detecting all three influenza A strains with detection limit of 0.01 PFU/reaction. In a study by Poon et al. (2005), LAMP assay was developed for detection of influenza A viruses. They first converted viral RNA into cDNA using reverse transcription (RT) step and afterward cDNA was used for LAMP reaction, making it a two step reaction. We used one-step RT-LAMP reaction for amplification of viral RNA with more rapid amplification. Typing RT-LAMP for influenza B virus was optimized using extracted viral RNA from reference strain (B/Brisbane/60/2008-like virus) and using published primers from nucleoprotein gene (NP gene) (Ito et al., 2006). The detection limit of the reaction was found to be 1 PFU/reaction. Both of the reactions were found to be highly specific and showed no cross-reactivity when applied on other related respiratory viruses including respiratory syncytial virus (RSV), human metapneumovirus (HMPV) and human parainfluenza viruses. For subtyping of influenza A viruses into H1N1, H3N2 and A(H1N1)pdm09, primers were selected from hemagglutinin gene (HA gene). For seasonal influenza strains (H1N1 and H3N2), primers were designed and detection limits of both the reactions were found to be 0.1 PFU/reaction each. For detection of A(H1N1)pdm09 strain, published primers for HA gene, were selected (Nakauchi et al., 2011) and the assay demonstrated limit of detection of 0.01 PFU/reaction. All three RT-LAMP reactions were highly specific for their corresponding influenza subtypes.
The positive RT-LAMP reactions were visualized by using agarose gel electrophoresis and visual inspection of green fluorescence because of added SYBR green I dye under UV light or blue light. The time of detection is reduced by using visual inspection of the RT-LAMP products as need of post-RT-LAMP processing is obviated (Mori and Notomi, 2009; Parida et al., 2011). Earlier, SYBR green dye has been used for detection of positive amplifications in the RT-LAMP reactions (Parida et al., 2005; Tao et al., 2011). Other dyes like calcein and hydroxy naphthol blue (HNB) have also been used to inspect RT-LAMP products (Tomita et al., 2008; Ma et al., 2010; Peng et al., 2011; Nie et al., 2013). In a study, Wastling et al. (2010) have reported that calcein dye was less sensitive than SYBR green for detection of LAMP reaction. Moreover, the results of calcein and HNB detection may vary due to deviation in reaction composition as these dyes don’t measure amplified DNA directly and rely upon concentrations of metal ions in the reaction (Zhang et al., 2013b). Therefore, in our study we have used SYBR green I dye for visual detection of RT-LAMP amplicons.

PCR-based methods like conventional RT-PCR and real-time RT-PCR have become popular choice for influenza diagnosis nowadays. Conventional monoplex and multiplex RT-PCR have been used for diagnosis of influenza viruses (Atmar et al., 1996; Pregliasco et al., 1998; Poddar, 2002; Gall et al., 2008). We optimized typing and subtyping conventional RT-PCRs using extracted viral RNAs of reference strains and using published primers sequences. The typing monoplex RT-PCRs demonstrated detection limits of 0.1 and 10 PFU/reaction for influenza A and B viruses, respectively. Similarly, subtyping monoplex RT-PCRs showed limit of detection of 1, 0.1 and 1 PFU/reaction for A(H1N1)pdm09, H3N2 and H1N1, respectively. We found that RT-LAMP assays were 10-100 times more sensitive than conventional RT-PCR, when applied on reference strains in parallel reactions (Sharma and Kaushik, 2016). Similar observations have been reported regarding the sensitivity comparison of RT-LAMP and conventional RT-PCR for detection of influenza viruses. According to Poon et al. (2005), detection limit of RT-LAMP assay was 10-fold more than conventional RT-PCR for influenza A virus detection using M gene primers. Imai et al. (2006) have reported that RT-LAMP was 100-fold more sensitive than RT-PCR for detection of avian influenza H5 subtype. Similarly, other research groups have reported high sensitivity of RT-LAMP
than RT-PCR for detection of swine influenza H3 (Gu et al., 2010) and avian influenza H7 viruses (Bao et al., 2014).

