RESEARCH ARTICLE

Detection of NEP Gene Among Patients Suffering from Flu-A viruses Isolated From Different Clinical Specimens

Mays Hadi Jebur[1], Hiba Jasim Hamza[2], Zahraa Mohammed[3]

[1]Basic and Medical Science Department, College of Nursing, University of Babylon, Iraq
[2]Basic and Medical Science Department, College of Nursing, University of Babylon, Iraq
[3]Department of Microbiology, College of Medicine, Mustansiriyah University, Iraq

ARTICLE INFO	ABSTRACT
	Influenza A viruses are medically directed viral pathogens that continue to cause significant mortality and morbidity in people worldwide. In migratory disease epidemics (known as influenza season), influenza A at a specific geographic location act as a catalyst for the unpredictability of the pathogen and the evolution of new pandemic viruses. This study included (40) patients ranging in age from (3 to 10) years(22patients carry flu-A and 18 control). (7)pharyngeal wash samples, 9 throat swabs, 14 blood samples, and 10 pharyngeal swabs were collected. it was found that the percentages were (17.5%, 22.5%, 35%, and 25%) sequentially.Influenza A patients visited Al Hilla Teaching Hospital in Babylon Governorate, for the period between January 2022 to March 2022. The aim of this study was to detect the NEPgene in influenza A viruses using the Latest rapid and modern methods and techniques by conventional and rRT-PCR Influenza. A 40 specimens carried with rapid tests now Influenza A detected byEasyPure® Viral RNA Kit for virus genomic extraction using Conventional-PCR and rRT-PCR Virus detection using nuclear export protein GENE (NEP) and matrix protein (M1) genes for virus diagnosis In the results, (22) sample carrying IGM Ab for virus A and 15 (68.1%) out of 22 samples carrying the (NEP) gene while (M1) gene was found in 10 (45.4 %) out of (22) sample. Bilirubin in urine test was also conducted for all samples, and the percentages 22(55%)were positive results and18(45%) negative results out of 40 samples. In conclusion, Influenza A is considered an epidemic spreading for everyone all over the world, and the gene is considered a diagnostic gene for this virus due to its high percentage in samples.The gene (M1) is used to describe the protein that forms a layer within the viral envelope.

INTRODUCTION

Influenza A is a standard respiratory infection that affects humans. There is an annual plague of sickness called flu season caused by human influenza A viruses. Only influenza A viruses have been linked to influenza pandemics. A pandemic may develop if a new and different influenza A virus evolves that both contaminate humans and has the potential to effectively spread among people [1]. Depending on the surface proteins, the virus is divided into secondary divisions, where there were 18 divisions of the and 11 divisions of the also found more, there were 130 other species, and its ability to divide is due to its ability to reassortments. The flu belongs in orthomyxoviridae family and you have a divided genome (negatively single strand) and it's coated and their genome has five subdivided[2]. A protein NEP represented in the multiplication of the virus and the emergence of

differents types, so there was a need to intensify studies on this gene and its effective role in finding unique types[3]. The M1 protein is a part of the influenza virus that helps it stay together. It plays a big part in getting other viral parts together during replication. It reacts with viral (vRNPs) [4]. It was found in most studies that the rates of gene M1 development between viruses that infect different types are high. It has been observed that there is more than one identity for this gene [5]. During the epidemiological history of this virus, advanced research indicates that this virus represents an epidemic in children who are less than years old in the absence of immunization[27]. In injuries by influenza A, liver problems occur, and one of the most indications of liver problems is Bilirubine that which is a strong indicator of liver injury and a defect in its functions as a result of exposure of the liver to this virus[28]. On the basis of the presence of bile pigments in the urine, it is considered one of the biggest indications of liver weakness as a result of excessive production of bilirubin and caused by the exposure of the liver to a viral infection[29].

AIM of the Research

The goal of this Research was to detect the NEPgene in influenza A viruses using the Latest rapid and modern methods and techniques by conventional and rRT-PCR technique.

Ethical approve

The patients' valid consent was obtained before they were included in the report. Each patient was told of the technique before any samples were obtained, ensuring that they were aware of what was going to happen. Patients were aware of their legal right to refuse.

MATERIALS AND METHOD

Producers

The researched included 40 patients with age ranging from(3-10)years. Patients with InfluenzaA visited to Al-Hilla Teaching Hospital in Babil Governorate, for the period between January 2022 to March 2022. (7)pharyngeal wash samples, 9 throat swabs, 14 blood samples, and 10 pharyngeal swabs were collected.

