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FACTORS AND OUTCOMES ASSOCIATED WITH INFLEUNZA VIRUS LOADS IN HOSPITALIZED PATIENTS

by
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ABSTRACT
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Title: Factors and outcomes associated with influenza virus loads in hospitalized patients

Annual influenza outbreaks constitute a substantial economic and public health burden accounting for 3-5 million hospitalizations and up to 645 000 deaths worldwide. Two influenza A subtypes (H1N1pdm09 and H3N2) and two influenza B lineages (B/Yamagata and B/Victoria) circulate around the globe causing seasonal outbreaks. In this study, we aimed at performing clinical and virologic characterization of patients hospitalized with severe influenza disease during the 2018-2019 and 2019-2020 seasons. We enrolled 1654 patients within 72 hours of being hospitalized with acute respiratory symptoms and who had symptoms consistent with influenza within the seven days before admission. The study was approved by IRB. Demographic and clinical data were collected from individual patients along with nasopharyngeal (NP) and oropharyngeal (OP) swabs. The samples were screened for influenza A, B and their subtypes/lineages using reverse transcriptase real-time PCR (RT-PCR) following RNA extraction. The agreement between sample types was assessed. The viral titers were compared for patient demographics, respiratory symptoms, patient health characteristics, and clinical outcomes. NP and OP samples had minor agreement for influenza A (kappa=.464, p<.001) and moderate agreement for influenza B (kappa =.732, p<.001). NP swabs had better sensitivities in detecting both influenza A (91.2% vs. 70.4%) and B (83.3% vs. 61.5%) viruses and yielded higher viral loads compared to OP. Higher viral load was associated with asthma for influenza B/Victoria, and runny nose for influenza A and A/H1N1. A lower viral load, however, was found to be associated with ARDS (Acute Respiratory Distress Syndrome) and bacterial-co-infection for influenza A, and oxygen supplementation for A/H1N1. In conclusion, NP and OP swabs are complementary in detection influenza, however, with limited resources NP swabs are preferred. Further studies with a more appropriate sample size are needed to validate the relationship between viral load and asthma as well as vaccination status.

Keywords: influenza, surveillance, burden, concordance, viral load, Lebanon

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ABBREVIATIONS

SARI: Severe Acute Respiratory Infections
EMR: Eastern Mediterranean Region
NP: Nasopharyngeal
OP: Oropharyngeal
HA: Hemagglutinin
NA: Neuraminidase
SA: Sialic acid
RT-PCR: Real-time polymerase chain reaction
RNA: Ribonucleic Acid
vRNA: Viral ribonucleic acid
vRNP: Viral ribonucleoprotein complex
cRNA: Complementary ribonucleic acid
PA: Polymerase acid
PB1: Polymerase basic 1
PB2: Polymerase basic 2
NS: Non-structural protein
ILI: Influenza-like illness
GIHSN: Global Influenza Hospital Surveillance Network
WHO: World Health Organization
WHOE: World Health Organization Europe
LAIV: Live attenuated influenza vaccine
IIV: Inactivated influenza vaccine
NAI: Neuraminidase inhibitor
M1: Matrix protein 1
M2: Matric protein 2
BM2: influenza B matrix protein 2
CM2: influenza C matrix protein 2
CDC: Center for Disease Control and Prevention
NS: Non-significant
ARDS: Acute respiratory distress syndrome
CV: cardiovascular
DM: Diabetes mellitus
NEP: Nuclear export protein
HEF: hemagglutinin esterase fusion protein
P3: polymerase acidic influenza C
URT: Upper respiratory tract
mRNA: Messenger ribonucleic acid

CHAPTER I

INTRODUCTION

Influenza viruses constitute a major economic and public health burden accounting for 3-5 million hospitalizations and up to 645 000 deaths worldwide (Perera, 2017; Troeger et al., 2019). It was estimated that influenza alone accounts for 0.26% of all-cause mortality globally (Collaborators GBDI, 2019). Influenza viruses cause annual outbreaks (seasonal or inter-pandemic) and occasional pandemics (Bennett, Dolin, & Blaser, 2015, Paules, & Subbarao, 2017). Influenza viruses evolve constantly *via* mutations or reassortment of their genome segments which could result in antigenic drift or antigenic shift. Both influenza A and B undergo antigenic drift, which enables the virus to escape vaccine-generated immunity and results in seasonal epidemics (Paules & Subbarao, 2017). Influenza A and B cause annual epidemics of influenza illness such that influenza A/H1N1 (H1N1pdm09) co-circulates with A/H3N2 and influenza B viruses(Cox et al., 1999; Paules & Subbarao, 2017).

