Mutation analysis of Influenza RD8 from chicken egg.

Introduction

Influenza viruses are segmented RNA viruses that belong to the orthomyxovirus family. Influenza viruses can be divided into three categories based on their genetic makeup: types A, B, and C. (Henry et al., 2010). Pandemic influenza is always caused by H3N2 influenza viruses, while influenza B and C viruses only cause occasional sickness and small outbreaks (Treanor, 2005).

Seasonal epidemics caused by influenza A viruses (IAVs) are responsible for 30-50 lac cases of severe influenza in humans and more than 250,000 fatalities annually over the world (Yang et al., 2021). Pandemic potential is achieved when a circulating IAV obtains changes in its hemagglutinin (HA) protein that allow immune evasion, and such a virus can disseminate efficiently in individuals that lack preexisting antibodies against the variation (Schmier et al., 2015). We need to constantly monitor human and animal reservoirs to give us an idea of the likelihood of outbreaks of novel IAVs, and we need to reformulate our seasonal IAV vaccinations every one to three years.

Despite developments in pharmacology and vaccinology, this virus continues to impact morbidity and mortality around the globe in people of all ages (Kuszewski & Brydak, 2000). Low vaccine efficacy from unanticipated outbreaks of next epidemic strains or the introduction of pandemic viruses continues to make influenza one of the world's most pressing public health concerns (Lee et al., 2014). Vaccines against the flu nowadays are designed to protect against strains of the virus that differ greatly from one another, specifically the hemagglutinin antigen (Miller, King, Afonso, & Suarez, 2007).

The current study's objective is to isolate influenza virus from the chicken egg and process it for sequence analysis. This analysis will confirm the variations in the particular virus and will serve as a source of production of recombinant vaccines on the basis of the mutations. This will serve as a source to provide updated versions of vaccines against influenza.

2. Methods

2.1. RNA Extraction

Amniotic fluid was taken from the infected eggs and the RNA of influenza RD8 virus was extracted. QIAamp RNA extraction mini kit was utilized for the RNA extraction.

- Amniotic fluid sample was treated with AVL buffer ethanol was added.
- 630 µl viral RNA solution and 500 µl of AW1 buffer was added followed by centrifugation and addition of AW1 buffer.
- 500 µl AW2 buffer and AW2 buffer was added.
- Replaced collection tube with spin column and added 50 µl of RNase-free water and preserve it for future use.

2.2. RNA Quantification

For the RNA quantification Nanodrop was done. The sample reader was first washed molecular grade water and dried. Elution water was used as blank and then 2µL sample was loaded. The readings were calculated at 250, 260 and 280 nm.

2.3. cDNA Synthesis

- A 5 µl solution of influenza viral RNA was added to tubes and 5 µL of DEPC water into the fourth mastermix. 1 µL primer solution (NNNAGCAAAAGCAGG) was added to tubes 1 and 4.
- 1 ul of oligo (dT)18 primers was added into tube 2 and 1 µL of a random hexamer primer mixture into tube 3. Placed tubes 1, 2, and 4 in an incubator at 45 °C.

2.4. PCR Analysis

For the PCR reaction the cDNA prepared was used primers were used in sets.

- In master mix tubes 1 and 2. 2 µL of primer set 1 solution was added.
  7024NAFor1: AGCGAAAGCAGGGGTTTAAAATG
  7024NARev1: AGTAGAAACAAGGAGTTTTTTGAAC
  7024HAFor1: AGCAAAAGCAGGGGAAAATAAAAAC
  7024HARev1: AGTAGAAACAAGGGTGTTTTTCC
- PCR DNA preparation (0.5 µg/ µL) was added 2 µL in each of the four master mix tubes.
- Added 1 µL of molecular grade water added to all the tubes.