Real-time RT-PCR (rRT-PCR) is a highly sensitive and specific molecular diagnosis method utilized for detection of influenza viruses as well as their typing and subtyping (Carr et al., 2009; Wang and Taubenberger, 2010; Landry, 2011). Schweiger et al. (2000), have developed TaqMan rRT-PCR assay which was able to detect influenza A and B viruses using primer/probe sets against $M$ gene and $HA$ gene respectively. They further used HA primers/probes for subtyping of influenza A viruses into H1 and H3. Di Trani et al (2006), have developed a highly sensitive rRT-PCR assay (limit of detection, 0.001TCID<sub>50</sub>/reaction) for universal detection of influenza A viruses using Minor Groove Binder (MGB) probe, targeted against $M$ gene. CDC has recommended the use of TaqMan rRT-PCR for diagnosis of pandemic influenza A(H1N1)pdm09 during swine flu outbreak in 2009 (Carr et al., 2009; WHO, 2009b). In an earlier study, rRT-PCR has been developed in multiplex format to detect influenza virus type A, B and subtype H5 in a single reaction (Wu et al., 2008). WHO has developed protocols for molecular detection, typing and subtyping of influenza viruses using conventional RT-PCR and rRT-PCR assays that are being updated from time to time (WHO, 2014). In the current study, analytical sensitivities of typing and subtyping TaqMan rRT-PCR were computed and compared with RT-LAMP assays. The results showed that both RT-LAMP and rRT-PCR have comparable analytical sensitivities for detection of influenza viruses. For detection of influenza A viruses and subtype H1N1, rRT-PCR was found to be ten-times more sensitive than RT-LAMP assay, whereas for detection of A(H1N1)pdm09, H3N2 and influenza B virus, both RT-LAMP and rRT-PCR exhibited similar sensitivities (Sharma et al., 2017). Kubo et al. (2010) have reported that both RT-LAMP and TaqMan rRT-PCR were equally sensitive for detection of influenza A(H1N1)pdm09 with the limit of detection of 10 copies of RNA per reaction. According to Lee et al. (2011), the detection limit of RT-LAMP and TaqMan rRT-PCR for influenza A(H1N1)pdm09 was 10 copies/μl of viral RNA. Similar observations were reported in another RT-LAMP study for avian influenza H5N1 diagnosis (Dinh et al., 2011). Parida et al. (2011) have reported that analytical sensitivity of RT-LAMP (0.1 TCID<sub>50</sub>/ml) was ten times more than rRT-PCR (1 TCID<sub>50</sub>/ml) for detecting swine-origin influenza H1N1 viruses.
Clinical evaluation of optimized RT-LAMP assays was done by comparing sensitivities and specificities of the reactions with conventional RT-PCR, TaqMan rRT-PCR and virus isolation. To accomplish this, 252 nasal/throat swab samples were collected from ILI patients enrolled in PGIMS, Rohtak, from September 2014 to August 2017. Virus isolation was considered as ‘gold standard’ to detect and identify influenza viruses for this study. A total of 35/252 (13.8%) samples were found positive for influenza viruses by observing cytopathic effect (CPE) during virus isolation in MDCK cells. Hemagglutination inhibition (HI) assay was performed to type and subtype the influenza virus isolates. Out of influenza positive samples, 31/35 (88.6%) samples were typed as influenza A and 4/35 (11.4%) samples were typed as influenza B type, indicating more prevalence of influenza A than influenza B viruses. Subtyping results demonstrated that subtype A(H1N1)pdm09 (74.2%) was the leading strain circulating in the community during 2014-2017 followed by H3N2 (25.8%).