Antigenic shift is a rare event that only takes place in influenza A viruses. It refers to the introduction of a novel virus strain into human beings to which most or all of the population does not have immunity. If spread efficiently, it can cause a global pandemic (Bennett, Dolin, & Blaser, 2015). Four major global pandemics have occurred as a result of antigenic shift within the past 100 years: the H1N1 Spanish Influenza 1918 (at least 50 million deaths); the H2N2 Asian Influenza 1957 (1.2 million deaths); the H3N2 Hong Kong Influenza 1958 (1 million deaths); and the H1N1 Swine influenza 2009 (151,700-575,400 deaths) (Pandemic Influenza | CDC; 2019). Sixteen hemagglutinin (HA) and nine neuraminidase (NA) subtypes have been isolated from

birds, and two additional HA and NA subtypes have been identified in bats (Tong, 2013). The large genetic diversity in the avian reservoir and high mutation rate and potential for reassortment provides an environment for the emergence of novel and potentially pandemic influenza A viruses. Influenza B viruses do not have an animal reservoir and possess a relatively lower rate of mutation compared to influenza A. Two antigenically distinct B lineages —Victoria (B/Victoria/2/87-like) and Yamagata (B/Yamagata/16/88-like) — have co-circulated in human beings since 1983 (Bennett, Dolin, & Blaser, 2015) (Chen, 2016). The continuous evolution of influenza viruses has a significant effect on vaccine effectiveness, the efficacy of antiviral drugs, and diagnosis (Kim & Poudel, 2013).

Several diagnostic tests are used in the detection of the virus including immunofluorescence, serological testing, and viral culture; however, rapid influenza detection and real-time polymerase chain reaction remain the ones most commonly used, based on timelines and sensitivity (Overview of Influenza Testing Methods. CDC, 2020) (Kim & Poudel, 2013). This study aims to assess the correlation between influenza viral load and patient factors and outcomes during two influenza seasons in Lebanon.

A. Influenza Taxonomy:

1. Classification

Influenza viruses belong to the *Orthomyxoviridae* family. The family represents enveloped particles that contain single-stranded, segmented negative-sense RNA genomes. It is classified into four types A, B, C, and D (Gideon Informatics, Inc., & Berger, 2018).

2. Structure

Under an electron microscope, influenza A and B are virtually indistinguishable in their morphology (Bouvier & Palese, 2008). Lab-adapted strains tend to be spherical or elliptical, whereas clinical isolates are generally filamentous. The genome is comprised of eight RNA segments (Paules & Subbarao, 2017). Influenza C is known to form cord-like structures in virus-infected cells. It has 7 segments of single-stranded RNA encoding 9 proteins (Muraki et al., 2004). Influenza D has an identical genome structure as influenza C; it consists of 7 gene segments of single-stranded RNA (Su et al., 2017).

The HA protein is the main antigenic glycoprotein on the surface of the virus that elicits neutralizing antibody responses (Sriwilaijaroen & Suzuki, 2012). It is coded by RNA segment 4 (Palese & Schulman, 1976). HA is a homotrimer with a globular domain containing a receptor binding site specific for sialic acid (SA). Infection is initiated when HA attaches the virus to SA-conjugated receptors and possibly other receptors on the cell surface (Liu et al., 1995, Stray et al., 2000). Avian influenza viruses such as H5, H7, and H9 can occasionally infect humans, crossing the species barrier. However, due to the difference in the receptor-binding specificities of human and avian influenza viruses, these viruses do not spread efficiently from person to person. To acquire efficient person-to-person transmission, the HAs of avian viruses adapt to human receptors and regulate pathogenicity by modulating IFN (interferon) type 1 responses (Imai & Kawaoka, 2012; Russier et al., 2019).

NA is a tetramer with a box-shaped head made of four coplanar and a centrally attached stalk and is coded for by RNA segment 6 (Air & Laver, 1989). A major role of NA is involved in the last stages of infection to remove decoy receptors from cilia,

mucins, and glycocalyx, which obstruct the virus from reaching its target receptors on the host cells (Matrosovich et al., 2004). NA also cleaves the sialic acid receptors on the cell surface which releases the progeny virions (Zhu et al., 2012).