Steps	Time	Cycles	Temperature
Initial Denaturation	30 sec		94°C
Denaturation	30 sec	30 Cycles	95°C
Annealing	30 sec		50°C
Elongation	90 sec		72°C
Final Extension	10 min		72°C

2.5. Gel Electrophoresis of PCR DNA

- Added 2 µL of loading dye to 10 µL of eluted DNA in an Eppendorf tube and samples were loaded in gel with a DNA ladder in one well.
- Kept the voltage at 100 for 45 minutes. Took a picture using the UV detection system.

2.6. Cloning

The amplified gene fragment was inserted in a PCR 2.1. vector.

- Ligation mixture was prepared by adding 2 µl 3X Fresh PCR product, 1 µl of 10X Ligation Buffer, 2 µl of pCR ® 2.1 vector (25 ng/µl), 4 µl of water and 1 µl T4 DNA Ligase.
- Mixture was incubated for 4 hrs at 14°C.

2.7. Transformation

The cloned vector was transformed into *Escherichia coli.*

- The ligation mixture was prepared and the competent cells were mixed and 30 sec heat shock at 40°C was given. S.O.C medium was added.
- Prepared LB plates containing X-Gal and IPTG. Transformed samples were spread on the LB plates and incubated at 37°C.

2.8. Analysis of Transformants

- Colonies were isolated from LB agar plates and transferred LB broth (ampicillin).
- Spin column was washed and the solution was added to the Qigen miniprep column.
- DNA was eluted into a fresh tube. Agarose gel electrophoresis was used to examine the digested DNA.

2.9. Sequencing

- The DNA was treated with sodium acetate, EDTA, and ethanol and 70% ethanol.
- The sequencer was used for sequencing and BLAST analysis was used to compare the acquired sequence to identify mutations in the isolated gene.

3. Results

3.1. RNA Quantification

The graph shows the absorbance by the samples at certain wavelengths. The absorbance value is displayed on the y-axis, while the nm wavelengths are displayed on the x-axis. The nucleic acid concentration obtained was 68.0 ng/µl. The concentration obtained is good and shows that the extracted RNA can be used further for the cDNA preparation.

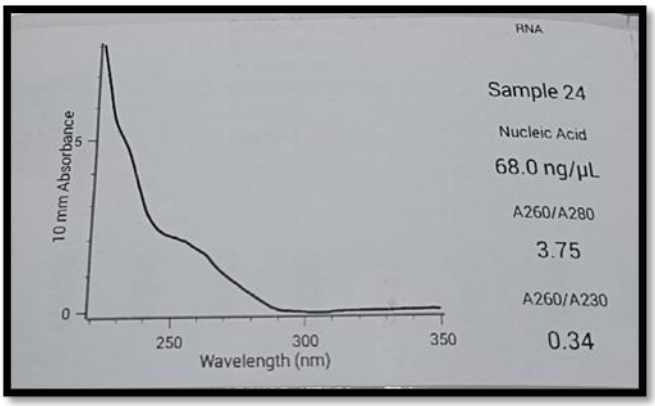

Figure 1. RNA Quantification through Nanodrop at A260/280 and A260/230

A260/280 value shows the purity of the extracted RNA. For the ratio A260/280 the value obtained is 3.75. The value is higher than 2 which shows that there are some impurities in the extracted RNA. The A260/A230 value was 0.34 which is lower than 1.8 which showed the presence of high density of organic contaminants.

3.2. PCR Results

As standard the DNA ladder was run along with the samples. The image (Figure 2.) obtained from the gel electrophoresis showed the presence of ladder in 1st well. in 2nd and 3rd well HA and NA samples were loaded respectively. The well 2 showed a smear and primer dimers. For the 3rd well primer dimers were observed.

