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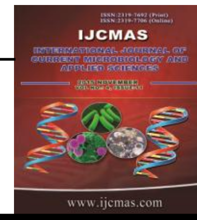

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Original Research Article

Early Detection for the Swine Flu Virus in the Mecca Area using Modern Methods of Molecular Genetics

Adel E El-Tarras[1,2], Nabil S Awad[1,3]*, Abdulla Alharthi[4] and Mohammed Ibrahim[5]

[1]Biotechnology and Genetic Engineering Unit, Scientific Research Deanship, Taif University, Saudi Arabia
[2]Genetics Dep., Faculty of Agriculture, Cairo University, Cairo, Egypt,
[3]Department of Genetics, Faculty of Agriculture and Natural Resources, Aswan University, Aswan, Egypt
[4]Faculty of Medicine, Taif University, Saudi Arabia
[5]Misr Univ. for Science and Technology, Cairo, Egypt
**Corresponding author*

A B S T R A C T

Periodically, complete novel antigenic subtypes of influenza viruses have been introduced in human population, causing large-scale global outbreaks with high death tolls. As a consequence of all this, pandemic preparedness has become an important issue worldwide and specially for Kingdom of Saudi Arabia. Theses Pandemic plans should includes proper approaches that allow early recognition of novel influenza viruses infecting humans in the future. Therefore, this study aimed at the development of a sensitive and rapid real time RT-PCR protocols specific for the detection of the KSA AIV circulating strains. Full length Neuraminidase (N1) genes have been downloaded from the Flu database (www.ncbi.nlm.nih.gov). Sequence database of target gene have been constructed and aligned. Moreover, conserved regions have been determined using the bioedit sequence alignment software. In addition, oligonucleotide primers specific for N1 gene of KSA strains have been designed using primer express software. Nevertheless, optimization of real time PCR protocol specific to the KSA H1N1 strains has been carried out. Finally, sensitivity test of the optimized protocol have been performed. This scientific article represents the first research to design and test specific oligonucleotide primers for the detection of local H1N1 viral strains in Kingdom of Saudi Arabia.

Keywords

Real time PCR, H1N1, diagnostic, Kingdom of Saudi Arabia, Haemaglutinine, Neuraminidase

Introduction

Influenza virus is a member of the genus influenza A virus, family Orthomyxoviridae, and contains negative sense single-stranded RNA (Lamb and Krug, 1996).

There are 18 different subtypes of hemagglutinin (HA) and 9 different subtypes of neuraminidase (NA) that can be differentiated both antigenically and genetically (Tong *et al.,* 2013).

Influenza A H1N1 is a novel influenza virus that is of swine source. Clinical manifestations of this virus vary from mild respiratory symptoms to fatal respiratory or/ and cardiovascular complications.

The most devastating influenza pandemic in modern recorded history, known as the “Spanish flu”, occurred in 1918–1919, killing up to 100 million people worldwide. The new influenza A H1N1 pandemic was first identified in April 2009 in the United States and Mexico, and then spread globally. In September 10 2010, the World Health Organization announced that the influenza A H1N1 pandemic had moved into the post-pandemic period and is no longer considered a dangerous global disease.

In November 2007, Saudi Arabia’s Agriculture Ministry announced that it had culled 50,000 birds after a deadly H5N1 strain of bird flu was detected at a poultry farm in Al-Kharj, 150 kms south of Riyadh. Tests were carried out after 1,500 birds died in the farm, which had a total of 50,000 birds. According to the Saudi Ministry of Health, the number of laboratory-confirmed cases in Saudi Arabia as of December 30, 2009 was 15850, with 124 deaths. On the 15th of August 2010, the Ministry of Health in Saudi Arabia declared that the cases of influenza A H1N1 in 2010 have drastically declined to 874 cases with no deaths or serious complications. However, positive H1N1 cases still exist up to 2013.