Clinical samples were also assessed for detection, typing and subtyping of influenza viruses by using WHO recommended TaqMan rRT-PCR. Out of 252 clinical samples collected during the study period, 61 (24.2%) samples were detected positive for influenza viruses by using rRT-PCR as compared to only 35 (13.8%) using virus culture. This showed the high clinical sensitivity of rRT-PCR as compared to virus culture. Most of the samples were collected from adult population and it has been reported earlier that in adults the viral load was less as compared to children samples (Cruz et al., 2010; Sakai-Tagawa et al., 2010). It might be a reason for high sensitivity of rRT-PCR as compared to virus culture. Earlier studies have also reported high sensitivity of rRT-PCR assays over virus isolation methods for detection of influenza viruses (Habib-Bein et al., 2003; Gooskens et al., 2008; Legoff et al., 2008). Typing rRT-PCR has detected 55/61 (90.2%) samples positive for influenza A viruses and 6/61 (9.8%) samples positive for influenza B viruses. During subtyping of influenza A viruses, rRT-PCR detected 37/55(67.3%), 14/55 (25.4%) and 4/55 (7.3) for A(H1N1)pdm09, H3N2 and H1N1 respectively. For detection of influenza viruses, TaqMan rRT-PCR has demonstrated sensitivity, specificity, PPV and NPV of 100%, 88%, 57.4% and 100%, respectively in comparison to virus isolation. After the 2009 pandemic, the influenza A(H1N1)pdm09 strain has established itself as seasonal strain in the community as sporadic cases and outbreaks were reported from
India along with major outbreak in 2014-15. In the present study, we found that most of the infected individuals were from age group of 21-40 years, signifying high infection rate in adult population. In surveillance study from Pune, India during pandemic and post pandemic period, Chadha et al. (2012) reported high prevalence of A(H1N1)pdm09 and seasonal influenza viruses among the individuals belonging to age group of 2-29 years. Koul et al. (2013) have reported the resurgence of influenza A(H1N1)pdm09 strain in Kashmir, India during 2012-13 and found 184/751 (24.5%) positive samples for influenza viruses. They have reported that A(H1N1)pdm09 (57%) was the major strain of influenza circulating in the local community during 2012-13. In a study from Puducherry, India, clinical samples were assessed for influenza activity form 2009-2013 using rRT-PCR. The study has reported 12.7%, 4% and 4.3% positive cases of A(H1N1)pdm09, H3N2 and influenza B, respectively with higher infection rate in adults than in children (Nandhini and Sujatha, 2015). In another study from India, Mukherjee et al. (2016) have reported the prevalence of influenza A(H1N1)pdm09 with 23.3% positive cases among the population of Kolkata, India, during 2015 outbreak and described high infection rate among children (1-5 years) than in adults (18-36 years). Malhotra et al. (2016) have reported 34.1% cases positive for influenza A(H1N1)pdm09 during January to March 2015 from Rajasthan, India and reported the high infection (47.6%) and death rate (52.1%) among individuals from age group of 26-50 years. Murhekar and Mehendale (2016) have reported more than 62,000 cases of influenza and about 5,000 deaths from 2010 to March 2015 in India. Maximum numbers of cases were reported in the year 2015 during the influenza A(H1N1)pdm09 outbreak.

In the present study, clinical symptoms and epidemiological data of influenza positive patients indicated fever (94.3%) as the most common symptom followed by cough (85.7%) and fatigue (80%). Less common symptoms were vomiting (11.4%) and diarrhoea (5.7%) which were more common in children (<5 years). Primary symptoms like fever ($P = 0.008$ Fisher’s exact) and cough ($P = 0.018$ Fisher’s exact) were found to be significantly associated with occurrence of influenza. In an earlier study, Cheng et al. (2011) have reported similar clinical features for seasonal as well as pandemic influenza patients with common symptoms including cough (92%) and fever (80%). They have computed significant association of fever and sore throat with frequency of influenza
infections. In a study from India, reported common clinical symptoms of influenza A(H1N1)pdm09 positive patients were fever, cough, sore throat and shortness of breath (Siddharth et al., 2012). However, it is difficult to confirm influenza infections solely based on clinical symptoms and only laboratory tests can provide accurate diagnosis (Petric et al., 2006; Stefanska et al., 2013).