1 2 3 4 5 6 7 8 9 10 11 12

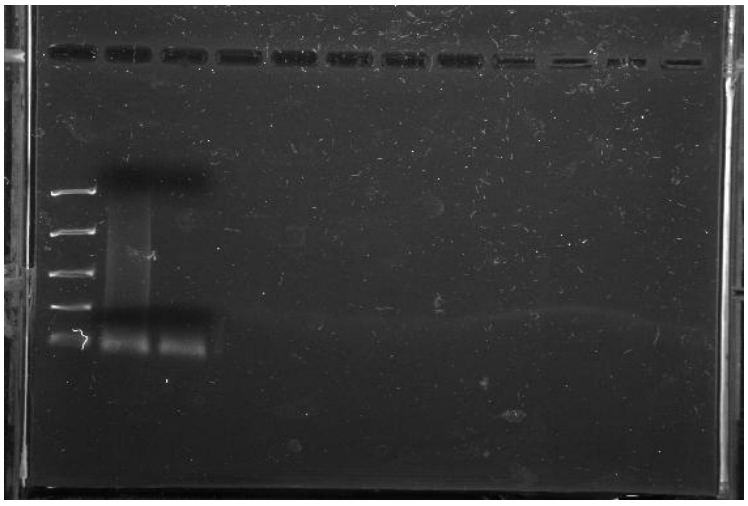

Figure 2. PCR analysis of HA sample

3.3. NA sample Nanodrop

A spectrophotometric examination was performed on the NA sample using a Nanodrop. The concentration of nucleic acids found was 15.6 ng/µl.

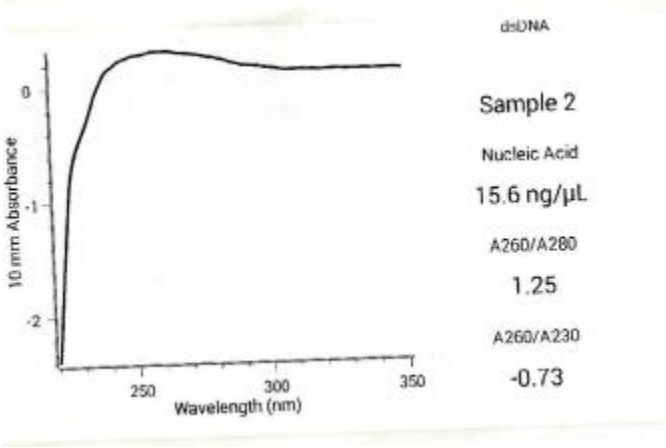

Figure 4. RNA Quantification of NA through Nanodrop at A260/280 and A260/230

For the ratio A260/280 the value obtained is 1.25. The value showed that there are some impurities in the NA DNA. The A260/A230 value was -0.73 which is lower than 1.8 which showed the presence of high density of organic contaminants.

3.4. Transformation Efficiency

Screening of the transformed cell was done using blue-white screening technique. The standard DNA value 0.001ng was used to calculate the transformation efficiency

Transformation efficiency is calculated as:

TE= (cfu on control plate/ng of competent cells) x $1x10^3$

=100/0.001 x 1 x 10^3

=$1x10^8$ cfu/ng

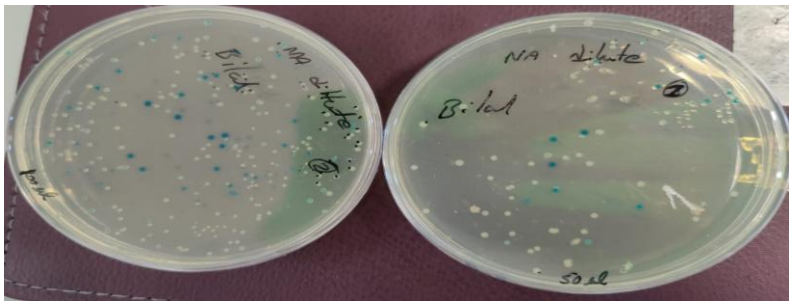

Figure 5. LB agar plates containing blue and white E.coli colonies

Table 1. Number of blue and white colonies from different volumes

Volume	Blue Colonies	White Colonies
50µl	9	62
100µl	39	196
100µl	37	235
250µl	55	254

(No. of blue colonies/Total number of colonies) x 100

Blue Colonies = (140/887) x 100 = 15%

White Colonies= (747/887) x 100= 84.2%

3.5. Transformation analysis

The gel was run for the digested and undigested samples. The standard DNA ladder was also run along with the samples to determine the size of the sample DNA.