Blood Sample:

A total of 5 ml of blood was taken from 14 patients out of 40 sample. 2ml from the sample were put in an EDTA tube, to obtain whole blood, All samples were stored in freezing at (-20°C) until used, put down gradually into dispensable tubes containing isolating gel, After 30 minutes of clotting at room temperature, the serum was centrifuged at 2000g for about 10 minutes, then partitioned into small aliquots and stored at (-20°C).

Pharyngeal and Nasal Swabs:

Pharyngeal and nasal swabs samples are collected from the end of the nose and pharynx using these swabs, which are wooden or nylon sticks covered at the end with cotton, 10 samples from the nasopharynx and 9 samples from the throat.

### Identification of InfluenzaA virues by Rapid tests technique:

Rapid tests technique for the simultaneous detection in IgM antibodies or IgG antibodies to the main etiological agents of influenza A viruses. The rapid tests technique is based on the reactivity of antibodies with antigen on the slide surface. If antigen-antibody complexes appeared, it investigates infections news when appears IGM while IGG is detected past infection can be used to investigate it. The test required 10 minutes to complete the reaction.

Fouchet's Test(Detection of Dilirubin in Urine):

10 ml of urine with a few ml of barium chloride, filter, and filtering remain on filter paper after that add one drop of (FeCL3), green spot positive results in this study 55% positive results while 45% negative results.

Distribution of Flu-A among specimens in this study:

In this research, influenza A was detected from samples of pharyngeal lavage, throat swab, blood, and finally a nasal swab, where it was found that the percentages were (17.5%, 22.5%, 35%, and 25%) as shown in the figure (1).
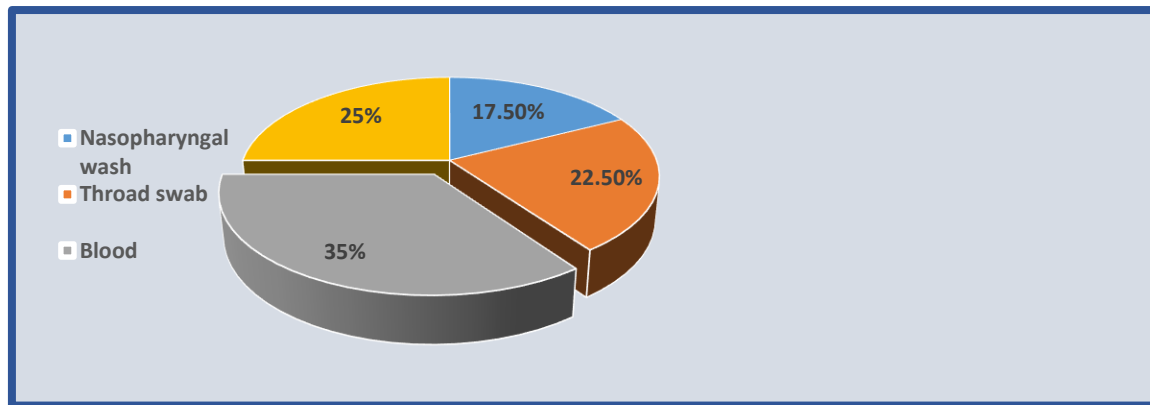
Figures (1):Distribution of Flu-A among specimens

Molecular Detection of Virus

   1- Extraction of Genomic Information:

RNA Extraction

EasyPure® Viral RNA Kit for Extraction

Genomic from blood samples were extracted by using gSYAN RNA mini kit extraction kit USA. and extracted. For future usage, RNA samples were kept at -20 °C.

   2- Real-Time PCR:

Syper Green qPCR SuperMix

The qPCR master mix reaction was made with a (one taq quick-load)PCR Kit and this master mix done as the company said.

qPCR Thermocycler Conditions

qPCR thermocycler conditions were happened by using Real-Time PCR thermocycler system listed in Table(1) and Table (2).

   4- Genes Amplification in q PCR .

In real time, the threshold cycle number (CT value) that showed the positive amplification in the Real-Time PCR cycle was the best way to get the information. Amplification of genes in Real-Time PCR is shown in the figures (2) and (3) for genes that were used in this study, as shown.