Diagnostic tests that detect antigens, such as immune fluorescent staining and enzyme linked immune sorbent assays have been reported (Takimoto *et al.,* 1991). Direct fluorescent antibody (DFA) staining of cells derived from nasopharyngeal swabs or nasopharyngeal aspirates (NPA) became the mainstay for many laboratories and provided a rapid test result in about 3 h. A number of rapid EIAs for influenza A and B viruses have been introduced over the past 10 years (WHO 2005; Bai *et al.,* 2006; Chan *et al.,* 2007; Chan *et al.,* 2002, Gavin and Thomson, 2003; Uykei *et al.,* 2003 Mahony, 2008. However, these tests lacked sensitivity and were usually relegated to point-of-care testing in defined settings. Nucleic acid amplification procedures including PCR, nucleic acid sequence-based amplification (NASBA), and loop-mediated isothermal amplification (LAMP) were also developed (Fakruddin *et al.,* 2013). In the case of RT-PCR, nucleic acid is reverse transcribed into cDNA using virus-specific oligonucleotide primers or random hexamers. Random hexamers have the advantage of generating cDNA for multiple viruses present in a specimen and for use in virus discovery, as cDNA is more stable during storage than extracted RNA or intact clinical specimens (Logan *et al.,* 2006). Several RT-PCR assays for influenza virus infection have been described since the first report by Zhang and Evans (1991) and Mahony (2008). Real time PCR represents the most common diagnostic approach in routine laboratories due to its sensitivity and accuracy (Lee, 2012).

To date, the total number of infected humans with the highly pathogenic avian influenza virus (HPAIV), H5N1, was 106 cases with 34 deaths. Although there is no evidence for human to human transmission of the H5N1, continued circulation of such influenza strains, emergence of new subtypes,

reassortment among various subtypes and spontaneous sequence changes that might occur in key viral molecules (hemagglutinin (HA), neuraminidase (NA), matrix (M) and polymerases) might result in emergence of new more adapted variant to infect mammals including humans. Such variant might also cross the human to human transmission barrier. Moreover, improve the diagnostic capacity of high risk countries is very important in order to control, quarantine the spread of avian influenza viruses as a potential global threat. Thus, there is an urgent need for pandemic preparedness plans relying on sensitive and specific molecular tools for surveillance of any newly emerging influenza strains.

Materials and Methods

Viral RNA extraction

Viral nucleic acid of the positive control sample has been carried out via RNA extraction kit (Qiagen, Germany) as described by the manufacturer protocol. Both qualitative and quantitative analysis of the extracted total viral RNA has been carried out via spectrophotometery (Nanodrop, invitrogen, USA).

In *silico* Analysis

Saudi Arabian Type A H1N1 nuraminidase (H1) gene sequences have been downloaded from the flu database (www.ncbi.nlm.nih.gov) and a sequence database has been constructed by the use of bioedit software (www.bioedit.com). Sequence alignments of the collected DNA sequences have been carried out by the use of bioedit program. Conserved regions were used as a template for primer design by the use of primer express software (Applied biosystem, USA) (Table 1). Finally, Designed oligonucleotide primers have been tested against the downloaded Saudi Arabian Type A H1N1 nuraminidase (N1) gene sequences in order to test the efficiency of the designed primers (Figure 3–7).

Full genome C-DNA construction

Six microliters of extracted RNA was added to 4ul of reverse transcription solution (15 uM random hexamers and 0.9 U of avian myeloblastosis virus reverse transcriptase enzyme). Reaction mixtures were incubated at 25°C for 5 min, 42°C for 10 min, 50°C for 20 min, and 85°C for 5 min and were then kept at 4°C until amplification.

Optimization of real time PCR protocol

Several real time PCR trials have been carried out in order to optimize the diagnostic protocol that employs the designed primers and to test the efficiency of the proposed diagnostic protocol *in vitro*. Master mix components were 2x universal standard sybergreen chemistry (Applied biosystem, USA), 150 pmoles of each of the forward and reverse oligonucleotide primers (Applied biosystem, Egypt). Thermal cycler program was 95ºC for 30 seconds preheating step, 35 cycles of 15 seconds at 95ºC and 60ºC for 1 minute. Melting curve analysis at 65–90ºC on 2ºC intervals.