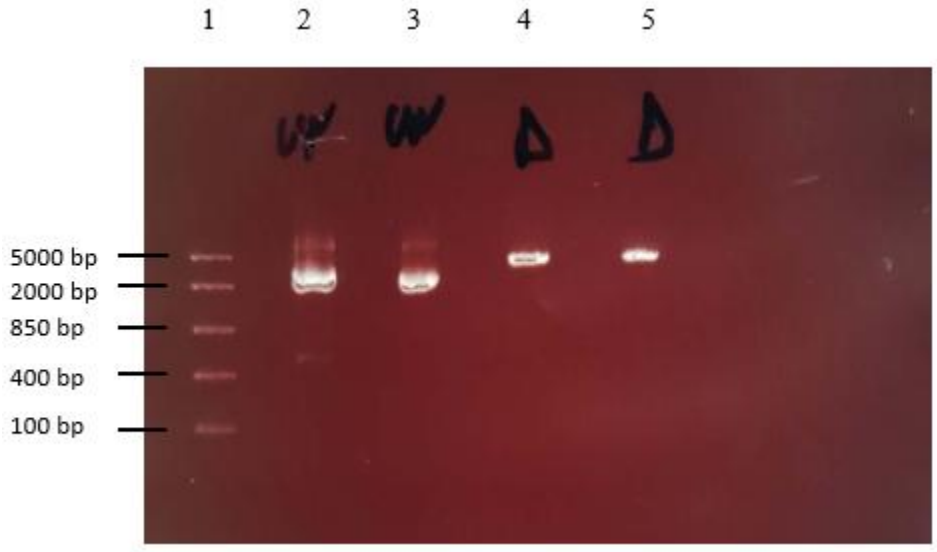

Figure 6. PCR results for digested and undigested samples

According to figure 5. well no 1. contained the DNA ladder, well no. 2, 3 contained undigested DNA while well 4,5 contained digested DNA. The bands for digested DNA were observed at 5000 bp and the bands for undigested samples were obtained at 2000 bp.

3.6. Sequencing and BLAST Analysis

3.6.1. Sequencing Results of NA

ATCCCCGAATTCGCATGGGGTTAAAAGAAAGAGCGAAARCTCGKGTTCWMTATCTCCCCGC
CKACTTCCASCTGCTGGCCGCCGTTACTGGTGGCTCCCAGCTCGCYACCCAKGTTGGARTAA
TCATGGTCATTGCTGTTTCCTGTGTATTATTGTTATCCGCTCACTATTCCMCACAACATACSA
GCCAGAATCATATAKTGTACCTGCTGTGCCGCCTGATGGATGAGCTACCTCACATTAGTTGC

GCTGTCCTTGCTGSCTTTTTTCCAGTCGGGCATCCTGTCCTGCCTGCTGCATTGATTAATCGAC
CGCCSCGCGAGAAGCGGTGGGTTTCKTGTTGGGTGCTCTTCCGCTTCCTTSATCGATTACTTG
CTGCSCTCGGTCCTTCTGCTGCGASSAGYGGTATCTSCTCACTCAGAGGCRGTAATACGGTTA
TCCACAKAATCRGGKGATAACGCARGAAASATCTTGTGAAWAAGASGGCCAAAARAAGGCC
AASAACCGWAAAAACGCCGCGTTGCTGGYKTTTTTYCATMGGCTCCSCCCCCGMTGASSAGC
ATMAMAAAMAWCGAYSMTCARTAAGGAGGYGGAMSAAACCCGAGASGAYTATGAARATA
CSAGTKTTYCCYCCTAGGAAGCTTCSTGGKGYGYTCTGYTGYTACSAYGCTGGCTGMTTASA
KGAMTRSYTGTCCKTCYTTTCCTYCCKTSRAGAARGGGYGGCTGYTTTMYTMATARCMTSAC
GCTAGTAGTWACTYTMGKCGKAGGTAGGTYCGTYSCCWCCAAGCKGTGSKGKGKMGCAAS
MACCCCSTCKATTCAGGACGACTGCTGCKTCATTYWKCGAGATATCTATCTGTTCGATGGSA
ATCCAGCGCGAGGTAACAGACACACGTACWTTCART