Table (1) Thermal cycler steps:

Table 1: Stages and Temperature of qPCR for NEP gene

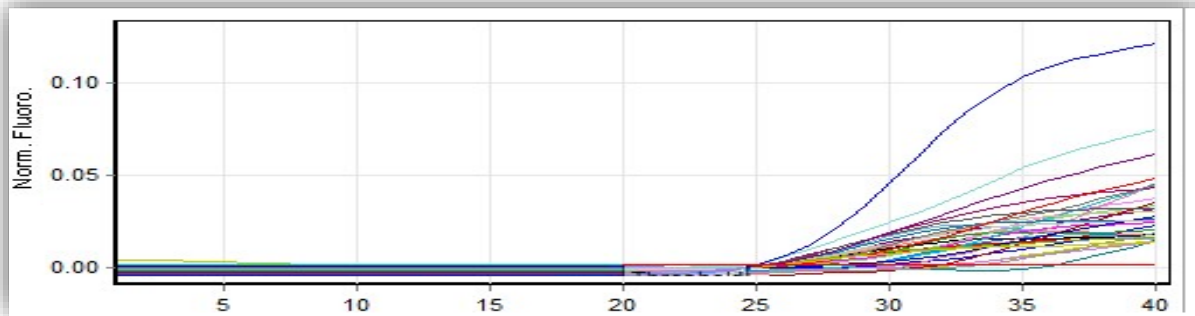|		Temperature	Time	Cycle
Stage 1	Denaturation	94	30	1
Stage 2	Denaturation	94	5	
	Annealing	53	15	35
	extension	72	20	
Stage 3	Dissociation	78-98	1	1

Figure 2: NEP gene amplification plots by qPCR. Samples included all samples. The photograph was taken directly from qPCR machine

Table (2) stages and temperature of qPCR for The *M1* -matrix protein gene

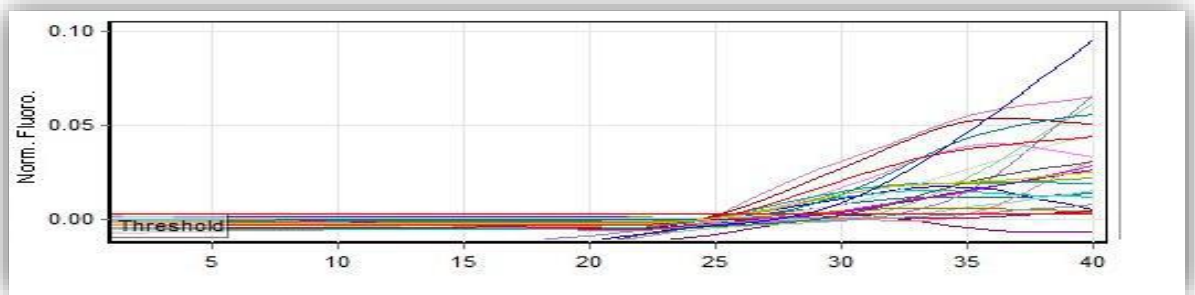|		Temperature	Time	Cycle
Stage 1	Denaturation	94	30	1
Stage 2	Denaturation	94	5	
	Annealing	52	15	35
	extension	72	20	
Stage 3	Dissociation	78-98	1	1

Figure (3) Matrix protein- Gene amplification plots made by qPCR are shown here. Samples had all samples in them. The picture was taken right from the qPCR machine.

5- PCR (Convensional- Polymerase Chain Reaction) Technique:

PCR was done with the(25 µ) reaction mixture from the cDNA reaction. The PCR mix was made. In a PCR machine, the amplification was done 40 times (Biomed, Ditfurth, Germany). Each cycle

consisted of denaturation at 94°C for 2 min, annealing of the primers at 53°C and 52°C for 2 min, and chain elongation at 70°C for 5 min. On positive influenza A samples, the PCR technique was used to perform RNA analysis. This procedure was carried out according to E.C.J. Claas *et al.*, (1992), Hendrixson *et al.*, (1997) with influenza A specific primer NEP gene and Matrix protein gene the primer sequence in this shown in Table (3).