Sensitivity test

In order to test the lowest concentration of the target H1N1 viral nucleic acid that could be detected by the optimized protocol, a sensitivity test of the

developed real time PCR protocol has been carried out. Briefly, serial dilutions of the original viral nucleic acid concentration (40ug/ul) have been prepared. These serial dilutions were used as a template against the designed oligonucleotide primers in a sybergreen real time PCR format as described above.

Results and Discussion

In *silico* Analysis

Based on the nucleotide sequence database of Saudi Arabian H1N1 strains that has been construted at this study (Figure 1) and sequence alignment of the full length H1 gene to identify conserved regions (Figure 2), sequence alignment of the designed oligonucleotide primers showed a high specificity to Saudi Arabian strains These data confirm the value of designing oligonucleotide primers specific to the local strains in order to improve the efficiency of the diagnostic protocols and, hence, increase the chance of control and quarantine epidemic spread of infectious viral pathogens.

Optimization of real time PCR protocol

In order to test the efficiency and accuracy of the designed oligonucleotide primers, a specific real time PCR protocol has been optimized. Generated Cycle threshold (C.t.) values showed that oligonulcleotide primers generated in this study have appropriate C.t. values compared to the commercially CDC recommended protocol (Table 1), Figures (8–11). The results showed that the best primer combination is primer H1N1 KSA F2R2 which generated a Ct value of 16.6. Also, results showed that the rest of primer pairs are more efficient than the commercial kit (applied Biosystem, USA) which produced a much more late C.t. value (26.03). These results indicated that among all combinations of oligonucleotide primers that are specific to the KSA circulating H1N1 viruses only H1N1 KSA F2R2 primer is the most efficient one compared to the commercially available kits. These results could be attributed to the fact that oligonucleotide primers that are designed specific to local strains might be more specific and, hence, generates a more efficient data and more sensitive results than generic oigonucleotide primers. Specially, with RNA genome viruses in which a high rate of mutation events occurs.

Sensitivity test

In order to test the efficiency of the developed protocol a sensitivity test has been carried out using serial dilutions of the initial concentration (40ug/ul). The results of this experiment showed a gradual increase of the C.t. values parallel to the increase of the dilution factor (Table 3) occurred with all primer combinations except for the primer H1N1KSA F2R3 which produced a fluctuated result among tested serial dilutions. Sensitivity test represents the determination of the lowest concentration of the viral nucleic acid that could be detected by the use of the recommended protocol.

The lower the viral nucleic acid that could be detected, the higher the sensitivity of the implemented protocol. Therefore, data generated at this experiment indicated that both fidelity and accuracy of the recommended protocol is acceptable and the described real time PCR conditions are efficient.

Table.1 Oligonucleotide primers designed at this study

Primer name	Primer sequences
H1N1 KSA F1	GCATAACGGGAAACTATGCAA
H1N1 KSA R1	TGAGCTTGCTGTGGAGAGTG
H1N1 KSA F2	GAAGACAAGCATAACGGGAAA
H1N1 KSA R2	CATGAGCTTGCTGTGGAGAG
H1N1 KSA R3	CCACAATGTAGGACCATGAGC

Table.2 List of Ct values produced by designed primers in addition to diagnostic kit

Primer name	C.t. value
H1N1 KSA F1R2	29.2
H1N1 KSA F1R3	31.7
H1N1 KSA F2R1	30.00
H1N1 KSA F2R2	26.97
N1 positive control kit	26.03
H1N1 KSA F2R2	16.61

Table.3 List of Ct values produced by real time PCR sensitivity test analysis of sample 1 against all tested primers

Dilution	Primer	H1N1 KSA F1R2	H1N1 KSA F1R3	H1N1 KSA F2R1	H1N1 KSA F2R2	H1N1KSA F2R3
1/10		15.65	13.88	19.08	18.05	undetermined
1/100		16.16	22.01	31.38	29.86	35.27
1/1000		16.65	23.4	35.67	32.95	undetermined
1/10000		16.49	undetermined	undetermined	undetermined	undetermined

Figure.1 Construction of a nucleic acid sequences database of the H1 full length gene