Table 1: Functions of proteins encoded by influenza virus genome

Protein	Function	Coding gene	Unique to	Reference
Hemagglutinin	-Receptor binding and entry	RNA segment 4	Influenza A and B	(Sriwilaijaroen & Suzuki, 2012) (Palese & Schulman, 1976)
Neuraminidase	-Cleaves decoy receptors from cilia, mucins and glycocalyx -Destroys the hemagglutinin receptors which permits for the release progeny virions from the cell	RNA segment 6	Influenza A and B	(Matrosovich et al., 2004) (Air & Laver, 1989)
Matrix M2 (AM2)	Ion channel traversing the lipid envelope which equilibrates the PH across the viral membrane during cell entry and across the Golgi membrane during viral maturation.	RNA segment 7	Influenza A	(Pielak & Chou, 2011) (Lamb & Lai, 1981)
Matrix M1	Coordinates the assembly of viral components by binding influenza genetic material, lipids in the inner leaflet of the PM, transmembrane proteins, and other M1 molecules to form the structure of the underlying the envelope.	RNA segment 7 RNA segment 6	Influenza A and B Influenza C and D	(Imai et al., 2004) (Lamb & Krug, 1996) (Lamb & Lai, 1981) (Strauss & Strauss, 2008)

Nuclear Export Protein NEP	Export of vRNPs from the host cell nucleus	RNA segment 7	Influenza A Influenza C	(Hongo et al., 1992)
NB and BM2	Have the same role as M2 proteins	RNA segment 8	Influenza B	(Lamb & Krug, 1996) (Briedis et al., 1982)
Hemagglutinin-esterase-fusion protein (HEF)	Combines the roles of HA and NA	RNA segment 7	Influenza C and D	(Lamb & Krug, 1996) (Gao et al., 2008) (Su et al., 2017)
PB1 and PB2 PA	Form the trimeric RNA dependent RNA polymerase responsible for the transcription and replication of the viral RNA genome PB1 contains the polymerase active site PB2 cap-binding site PA is the endonuclease binding site	RNA segment 2(PB1) RNA segment 1(PB2) RNA segment 3 (PA)	Influenza A, B, C, D (PB1, 2) Influenza A and B (PA)	(Engelhardt & Fodor, 2006) (Bouvier & Palese, 2008) (Su et al., 2017)
P3	The form of PA in influenza C	RNA segment 3	Influenza C, D	(te Velthuis & Fodor, 2016) (Su et al., 2017) (Hause et al., 2013)
CM2	Peripheral membrane protein that exhibits proton channel activity	RNA segment 6	Influenza C	(Wang & Veit, 2016)

				(Hongo et al., 1994)
NS1 and NS2	NS1 counteracts the cellular interferon response NS2 mediates the nuclear export of ribonucleic proteins	RNA segment 8 RNA segment 7	Influenza A and B Influenza C and D	(Lamb & Krug, 1996). (Lamb & Lai, 1980) (Lamb & Choppin, 1979) (Wang et al., 2015) (Krug, 2015) (Paterson & Fodor, 2012) (Su et al., 2017)

B. Types of Influenza:

1. Influenza A

Influenza A virus originates from its ancestors, avian influenza viruses (Webster et al., 1992; Taubenberger et al. 2006). The natural host reservoirs of avian influenza viruses are water birds of the order Anseriformes (geese, ducks, swans, etc.) and Charadriiformes (mainly gulls and waders) (Webster et al., 1992; Olsent et al., 2006). They are transmitted to humans through the avian reservoir itself or an intermediate mammalian reservoir (e.g. swine) (De Jong et al., 2000; Molinari et al., 2007). Influenza A viruses are classified into subtypes based on the antigenic properties of their two surface glycoproteins, HA and NA (Paules & Subbarao, 2017). At least 16 subtypes of HA and nine NA subtypes exist. This provides influenza A with the potential to cause

global pandemics achieved when animal viruses acquire mutations or re-assort with human viruses adapting them to replicate and spread in human hosts (Jackson et al., 2011).