Table 3: The PCR condition for all genes and sequences genes was used in this study.

| Gene name	genes sequences (5'-'3)	Size of Bp	PCR Conditions	References
NEP gene	F 5'AAGGGCTTTACCGAAGAGG'3 R 5'CCCATTCTCATTACTGCTTC'3	**190**	1: 94C, 2 min. 　　2 : 94ºC, 30 sec. 3: 53.3ºC, 30 sec. 　　4: 70ºC, 20 sec. 5: 70ºC, 5 min.	E.C.J. Claas *et al.*,1992
M1 gene	F5'- AGCAAAAGCAGGTAGATATTGAAAGA-'3 R 5'-AGTAGAAACAAGTAGTTTTTTACTC-'3	**459**	1: 94ºC, 2 min. 　　2: 94C, 30 sec. 3: 52.0ºC, 30 sec. 　　4: 70ºC, 50.0 sec. 5:　94C, 30 sec. 6: 45.0ºC, 30 sec. 　　7: 70ºC, 50.0 se 8: 70ºC, 5 min.	Hendrixson *et al.*, (1997)
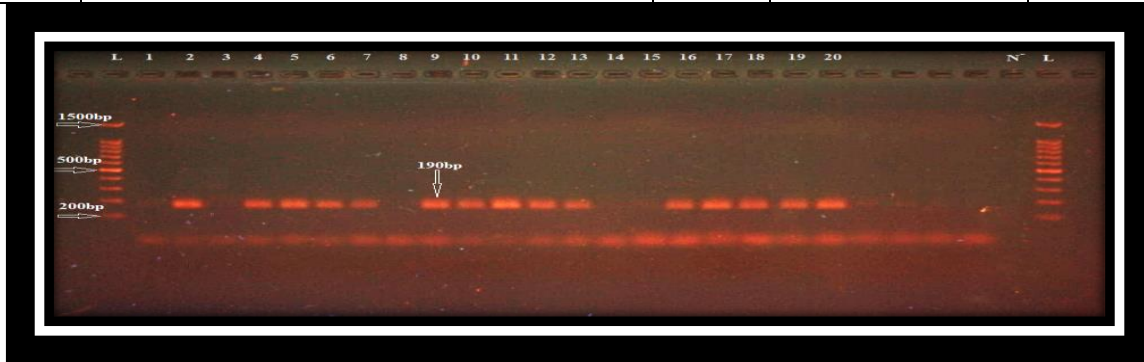
Figure (4) :- Tests for the (NEP gene) that show 190bp at 53°C when gel electrophoresis is done (Agarose 2 percent , 15min. at 100 voltage and then lowered to 70 volts, 60min.) After staining with ethidium bromide, this picture can be seen under UV light. It was in Lane L that the DNA ladder (1500-100)bp was shown, as well as Lanes 2, 4, 7, 9, 13, and 16-20. Lanes 1, 3, 8, and 14-15 were Negative results, and Lane (N) was a Negative control.
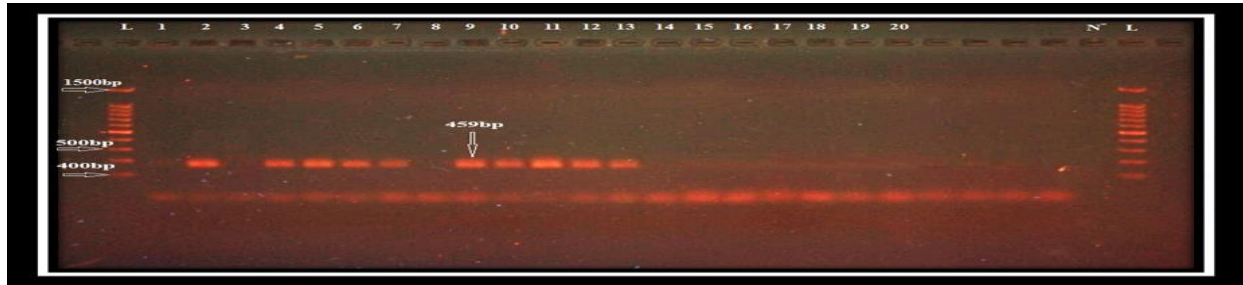
Figure 5:- This is gel electrophoresis for the PCR product of (M1gene) that shows 459 bp at 52°C (Agarose 2 percent , 15min. at 100 voltage and then lowered to 70 volts, 60min.) After staining with ethidium bromide, this picture can be seen under UV light. In Lane L, there was a DNA ladder (1500-100)bp. Lanes (2,4-7,9-13) were positive results, Lanes (14-20) were negative results, and Lane (N) was a Negative control.