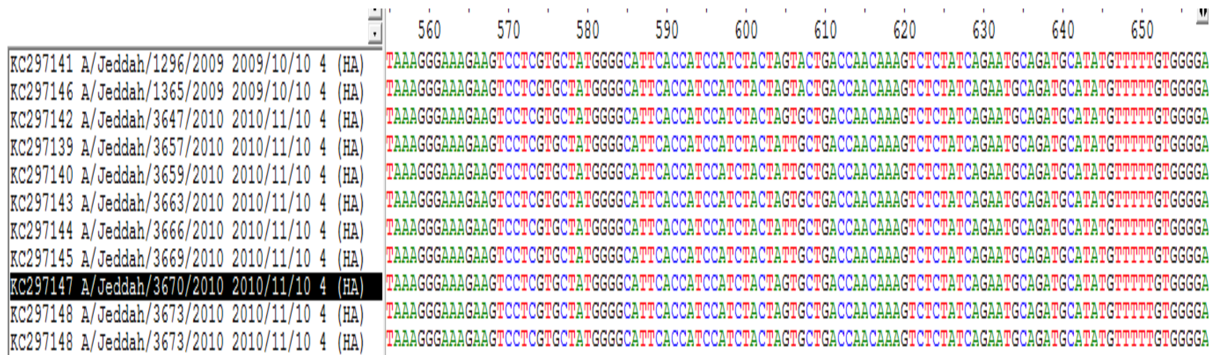


Figure.2 Alignment of a nucleic acid sequences database of H1 full length gene belongs to KSA strains