2. Influenza B

Despite the absence of subtypes for influenza B virus, two genetically and antigenically distinct lineages have been identified based on of the surface HA glycoprotein namely B/Victoria and B/Yamagata (Rota et al., 1990). Humans are the only identified influenza B virus host. Although it was also identified in seals, no genetic reassortment has been observed, and the detected influenza B was, in fact, identical to that infecting humans (Osterhaus et al., 2000). This explains its limited potential for causing pandemics (van de Sandt et al., 2015).

3. Influenza C

Influenza C is a less common type of influenza. It commonly causes cold-like symptoms, and may be the cause of lower respiratory tract infections especially in children below 2 years of age (Matsuzaki et al., 2006). It is mainly a human virus; however, it has also been detected in cattle, pigs, dogs (Kimura et al., 1997; Guo et al., 1983; Yamaoka et al., 1991; Manuguerra & Hannoun, 1992; Zhang et al., 2018). The HA and NA roles of influenza A and B viruses are fulfilled by hemagglutinin esterase fusion protein (HEF) possessed by influenza C virus (Herrler et al., 1988). Several studies have indicated a winter-spring seasonality of the virus (Pabbaraju et al., 2013, Gouarin et al., 2008).

C. Viral Life Cycle:

The viral life cycle is initiated upon attachment of the HA protein to SA-conjugated receptors on the cell surface (Luo et al., 2011).

Serine protease cleaves HA during viral replication into HA1 and HA2. HA1 contains the receptor binding and antigenic sites and HA2 mediates fusion of the virus envelope with cell membranes (Bouvier & Palese, 2008).

Following attachment of HA (or HEF of influenza C) to SA, the virus is endocytosed. The low pH of the endosome triggers a conformational change in the HA, exposing a fusion peptide. This peptide mediates the fusion of the viral envelope with the endosomal membrane and therefore opening a pore through which viral RNPs (vRNPs) are released into the host cytoplasm (reviewed in (Stegmann, 2000) and (Sieczkarski & Whittaker, 2005)). vRNPs are released into the cytoplasm as a result of internal acidification through the M2 protein channel disrupting internal protein-protein interactions (Martin & Helenius, 1991).

Upon release from the virion, vRNPs are trafficked to the host cell nucleus through means of nuclear signaling proteins which in turn import the vRNPs into the cell nucleus (reviewed in Cros & Palese, 2003). In the nucleus, the viral RNA-dependent RNA polymerase uses the negative sense vRNA as a template to synthesize mRNA templates for viral protein synthesis, as well as a complementary RNA (cRNA) intermediate. RNA polymerase, then, transcribes more negative-sense, genomic vRNA (Bouvier & Palese, 2008).

The viral proteins HA, NA, and M2 are synthesized from viral mRNA. They are then folded and sent to the Golgi apparatus for further post-translational modifications (Shaw & Palese, 2013).

The proteins are then directed to the cell membrane through apical sorting signals. M1 is believed to have a role in bringing the RNP-NEP complex in contact with the envelope-bound HA, NA, and M2 proteins to package at the host cell membrane (Shaw & Palese, 2013). Evidence suggests that RNA packaging is a selective process in which discrete packaging signals on all vRNA segments ensuring most virus particles include a full genome (Bancroft & Parslow, 2002; Cros & Palese, 2003; Enami et al., 1991; Fujii et al., 2003). Unless the virions contain eight RNA segments, the influenza virus is not fully infectious.

Budding occurs at the cell membrane. HA spikes continue binding virions to the SA on the cell surface until viral particles are released by sialidase activity of the NA protein (Bouvier & Palese, 2008).

D. Transmission:

The main mode of transmission of influenza virus is *via* droplets. They can be inhaled and deposited in the upper respiratory tract (URT) (Bennett, Dolin, Blaser, 2015; Killingley & Nguyen-Van-Tam, 2013). The virus can remain infectious on the hands for a few hours and on non-porous surfaces for up to 48 hours (Nashimura et al., 1990; Paules C., Subbarao K., 2017).

E. Clinical Symptoms:

As for the clinical presentation of influenza virus infection, it varies depending on the characteristics of both, the host and virus. As such, symptoms can range from asymptomatic to severe. The incubation period of 1-2 days, and is followed by the onset of symptoms. They include systemic symptoms such as fever, chills, headache, myalgia,

malaise, and anorexia. The disease presentation also includes respiratory symptoms characterized by non-productive cough, nasal discharge, and sore throat (Bennett, Dolin, Blaser, 2015) (Cox & Subbarao, 1999) (Long, Pickering, Prober, 2012) (Nicholson, 1992). Ocular symptoms may also be present and include lacrimation, conjunctivitis, and photophobia (Nicholson, 1992). Fever and associated systemic symptoms usually last for around two days but can be longer (up to 8 days). Cough and malaise can persist for up to two weeks after the fever has resolved (Paules, Subbarao, 2017).