3.6.2. BLAST Analysis for NA

The BLAST results for NA sequence are shown in figure 6. The sequence showed 85% similarity with *Corynebacterium pseudotuberculosis* strain phoP. The result showed the isolated sequence belong to the bacterium and there was no similarity found with Influenza H1 virus.

```
>Corynebacterium pseudotuberculosis strain phoP, complete genome
Sequence ID: CP019768.1 Length: 2339296
Range 1: 941210 to 941967

Score:512 bits(567), Expect:9e-140,
Identities:557/776(72%),  Gaps:21/776(2%), Strand: Plus/Plus

Query  60      GCCKACTTCCASCTG-CTGGCCGCCGTTACTGGTGGCTCCCAGCTCGCYACCCAKGTTGG  118
               ||| | ||||| |   ||||| ||||||||| |||| ||| ||||||  ||| |  ||||
Sbjct  941210  GCCGAATTCCAGCACACTGGCGGCCGTTACTAGTGGATCCGAGCTCGGTACCAAGCTTGG  941269

Query  119     ARTAATCATGGTCATTGCTGTTTCCTGTGTATTATTGTTATCCGCTCACTATTCCMCACA  178
                 ||||||||||||| |||||||||||||||   |||||||||||||||| ||||| ||||
Sbjct  941270  CGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACA  941329

Query  179     ACATACSAGCCAGAATCATATAKTGTACCTGCTGTGCCGCCTGATGGATGAGCTACCTCA  238
               |||||| |||| ||| |||| | ||||    ||| |  |||| |||  ||||||| ||||
Sbjct  941330  ACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCA  941389

Query  239     CATTAGTTGCGCTGTCCTTGCTGSCTTTTTTCCAGTCGGGCATCCTGTCCTGCCTGCTGC  298
               ||||| ||||| ||   ||  ||| |   |||||||||||| | |||||| |||| |||||
Sbjct  941390  CATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGC  941449

Query  299     ATTGATTAATCGACCGCCSCGCGAGAAGCGGTGGGTTTCKTGTTGGGTGCTCTTCCGCTT  358
               ||| || ||||| ||  | |||| | || || || || | | ||||| ||||||||||||
Sbjct  941450  ATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTT  941509

Query  359     CCTTSATCGATTACTTGCTGCSCTCGGTCCTTCTGCTGCGASSAGYGGTATCTSCTCACT  418
               |||   ||  | ||| ||||| ||||||| ||| ||||||   || ||||||  ||||||
Sbjct  941510  CCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACT  941569

Query  419     CAGAGGCRGTAATACGGTTATCCACAKAATCRGGKGATAACGCARgaaasatcttgtgaa  478
               || |||| |||||||||||||||| |||| || ||||||||| |||| | | |||||
Sbjct  941570  CAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAG  941629

Query  479     waagasggccaaaaraaggccaasaaccgwaaaaaCGCCGCGTTGCTGGYKTTTTTYCAT  538
                || | |||||  | ||||||||  ||||| ||||| |||||||||||||  ||||| |||
Sbjct  941630  CAA-AAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCAT  941688

Query  539     MGGCTCCSCCCCCCGMTGASSAGCATmamaaamawCGAYSMTCARTAAGGAGGYGGAMSAA  598
                ||||||  |||||  |||  ||||| | ||| | |||    |||     |||| ||   ||
Sbjct  941689  AGGCTCCGCCCCC-CTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGG-CGAA  941746

Query  599     ACCCGAGASGAYTATGAARATACSA-GTKTTYCCYCCTAGGAAGCTTCSTGGKGYGYTCT  657
               |||||| | || ||| || |||| | |  || || ||| ||||||| | | | | |||
Sbjct  941747  ACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCT-GGAAGCTCCCTCGTGCGCTCT  941805

Query  658     GYTGYTACSAYGCTGGCTGMTTASAKGAMTRSYTGTCCKTCYTTTCCTYCCKTSRAGAAR  717
                 || | | |  || || | |||   || |   |||||  | ||  || || |   |||
Sbjct  941806  CCTGTTCCGACCCT-GCCGCTTACCGGA-TACCTGTCCGCCTTT--CTCCCTTCGGGAA-  941860

Query  718     GGGYGGCTGYTTTMYTMATARCMTSACGCTAGTA-GTWACTYTMGKCGKAGGTAGGTYCG  776
               | | ||| | |||  | ||| | | ||||| ||| ||  ||      ||   |||||| ||
Sbjct  941861  GCGTGGC-GCTTT-CTCATAGC-TCACGCT-GTAGGTATCTCAGTTCGG-TGTAGGT-CG  941914

Query  777     TYSCCWCCAAGCKGTGSKGKGKMGCAASMACCCCSTCKATTCAGGACGACTGCTGC  832
               |   | |||||| | |  | |  |||   |||||  |  |||||  |||| |||||
Sbjct  941915  TTCGCTCCAAGCTGGGCTGTGT-GCACGAACCCC--CCGTTCAGCCCGACCGCTGC  941967
```