RESULTS

In this study, the distribution of 40 samples was taken from pharyngeal and nasal swabs and blood samples sequentially. It was found that the best sample to obtain positive and accurate results is blood, through its ratio of about 35%, and it is illustrated in Figure No(1). Table (3), Virus was identified on 22 samples by using the Rapid test Flu-virus Card test, the results revealed that 22(55%) out of 40 specimens were related to virus infection, the sera of patients were shown anti-IgM to influenza antibodies. Influenza viruses from 22 samples who give positive results by Rapid test, it was found 15 isolated were related of influenza viruses, produced the specific (190bp) fragment for NEP genes when compared with ladder in Figure (4). Out of 22clinical specimens, 22 (55%) were positive Rapid test, while 15 (68.1%) had carried NEP gene, identified only 10 (45.4%) isolates having Matrix protein(M) in influenza viruses. The polymerase chain reaction methods depend on DNA polymerase's capacity to produce new strands of DNA that are complementary to the template strand, and the expected sequence will have been gathered in billions of copies by the end of the PCR reaction (Amplicon). For isolates previously identified as influenza A viruses, molecular identification of the NEPgene was performed. The results showed that 15 of the 22 isolates tested positive for this gene. When compared to an allelic ladder 100-1500bp), positive findings were discovered by the presence of (190 bp) bands, as illustrated in Figure (5). In addition, the Matrix protein (M1) gene was molecularly detected in 10 influenzas A viruses, with the results showing that only (45.4 percent) of the isolates had this gene. The existence of a (459 bp) band, when compared to an allelic ladder, revealed positive results forM1 the gene. The results of the study for the analysis of bilirubin in the urine also showed that 55% of the samples were positive for this test and 45%they were negative. Which proves that any defect in the liver due to viral infection leads to the production of excessive amounts of bilirubin.

DISCUSION

In this study, the result of researcher agreements with a study by [6]for the predominant of NEPgene in the influenza viruses while another study by [7] found the same result and agree with this study for this gene and other researchers show the relationship for the NEP with viral replication and ability to accept of mutation for produce other strain this show by [8] and by [9] agreement with this study. The study proved that a protein NEP appeared in the multiplication of the virus and the emergence of new types, so it became a target in finding treatments to combat influenza. while the other protein Matrix protein (M1) the results for this study agreement with the study by [10] and another study by [11], while another study by [12] shows the structure of matrix protein 1 of influenza A virus agreement with this study and study by [13] and [14] all this study show the role of matrix protein in influenza A by The influenza A virus budding process is facilitated by the matrix protein

M1 in infected cells. This protein binds to the inner leaflet of the PM and interacts with viral genetic material and envelope proteins. Thus, its oligomerization is strongly linked to viral component assembly and viriogenesis. Where the study indicated that it is necessary for its presence to increase the efficiency of the virus in reproduction, as its percentage was in the majority of viral isolates, and this proves its importance and its role in reproduction. All studies that dealt with this protein have dealt with its role in causing infection through the replication of the virus and its prominent role in this process. As its presence in the virus is considered one of the strength and pathogenic factors due to its ability to cause pathogenicity through its active role. Many studies have shown this, in addition to its high rate of influenza A virus.

The distribution in this study is consistent with a study conducted by the scientist [17] by collecting samples from the pharyngeal wash as well as another study by [18] was collected the specimens from blood and another study by [19] while a study by [20,21,22] agrees with this study for obtaining viruses from throat swab and syudy by [23,24,25] All these studies prove that the virus can be obtained from different clinical samples. This study agrees with a study conducted by a scientist [30] who clarified that any defect that affects the liver exposes it to excessive production of bilirubin and other study by [31] All studies have shown that liver dysfunction or failure disrupts the production of bilirubin and leads to its overproduction, which is a preliminary indication of infection and conclusive evidence of liver exposure to HAV infection. The presence of bilirubin in the urine, especially in children, is an indication of HIV infection, in addition to other secondary symptoms.

CONCLUSION

Influenza A is a virus with a distinctive feature, which is its possession of antigenic mutations to produce a rapidly spreading epidemic. This ability enables it to produce new and rapidly spreading viruses, and thus the occurrence of an epidemic. And the proteins that were dealt with in the above research show their importance and the proportion of their presence in the virus, and this proves their effective role in giving strength to the virus.