Having chronic medical conditions such as heart, lung, and rheumatologic diseases, diabetes mellitus, dementia, etc. is a risk factor for influenza complications. These high-risk factors increase the chances of hospitalization and death regardless of age (Glezen et al., 1987; Izurieta et al., 2000). The most common complication of influenza virus is pulmonary and it falls into four categories: primary influenza pneumonia, secondary bacterial pneumonia, exacerbations of chronic pulmonary diseases, and pneumonia due to unusual pathogens (Rothberg et al., 2008). Some miscellaneous complications of influenza include neurological complications (Reye's syndrome, encephalomyelitis, etc.) and heart complications (congestive heart failure, ischemic heart disease, etc.) (Studahl, 2003; Ison et al., 2005).

F. Diagnosis:

Viral shedding begins during the incubation period and peaks during the first or second day after the onset of symptoms. It then continues to decrease to undetectable levels after almost a week (Ip et al., 2016). Children and immunocompromised individuals tend to shed the virus for a longer duration than healthy immunocompetent

adults do (Memoli et al., 2014; Esposito et al., 2011). Memoli et al. reported that immunocompromised individuals exhibited viral shedding for a mean duration of 19 days in comparison to immunocompetent individuals who shed the virus for a mean of 6.4 days. (Memoli et al., 2014). In a study conducted by To et al., patients with a mean age of 15.4 years shed the virus for more than 5 days. However, in patients of mean age 24.7 years, viral shedding lasts for 4 days or less (To et al., 2010). Asymptomatic patients shed the virus for a shorter duration than symptomatic ones (Loeb et al., 2012; Ip et al., 2017).

Diagnosing influenza clinically can be challenging due to the overlap of symptoms between influenza virus and other respiratory viruses (Dwyer et al., 2006). It is difficult to differentiate between influenza and the common cold based on clinical symptoms. According to a systemic literature review by Jacek et al. in 2014, myalgia, cough, rhinorrhea, headache, sore throat and fever were present in 94%, 93%, 91%, 91%, 84%, and 68%, respectively in influenza patients. In common cold patients, 94% of patients endured myalgia as the most frequent symptom. 89% presented sore throat, 81% rhinorrhea, 84% sore throat, and 80% cough. Fever, however, was only reported in 40% of patients with the common cold (Jacek et al., 2021). Hence, laboratory diagnostics are available and provide an accurate diagnosis, allow appropriate treatment, prevent the unnecessary use of antibiotics, and aid in influenza surveillance (Bennett, R Dolin, MJ Blaser, 2015; Centers for Disease Control and Prevention, 2016; Kim, B Poudel, 2013; Peaper & Landry, 2014; Petrozzino et al., 2010). The diagnostic methods include antigen detection, nucleic acid testing using RT-PCR, and viral culture. Viral culture is not ideal for clinical diagnosis as it can take several days to obtain a

diagnosis when compared to minutes or hours for rapid antigen detection and RT-PCR, respectively (Vemula et al., 2016).

The World Health Organization (WHO) defines an influenza-like illness (ILI) case to have an acute respiratory with a measured fever of ≥ 38 C°, cough, and onset within the past 10 days (WHO, 2014). Hence, laboratory diagnosis should be done early on in the course of illness when viral shedding is at its peak which has important implications for antiviral therapy (Dwyer et al., 2006; Peaper & Landry, 2014). Nasopharyngeal swabs, nasal washes, and nasopharyngeal aspirates are the samples of preference (Paules C., Subbarao K., 2017). In individuals with pneumonia, lower respiratory tract samples like bronchoalveolar lavage and endotracheal aspirates can be more sensitive in diagnosis (N Ohkura, M Tani, M Nishitsuji, K Nishi, 2015)