Optimized RT-LAMP assays and conventional RT-PCR were applied on clinical samples and the results were compared with rRT-PCR and virus isolation. RT-LAMP assays and conventional RT-PCR have detected 57/252 (22.6%) and 49/252 (19.4%) influenza positive samples, respectively. Typing RT-LAMP assays have detected 51/57 (89.5%) samples positive for influenza A and 6/57 (10.5%) samples positive for influenza B viruses. Typing conventional multiplex RT-PCR was able to detect 44/49 (89.8%) and 5/49 (10.2%) samples positive for influenza A and B viruses, respectively. In comparison to conventional RT-PCR, RT-LAMP assay has detected 7 additional influenza A positive samples and one additional influenza B positive sample, which were also found positive during rRT-PCR detection. Similarly, subtyping RT-LAMP assays have detected 33/51 (64.7%), 14/51 (27.5%) and 4/51 (7.8%) samples as A(H1N1)pdm09, H3N2 and H1N1, respectively. Whereas 31/44 (70.5%), 11/44 (25%) and 2/44 (4.5%) samples were diagnosed positive for A(H1N1)pdm09, H3N2 and H1N1, respectively by using conventional multiplex RT-PCR. These results demonstrated higher clinical sensitivity of RT-LAMP than conventional RT-PCR for detection, typing and subtyping of influenza viruses. Similar observations have been reported from earlier studies. RT-LAMP assay was found more sensitive than RT-PCR for detection of avian influenza H9 subtype using clinical samples (Chen et al., 2008). According to Peng et al. (2011), clinical sensitivity of RT-LAMP assay was more than conventional RT-PCR for detection of influenza H3 subtype. In a similar study performed on swine samples, RT-LAMP assay was found more sensitive than RT-PCR for detecting swine influenza H3 (Gu et al., 2010). In an earlier study, Bao et al., (2014) evaluated clinical sensitivity of RT-LAMP and conventional RT-PCR for detection avian influenza H7N9. They have found that RT-LAMP assay was more sensitive method than RT-PCR and was able to detect two additional true positive samples which were missed out by conventional RT-PCR. Same research group have reported higher clinical sensitivity of RT-LAMP assay than RT-PCR
for detection of avian influenza subtype H10N8 in chicken swab samples (Bao et al., 2015).

In comparison with virus isolation, all three molecular assays i.e., RT-LAMP, conventional RT-PCR and TaqMan rRT-PCR have demonstrated 100% (CI 87.7-100) sensitivities for detecting influenza viruses as well as subtype A(H1N1)pdm09. We have also compared clinical sensitivity and specificity of virus isolation, conventional RT-PCR and RT-LAMP assay with WHO recommended TaqMan rRT-PCR taken as standard. All three methods were 100% (CI 97.54-100) specific for detection of influenza viruses as no false positive sample (PPV=100%) was detected by any of the methods. For detection of influenza viruses, sensitivities of 57.4% (CI 44.1-69.73), 80.3% (CI 67.78-89) and 93.4% (CI 83.25-97.88) were observed for virus isolation, conventional RT-PCR and RT-LAMP assays, respectively. Whereas, for detection of subtype A(H1N1)pdm09, virus isolation, conventional RT-PCR and RT-LAMP assays have demonstrated sensitivities of 62.2% (CI 44.79-77.06), 83.8% (CI 67.31-93.23) and 89.25% (CI 73.64-96.48), respectively. These results have shown that in comparison to rRT-PCR assay, virus isolation was least sensitive and RT-LAMP assays were most sensitive. Kubo et al. (2010) have compared RT-LAMP assay with rRT-PCR for detection of influenza A(H1N1)pdm09 and found that the sensitivity and specificity of RT-LAMP assay were 97.8% and 100%, respectively. Similarly, Nakauchi et al. (2011) have reported sensitivity and specificity of 96.3% and 88.9%, respectively for RT-LAMP assay in comparison to rRT-PCR for detection of influenza A(H1N1)pdm09. According to Liu et al. (2014), RT-LAMP assay exhibited 100% clinical sensitivity in comparison to rRT-PCR for detection of avian influenza H7N9. Clinical evaluation has signified that RT-LAMP assays performed well for detection of influenza viruses and identification of viral strains. The clinical sensitivity and specificity of these assays were higher than conventional RT-PCR and were comparable to rRT-PCR.

Some of the clinical influenza isolates were selected at random for sequencing and phylogenetic analyses. Partial HA gene sequences were obtained from six A(H1N1)pdm09 and four H3N2 influenza isolates. Predicted amino acid sequences of A(H1N1)pdm09 isolates were aligned with sequence of HA1 region of the reference strain (GenBank ID KU933485), which corresponds to globular head domain of
hemagglutinin protein. According to Caton et al. (1982), globular head domain of HA protein in influenza H1N1 viruses, has five antigenic sites viz., Ca1, Ca2, Cb, Sa and Sb. These sites are important in determining the antigenicity and even a single amino acid change can vary the immunogenic properties of the virus and influence the vaccine protection (Raymond et al., 1986; Tumpey et al., 2004).