REFERENCES

1- Skelton, R.M., 2021. *Investigating Effects of the Influenza NS1 Protein on Host Immune Responses Against Influenza a and D Viruses* (Doctoral dissertation, University of

2- Skelton, R.M., 2021. *Investigating Effects of the Influenza NS1 Protein on Host Immune Responses Against Influenza a and D Viruses* (Doctoral dissertation, University of

3- Stauffer S, Feng Y, Nebioglu F, Heilig R, Picotti P, Helenius A. 2014. Stepwise priming by acidic pH and a high K concentration is required for efficient uncoating of influenza A virus cores after penetration. J Virol 88:13029 –13046₃₆₉₆.

4- Manzoor R, Sakoda Y, Mweene A, Tsuda Y, Kishida N, Bai GR, Kameyama KI, Isoda N, Soda K, Naito M, Kida H. 2008. Phylogenic analysis of the M genes of influenza viruses isolated from free-flying water birds from their Northern Territory to Hokkaido, Japan. Virus Genes 37:144 –152

5- Long, J.S., Mistry, B., Haslam, S.M. and Barclay, W.S., 2019. Host and viral determinants of influenza A virus species specificity. Nature Reviews Microbiology, 17(2), pp.67-81.

6- Zhirnov, O.P., 2020. Unique bipolar gene architecture in the RNA genome of influenza A virus. Biochemistry (Moscow), 85(3), pp.387-392.

7- Zhirnov, O.P., 2020. Unique bipolar gene architecture in the RNA genome of influenza A virus. Biochemistry (Moscow), 85(3), pp.387-392.

8- Wang P, Zheng M, Lau SY, Chen P, Mok BW, Liu S, Liu H, Huang X, Cremin CJ, Song W, Chen Y. Generation of DelNS1 influenza viruses: a strategy for optimizing live attenuated influenza vaccines. MBio. 2019 Sep 17;10(5):e02180-19

9-   10-Cao, S., Liu, X., Yu, M., Li, J., Jia, X., Bi, Y., Sun, L., Gao, G.F. and Liu, W., 2012. A nuclear export signal in the matrix protein of Influenza A virus is required for efficient virus replication. Journal of virology, 86(9), pp.4883-4891.

10-   11- Guo, J., Chen, J., Li, Y., Li, Y., Deng, G., Shi, J., Liu, L., Chen, H. and Li, X., 2021. SUMOylation of matrix protein M1 and filamentous morphology collectively contribute to the replication and virulence of highly pathogenic H5N1 avian influenza viruses in mammals. Journal of virology, pp.JVI-01630.

11-   12- Peukes, J., Xiong, X., Erlendsson, S., Qu, K., Wan, W., Calder, L.J., Schraidt, O., Kummer, S., Freund, S., Kräusslich, H.G. and Briggs, J.A., 2020. The native structure of the assembled matrix protein 1 of influenza A virus. Nature, 587(7834), pp.495-498.

12-   13- Hom, N., Gentles, L., Bloom, J.D. and Lee, K.K., 2019. Deep mutational scan of the highly conserved influenza A virus M1 matrix protein reveals substantial intrinsic mutational tolerance. Journal of virology, 93(13), pp.e00161-19.

13-   14-Zhu, L., Zhao, W., Lu, J., Li, S., Zhou, K., Jiang, W., Duan, X., Fu, L., Yu, B., Cai, K.Q. and Gao, G.F., 2019. Influenza virus matrix protein M1 interacts with SLD5 to block host cell cycle. Cellular microbiology, 21(8), p.e13038.

14-   15- Claas ECJ, Sprenger MJW, Kleter GEM, van Beek R, Quint WGV, Masurel N. Type-specific identification of influenza viruses A, B and C by the polymerase chain reaction. J Virol Methods. 1992;39:1–13

15-   16- Baranovskaya, I.L., Sergeeva, M.V., Taraskin, A.S., Lozhkov, A.A. and Vasin, A.V., 2021. Mutations designed to modify the NS gene mRNA secondary structure affect influenza A pathogenicity in vivo. Microbiology Independent Research Journal (MIR Journal), 8(1), pp.1-9.

16-   17-Paradis, S., Lockamy, E., Cooper, C.K. and Young, S., 2021. Clinical evaluation of the molecular-based BD SARS-CoV-2/flu for the BD MAX™ System. Journal of Clinical Virology, 143, p.104946.