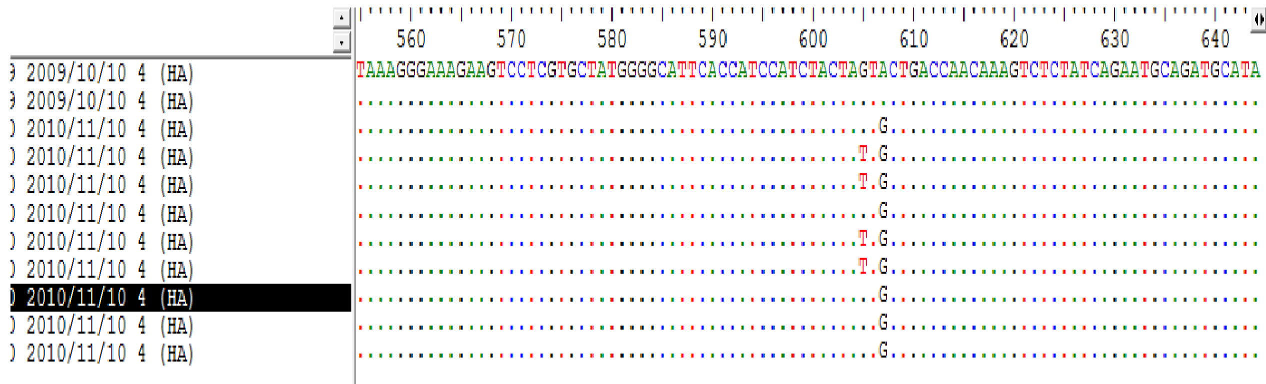


Figure.3 *In silico* test of primer H1N1KSAF1

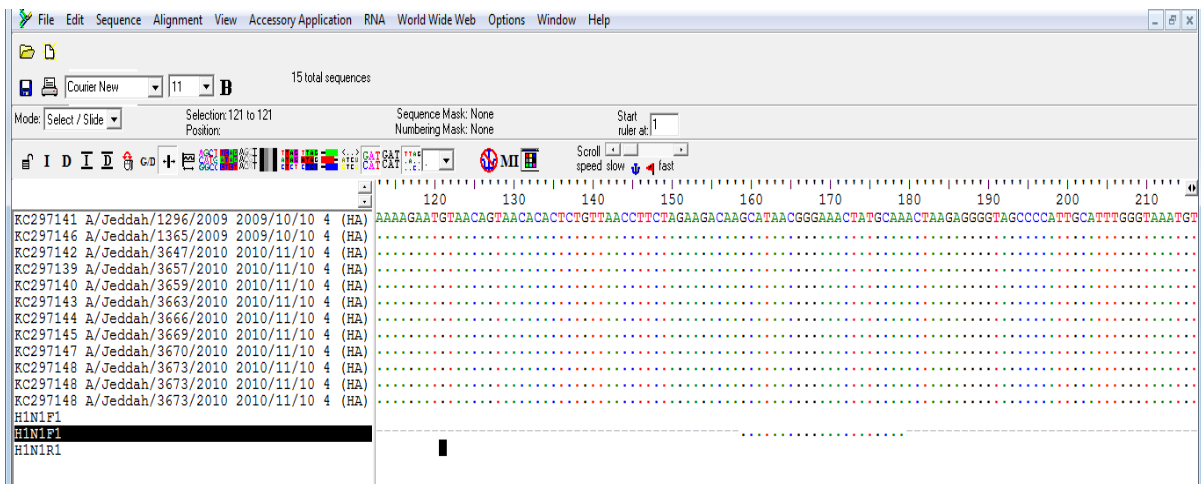


Figure.4 *In silico* test of primer H1N1KSAR1

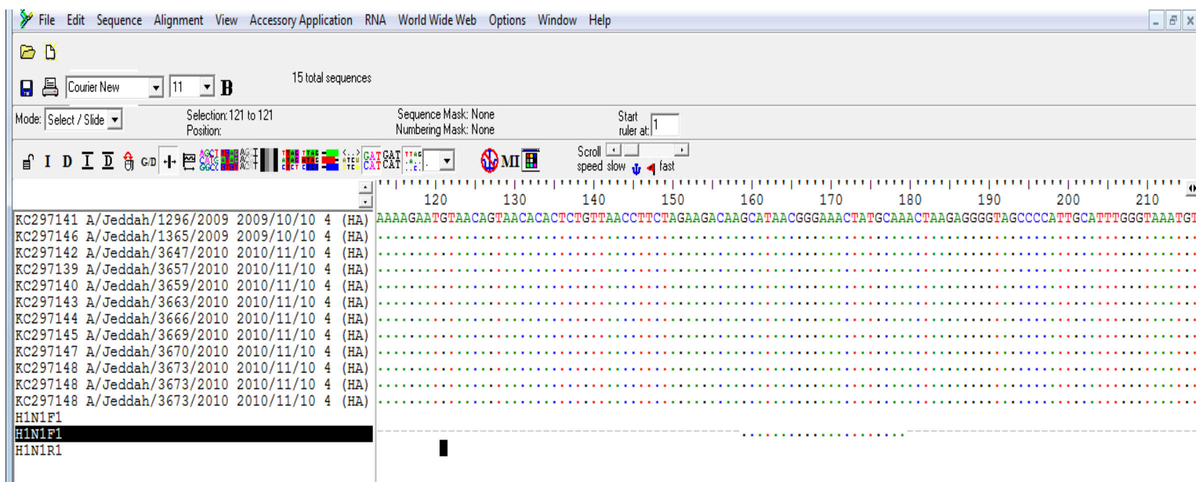


Figure.5 *In silico* test of primer H1N1KSAR2