Figure 7. BLAST Results for NA Sequence

3.6.3. Sequencing Results of HA

TMMYRGRGGCYTCCTARWGMAWGCTCGAGCGGCCGCCAGTGTGATGGATATCTGCAGAAT
TCGGYTTAGCAAAAGCAGGGGAAAATAAAAACACCCTTGTTTCTACTGGAAAAACACCAAG
CCGAATTCCAGCACACTGGCGGCCGTTACTAGTGGATCCGAGCTCGGTACCAAGCTTGGCGT
AATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATAC
GAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAAT
TGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAA
TCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACT
GACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAAT
ACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCA
AAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTG
ACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAGA
TACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACC
GGATACCTGTCGCCTTTCTCCCTTCGGAGCGTGCGCTTTCTCATAGCTCMGCTGTAGTATCTC
AGTCCGATGTAGGTCGTTCGCCTYCAAGCTGGGCCTGKGGTKGCCMSGAAAC

3.6.4. BLAST Analysis for HA

The BLAST analysis for HA sequence shows 89% similarity with cloning vector pX20pemIK-GW. There was no similarity found with influenza A virus.

```
Query: None Query ID: lcl|Query_96103 Length: 854


>Cloning vector pXF20pemIK-GW, complete sequence
Sequence ID: KX036765.1 Length: 7744
Range 1: 356 to 1079

Score:1280 bits(656), Expect:0.0,
Identities:713/725(98%),  Gaps:8/725(1%), Strand: Plus/Minus

Query  119   AAGCCGAATTCCAGCACACTGGCGGCCGTTACTAGTGGATCCGAGCTCGGTACCAAGCTT  178
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1079  AAGCCGAATTCCAGCACACTGGCGGCCGTTACTAGTGGATCCGAGCTCGGTACCAAGCTT  1020

Query  179   GGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACA  238
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1019  GGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACA  960

Query  239   CAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACT  298
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  959   CAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACT  900

Query  299   CACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCT  358
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  899   CACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCT  840

Query  359   GCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGC  418
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  839   GCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGC  780

Query  419   TTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCA  478
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  779   TTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCA  720

Query  479   CTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTG  538
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  719   CTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTG  660

Query  539   AGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCA  598
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  659   AGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCA  600

Query  599   TAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAA  658
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  599   TAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAA  540

Query  659   CCCGACAGGACTATAA-GATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCC  717
             |||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||
Sbjct  539   CCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCC  480

Query  718   TGTTCCGACCCTGCCGCTTACCGGATACCTGTC-GCCTTTCTCCCTTCGG--AGCGT-GC  773
             |||||||||||||||||||||||||||||||||| ||||||||||||||   ||||| ||
Sbjct  479   TGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGC  420

Query  774   GCTTTCTCATAGCTCM-GCTGTA-GTATCTCAGTCCGATGTAGGTCGTTCGCCTYCAAGC  831
             ||||||||||||||  |||||| |||||||||| || |||||||||||||| | |||||
Sbjct  419   GCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGC-TCCAAGC  361

Query  832   TGGGC  836
             |||||
Sbjct  360   TGGGC  356
```