G. Epidemiology

The WHO estimates that 5-15% of the world's population is infected with influenza virus every year. This accounts for 3-5 million severe cases and up to 650 000 deaths annually (WHO, 2018; Iuliano et al., 2018) excluding those resulting from other diseases underlying influenza (Iuliano, A. D., Roguski et al., 2018). Several studies reported that asymptomatic cases accounted for 14% up to 77% of influenza infections (Lau LL et al., 2010; Hayward AC et.al, 2014; Hsieh Y-H et al., 2014). In the Eastern Mediterranean Region (EMR), there is a scarcity of information about the burden of influenza (Kayali, G. et al., 2013). A review by Caini et al. reported that most of the EMR's countries had a primary peak between January and March. This falls in line with the Lebanese Severe Acute Respiratory Infections (SARI) sentinel surveillance that indicated that the primary peak extended from January till mid of March in Lebanon

(Caini S, El-Guerche Seblain C, Ciblak MA Paget J, 2018; Saleh M et al., 2018; Zaraket et al., 2019). We have recently shown that the incidence of influenza among subjects showing influenza-like illnesses (ILI) in Lebanon was 34% between 2016 and 2018 in outpatients (AlIbrahim, M. et al., 2019). During the same period, we showed that 23% of the hospitalized patients meeting the admission criteria ie. presenting influenza-like illness, tested positive for influenza virus (Yola et al., 2019). In a retrospective chart review extending over eight flu seasons between 2008-2016 in Lebanon, Assaf-Casals et al. reported that 26.6% of all identified influenza cases were hospitalized. However, the study was limited by its retrospective nature and in being limited to one tertiary hospital, which likely resulted in overestimation of the hospitalization rate. Children under 2 years of age and elderly ≥65 years were at the highest risk for severe disease and deaths (Assaf-Casals et al., 2020). In a study conducted in 2017, it was estimated that 0.6 per 100,000 population died from influenza in Lebanon (GBD 2017 Influenza Collaborators, 2019).

H. Prevention and Treatment

Vaccination is the most important preventative measure of seasonal influenza virus, while antivirals provide the first line of action for early outbreak response in case of a pandemic (Nguyen-Van-Tam et al., 2015). Antiviral drugs are also critical in the management and prophylaxis of seasonal influenza (WHOE, 2021).

Two main types of influenza vaccines exist: live-attenuated influenza vaccine (LAIV) and inactivated influenza vaccine (IIV). Both vaccines are traditionally formulated to protect against three seasonal influenza viruses commonly known as trivalent vaccines. The current trivalent influenza vaccine contains influenza

A/H1N1pdm09, A/H3N2 and one of the two influenza B lineage viruses. The repetitive failure to predict the influenza B lineage to be included in the trivalent vaccine poses a challenge to control and prevent influenza. This led to the recommendation and adoption of the quadrivalent vaccine which includes both influenza lineages (Ambrose & Levin, 2012).

The influenza vaccine is selected based on data collected from more than 100 influenza centers from more than 100 countries around the world. It involves receiving and testing thousands of influenza virus samples. Twice a year, the WHO organizes a consultation to review the results of surveillance, laboratory, and clinical studies, and the availability of vaccine viruses. The meetings take place in February to select the vaccine composition for the northern hemisphere and during September to select it for the southern hemisphere (CDC, 2020).

Influenza epidemics occur each year despite active vaccination programs. This is due to a mismatch between the circulating strains and vaccines and the low uptake of vaccination especially in young adults and children (Talbird et al., 2009).

Influenza vaccine coverage varies widely between countries around the world. According to data from National Health Interview Survey, 68.7% of elderly aged 65 and above received the vaccine in 2018 in the United States (National Health Interview Survey, 2018). In Europe according to WHOE (World Health Organization Europe), vaccination coverage among high-risk groups differed between countries ranging from <1% to more than 75% among the elderly. Less than 40% of people with chronic illnesses and healthcare workers had been covered by the vaccine in most countries (WHOE, 2018).

The WHO Eastern Mediterranean Region (EMR) consists of 22 countries accounting for almost 10% of the world's population. However, the region's share of influenza vaccines is roughly 2.2% (Iuliano et al., 2018). Almost half of the available reports on the vaccination rates in the EMR were done on healthcare workers. The median vaccination rate among healthcare workers in the MENA region was 28.2%. Most countries reported annual vaccination rates below 50% in high-risk groups (Zaraket et al., 2019). According to a cross-sectional review performed in 2019, influenza vaccine uptake was only 27.6% in the Lebanese adult population (El Khoury & Salameh, 2015).