17-   18-Zheng, D., Shuai, X., Li, Y., Zhou, P., Gong, T., Sun, X. and Zhang, Z., 2016. Novel flurbiprofen derivatives with improved brain delivery: synthesis, in vitro and in vivo evaluations. Drug Delivery, 23(7), pp.2183-2192.

18-   19-Henter, J.I., Palmkvist-Kaijser, K., Holzgraefe, B., Bryceson, Y.T. and Palmér, K., 2010. Cytotoxic therapy for severe swine flu A/H1N1. The Lancet, 376(9758), p.2116.

19-   20-Banerjee, D., Kanwar, N., Hassan, F., Essmyer, C. and Selvarangan, R., 2018. Comparison of six sample-to-answer influenza A/B and respiratory syncytial virus nucleic acid amplification assays using respiratory specimens from children. Journal of clinical microbiology, 56(11), pp.e00930-18.

20-   21-Dai, Q., Ma, W., Huang, H., Xu, K., Qi, X., Yu, H., Deng, F., Bao, C. and Huo, X., 2018. The effect of ambient temperature on the activity of influenza and influenza like illness in Jiangsu Province, China. Science of the total environment, 645, pp.684-691.

21-   22-Malekshahi, S.S., Azad, T.M., Yavarian, J., Shahmahmoodi, S., Naseri, M. and Rezaei, F., 2010. Molecular detection of respiratory viruses in clinical specimens from children with acute respiratory disease in Iran. The Pediatric infectious disease journal, 29(10), pp.931-933.

22-   23-Ma, P., Tang, X., Zhang, L., Wang, X., Wang, W., Zhang, X., Wang, S. and Zhou, N., 2022. Influenza A and B outbreaks differed in their associations with climate conditions in Shenzhen, China. International journal of biometeorology, 66(1), pp.163-173.

23-   24-Ranadheera, C., German, G.J., Steven, L., Eung, D., Lyubashenko, D., Pepin, J.C., Zivcec, M., Antonation, K. and Corbett, C.R., 2022. A four specimen-pooling scheme reliably detects SARS-CoV-2 and influenza viruses using the BioFire FilmArray Respiratory Panel 2.1. Scientific Reports, 12(1), pp.1-8.

24-   25-Shrestha, D., Bhattachan, B., Parajuli, H. and Shrestha, S., 2021. Avian/Bird flu: A review: H5N1 outbreaks in Nepal. Nepal Journal of Biotechnology, 9(1), pp.24-41.

25-   26-Truong, P.T., Saito, S., Takayama, I., Furuya, H., Nguyen, B.G., Van Do, T., Phan, P.T., Do, C.D., Dao, C.X., Dang, T.Q. and Ngo, C.Q., 2021. Respiratory microbes detected in hospitalized adults with acute respiratory infections: associations between influenza A (H1N1) pdm09 virus and intensive care unit admission or fatal outcome in Vietnam (2015–2017). BMC infectious diseases, 21(1), pp.1-9.

26-   27-American Academy of Pediatrics Committee on Infectious Diseases, 2004. Recommendations for influenza immunization of children. Pediatrics, 113(5), pp.1441-1447.

27-   28-Al-Jafar, H., Alkhaldi, J.A. and Termos, S., 2020. Etiology based sickle cell disease hepatopathy. Open Journal of Gastroenterology, 10(7), pp.187-201.

28-   29-Roy-Chowdhury, N., Wang, X. and Roy-Chowdhury, J., 2020. Bile pigment metabolism and its disorders. In Emery and Rimoin's Principles and Practice of Medical Genetics and Genomics (pp. 507-553). Academic Press.

29-   30-Thomas, M., Hardikar, W., Greaves, R.F., Tingay, D.G., Loh, T.P., Ignjatovic, V., Newall, F. and Rajapaksa, A.E., 2021. Mechanism of bilirubin elimination in urine: insights and prospects for neonatal jaundice. Clinical Chemistry and Laboratory Medicine (CCLM), 59(6), pp.1025-1033.

30-   31-Resmi, P.E., Raveendran, J., Suneesh, P.V., Ramanchandran, T., Nair, B.G. and Babu, T.S., 2021. Screen-printed carbon electrode for the electrochemical detection of conjugated bilirubin. Materials Letters, 304, p.1305