Figure 8. BLAST Analysis for HA Sequence

4. Discussion

H1N1 was one of several recent influenza pandemics that killed many people around the world. There may not be enough vaccines or medical staff to meet demand. Responses such as curtailing travel and public gatherings, shuttering businesses, schools, and other public spaces, and fewer people showing up for work because they are sick or caring for family members all have the potential to have far-reaching economic effects (Dixon et al., 2010). The virus' flexibility is largely driven by its ability to produce genetic variation through mutation and reassortment (Ison, 2011). Many influenza strains may eventually acquire and retain resistance to these drugs (Ison, 2011).

For the analysis of mutations in the influenza virus and to use the genes for the synthesis of novel vaccines against the Influenza RD8. The virus was isolated from the chick embryo by taking the fluid and isolating the RNA (Webster, Yakhno, Hinshaw, Bean, & Murti, 1978). After RNA extraction the quantification of the isolated RNA was done by spectrophotometric analysis using Nanodrop. Indicating the presence of contaminants in the extracted RNA. Indicators of RNA purity include an A260/280 ratio of 2.0 and an A260/230 ratio of 1.8-2.2, as stated in the Nanodrop technical bulletin (Wilfinger, Mackey, & Chomczynski, 1997). The concentration obtained was 68.0 ng/µl, the ratio between A260 and A280 is 3.75 and the A260/A230 ratio was 0.34, which is less than 1.8 and indicative of a significant concentration of organic contaminants. cDNA was prepared from the extracted RNA and analyzed using PCR. The PCR analysis showed no band for the target gene and the primer dimers were observed in the samples. The primer dimers are due to the increased concentration of the primer used in the PCR reaction mixture. The PCR conditions might be not suitable for the primers to anneal the target DNA region and due to complementarity the primers bind with each other forming primer dimers. Using the other primer as a template, primer dimers are thought to develop through random base pairing and extension (Poritz & Ririe, 2014). The quantification of NA sample cDNA was done which showed 15.6 ng/µl and there were impurities in the samples. The cDNA was ligated into PCR 2.1. vector and transformed into *E. coli* cells. The transformants were analyzed using blue-white screening (Chaffin & Rubens, 1998). The transformation efficiency was calculated as 1×10^8 cfu/ng (Panja, Saha, Jana, & Basu, 2006). The number of blue colonies obtained was 140 and 747 white colonies were observed with 84.2% of transformation efficiency.

The samples were digested and after that PCR was run for both digested and undigested samples. The PCR results showed undigested samples with 5000bp and digested with 2000 bp. There was no insert observed in the sample. The results indicated that the transformation was done but the lack of insert band in PCR showed the cDNA was not ligated in the vector. Following PCR the samples were sequenced and the sequence was then run on NCBI BLAST to find out the similarity and mutations with the influenza virus. The sequence was not found similar to the influenza virus but the *Corynebacterium pseudotuberculosis* HA sequence showed similarity with cloning vector pX20pemIK-GW. The results were not according to the expected results and the mutations for the influenza virus were not detected.

5. Conclusion

The influenza virus is associated with high rate of mutations and causing epidemic. The study aimed to analyze the mutations in Influenza RD8 virus. The RNA extraction was done and cDNA was formed and ligated into a vector. The vector was transformed in *E.coli* and the transformants were analyzed. The samples were digested and PCR was run but no insert was found. The sequencing results also showed no similarity with the influenza virus and hence no mutations could be detected. The study was incomplete due to the negative results and the mutation analysis was not completed for Influenza RD8.

References

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