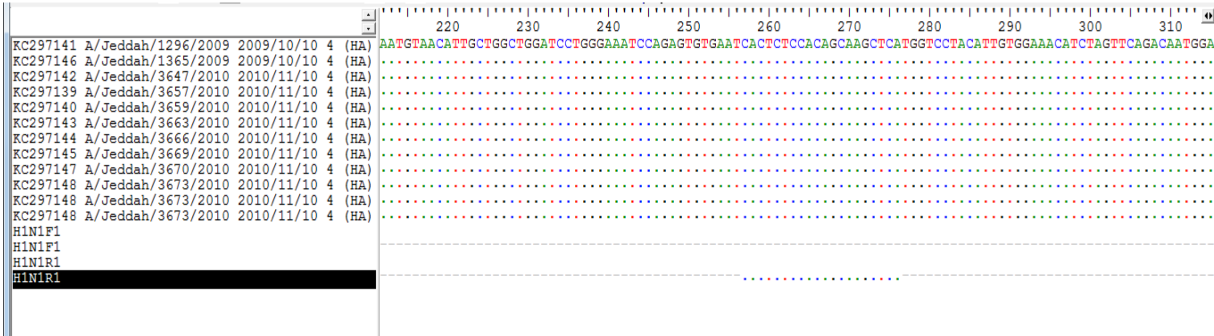


Figure.6 *In silico* test of primer H1N1KSAF2

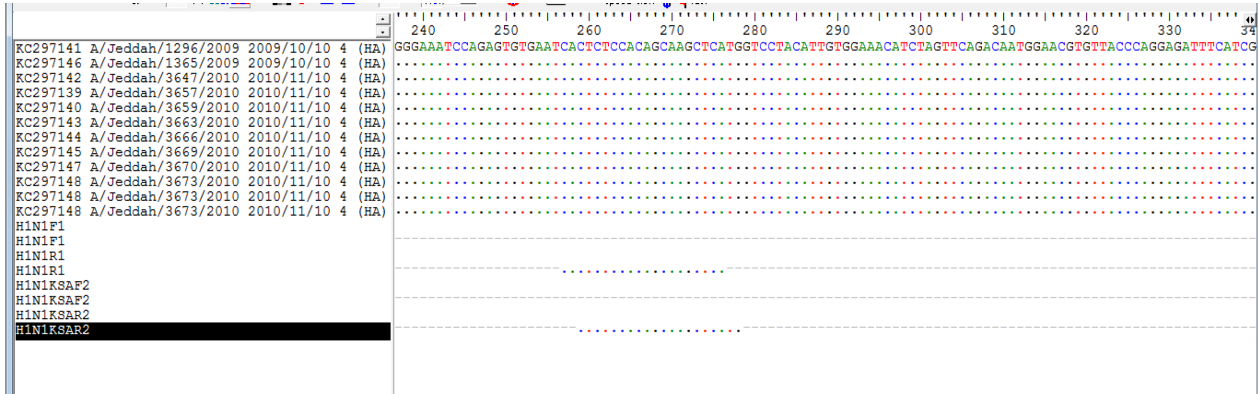


Figure.7 *In silico* test of primer H1N1 KSA R3

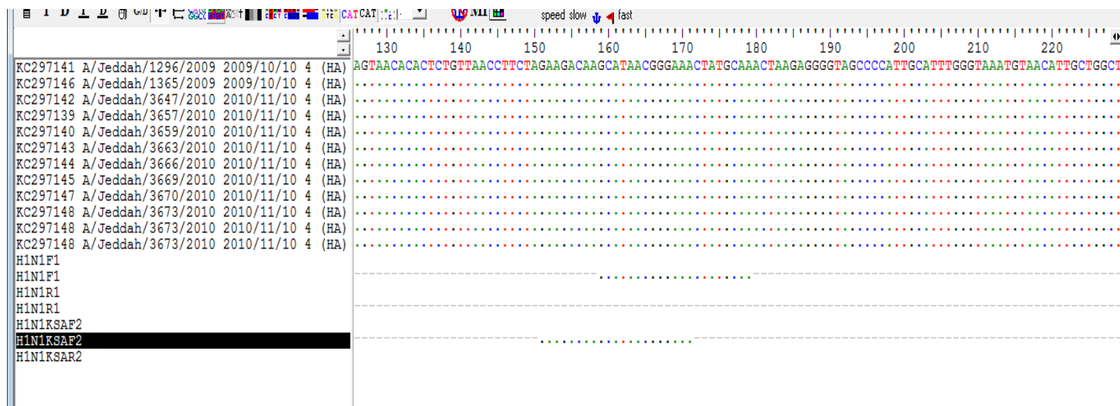


Figure.8 Amplification plot of primer H1N1 KSA F1R2 (C.t., 29.2)