Comparison of partial amino acids sequences of HA protein of A(H1N1)pdm09 isolates with the reference strain showed the presence of five amino acid substitutions which showed conserved pattern among all the six isolates. We found amino acid substitutions of D114N and S202T in Sb site, substitution of K180Q in Sa site and substitution of S220T in Ca1 antigenic site of the globular head domain of HA1 subunit. One mutation of P100S was also found in the intercalating region of Cb and Sa epitope. High virulence of A(H1N1)pdm09 during 2015 epidemic in India, may be due to accumulation of these mutations. Earlier, Potdar et al. (2010) have reported four major mutations in the HA protein of A(H1N1)pdm09 isolates from Indian population during 2009-2010. They found amino acid substitution of P100S and S220T along with T214A and I338V. Elderfield et al. (2014) have reported presence of mutation S202T in the A(H1N1)pdm09 isolates from United Kingdom during 2010-2011. In a recent study, Clark et al. (2017) have reported amino acid substitutions of P100S, D114N, K180Q, S202T and S220T in the A(H1N1)pdm09 isolates from study population of Rochester, New York in 2015-2016. Phylogenetic studies of A(H1N1)pdm09 isolates have shown that all the isolates were clustered along with global representative strains of clade 6B. This is in agreement with other earlier studies reported from India (Mukherjee et al., 2016; Parida et al., 2016; Nakamura et al., 2017). Influenza A(H1N1)pdm09 isolates form the present study have shown 97-98% nucleotide homology with the reference strain, A/California/07/2009 and 98-100% homology with each other. All six influenza isolates shared 98-99% homology with strain, A/India/Kol-018/2015, which also grouped in the genogroup 6B.

Phylogenetic analysis of four influenza H3N2 isolates showed the presence of two genogroups which were clustered away from the reference strain, A/Texas/50/2012. Out of four influenza H3N2 isolates, A/Haryana/015/2015 and A/Haryana/016/2015 were grouped together while A/Haryana/029/2015 and A/Haryana/032/2015 were grouped
together in the same genogroup. All H3N2 isolates have shared 97-98% nucleotide homology with the reference strain, A/Texas/50/2012 and 97-99% homology among themselves. Phylogenetic analysis has shown that isolates, A/Haryana/029/2015 and A/Haryana/032/2015 were more closely related with the reference strain than the other two isolates. Amino acid sequence analysis have shown different substitutions in the HA protein of the isolates. Five antigenic sites (A to E) have been reported in the HA protein of influenza H3N2 subtype (Webster and Laver, 1980; Wiley et al., 1981; Skehel et al., 1984). Comparison of HA gene sequences of A/Haryana/015/2015 and A/Haryana/016/2015 revealed substitutions of N144T, I156M, N160S, F175Y, K176T and N241D compared to A/Texas/50/2012. Whereas, in HA gene sequences of A/Haryana/029/2015 and A/Haryana/032/2015 amino acid substitutions of N144A, R158G and L173S were observed. Partial amino acid sequence comparison has shown conserved pattern of amino acid substitution of N161S and P214S in all four H3N2 isolates in the HA1 domain with respect to reference strain. Similar substitutions have been reported from a study from China during H3N2 outbreak in 2012 (Zhong et al., 2013). Resende et al. (2015) have reported non-synonymous substitution of N161S and P214S in the HA gene of H3N2 isolates from Brazil in 2014. H3N2 isolates have shown more variability in amino acid substitutions than A(H1N1)pdm09 isolates. These observations have shown the high antigenic drift rate in H3N2 than in A(H1N1)pdm09 subtype. This is also in accordance with previously reported studies (Bhatt et al., 2011; Falchi et al., 2011; Tewawong et al., 2015; Monamele et al., 2017). Occurrence of frequent mutations in the antigenic sites of both influenza A(H1N1)pdm09 and H3N2 isolates, support the need of routine surveillance for influenza viruses. The antigenic characterization and routine surveillance of influenza viruses are important in assessing the efficacy of influenza vaccines and in selecting suitable vaccine strains for annual influenza seasons.