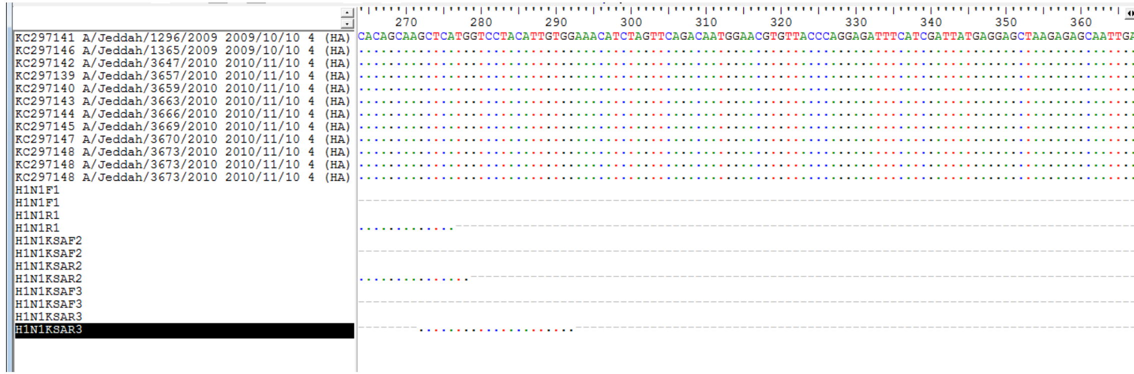


Figure.9 Amplification plot of primer H1N1 KSA F1R3 (C.t. 31.7)

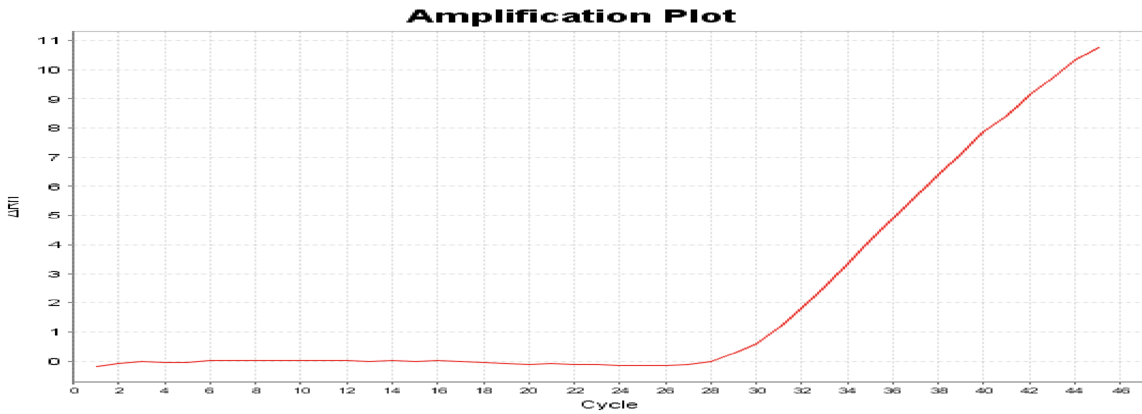


Figure.10 Amplification plot of primer H1N1 KSA F2R1 (C.t.30.00)

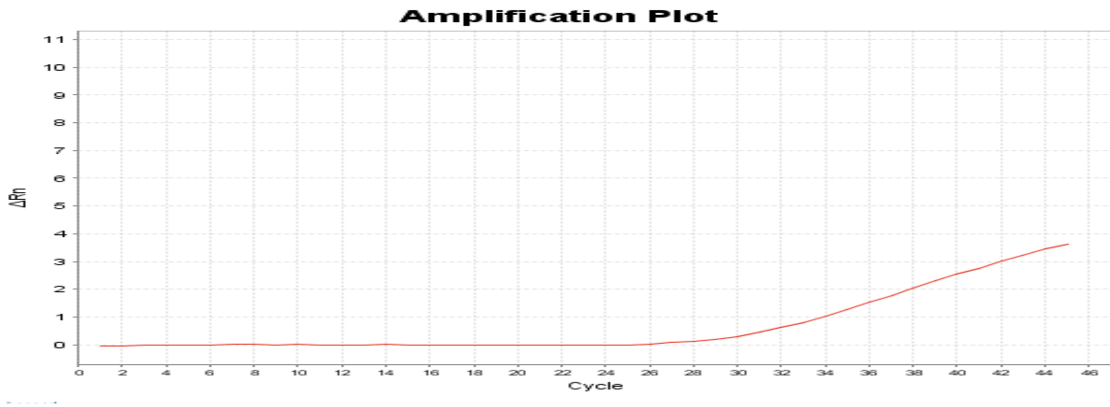


Figure.11 Amplification plot of primer H1N1 KSA F2R2 (C.t., 26.97)

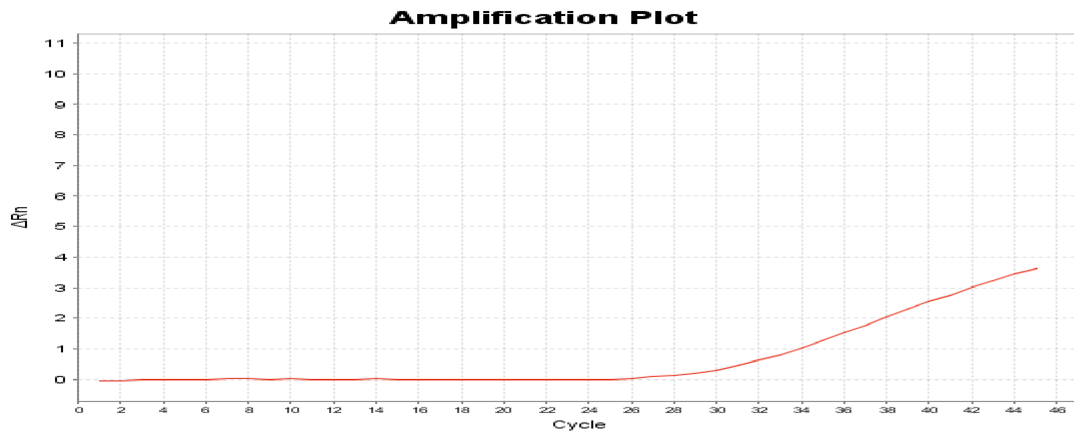
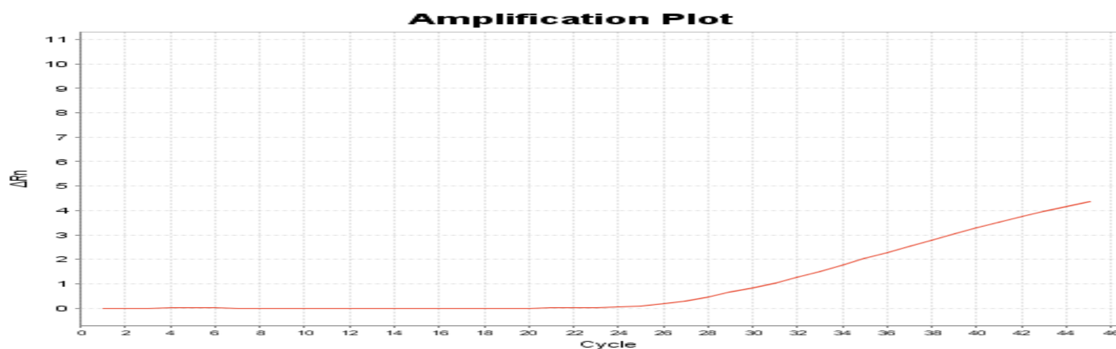


Figure.12 Amplification plot of N1 kit (C.t., 26.03)



Data generated from this study indicated that the recommended protocol is efficient and specific to the H1N1 KSA strains, specially the oligonucleotide primers H1N1 KSA F2R2 which produced an earlier C.t. value than the commercial kit described above. Also, the results of this study confirm the importance of design specific diagnostic protocols against local infectious agents in order to increase the accuracy and efficiency of the detection process and, hence, increase the chances to control the epidemic spread of H1N1 viruses. Finally, this scientific article represents the first research to design and test specific oligonucleotide primers for the detection of local H1N1 viral strains in Kingdom of Saudi Arabia.

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