Three classes of antiviral drugs are approved against influenza: M2 channel blockers, neuraminidase inhibitors (NAIs), and polymerase inhibitors.

The M2 ion channel blockers, adamantanes, were the first class of drugs licensed for influenza treatment use but they are only active against influenza A viruses (Nicholson et al., 2003). (Hayden et al., 1989). Adamantanes are not currently recommended for influenza treatment due to universal resistance among circulating viruses (Farrukee & Hurt, 2017).

NAIs are the second class of antiviral drugs approved for influenza (both A and B) treatment and prophylaxis (Duwe, 2017). NAIs block the release of progeny influenza virions from the cells preventing further replication cycles (Mawatari et al., 2019). There are four NAIs in use currently: oseltamivir (Tamiflu), zanamivir (Relenza), and peramivir (Rapiacta) which are available worldwide, in addition to laninamivir (Inavir) being exclusively available for use in Japan (Sugaya, 2011; Sugaya, 2016). Oseltamivir is available orally as a tablet whereas zanamivir and laninamivir are inhaled as a dry powder. Peramivir is administered intravenously (Zaraket & Saito,

2016). Oseltamivir has the highest global use of the four NAIs due to the convenience of oral administration and good safety profile (McClellan & Perry, 2001).

Polymerase inhibitor baloxavir marboxil, is the first-in-class antiviral drug targeting the endonuclease function of the viral PA polymerase subunit and prevents the transcription of viral mRNA (O'Hanlon & Shaw, 2019). It is considered superior to oseltamivir due to its ability to curb viral spread by reducing viral loads within 24 hours of treatment (Hayden et al., 2018). The polymerase inhibitor favipiravir (T705) is approved in Japan with limited licensure for severe influenza cases with novel viruses resistant to the currently administered therapies (Zaraket & Saito, 2016).

Antiviral drugs are used for prophylaxis, suppression, pre-emptive therapy, and treatment (Paintsil & Cheng, 2009).

Resistance to antiviral drugs may develop during treatment and could spread widely replacing the susceptible strains even in the absence of drug pressure (Hayden & de Jong, 2011). Resistance is usually acquired by the selection of single point mutations in the viral genome which alter the protein structure, and interfere with the binding of antivirals to the target protein. The segmented nature of the influenza virus genome can also promote the spread of resistant markers by reassortment with other viruses having improved fitness to replicate and spread (Simonsen et al., 2007). The global spread of amantadine-resistant A/H3N2 strains since 2003, oseltamivir-resistant A/H1N1pdm09 viruses since 2007, and adamantane-resistant pandemic A/H1N1pdm09 viruses in 2009 are examples of transmission of the resistant strain. They emphasize the unpredictability of the influenza virus and the increasing challenges of clinically managing it (Hayden & de Jong, 2011).

CHAPTER II

METHODS

A. Ethical Statement:

This study was conducted under the 1996 Helsinki declaration. It was approved by the Institutional Review Board (IRB) of the American University of Beirut. Signed consent was obtained from participants before sample collection after the appropriate information on the general surveillance characteristics was provided. The subjects were assigned unique study numbers to protect their privacy.

B. Study Population and Sample Collection:

This is a prospective surveillance that took place over the course of two influenza seasons (2018-2019 and 2019-2020) as part of the Global Hospital Surveillance Network (GIHSN).. Surveillance sites included the American University of Beirut Medical Center, Makassed General Hospital, Keserwan Medical Center, New Mazloum Hospital, Bekaa Hospital, and Rafik Hariri Hospital. To be considered for screening, hospitalized patients should present with admission diagnosiss possibly associated with an influenza infection, based on age. Patients over 5 years of age should present one of the following: acute upper or lower respiratory disease, acute myocardial infarction or acute coronary syndrome, acute asthma or exacerbation, acute heart failure, pneumonia, bronchitis and exacerbations of chronic pulmonary obstructive disease, acute respiratory failure, myalgia, acute metabolic failure (diabetic coma, renal dysfunction, acid-base disturbances, alterations to the water balance), altered consciousness, convulsions, febrile convulsions, syncope and collapse, dyspnea/respiratory abnormality, respiratory abnormality, shortness of breath,

respiratory abnormality not otherwise specified, respiratory symptoms/chest symptoms, fever or fever unknown origin or non-specified, cough, sepsis, systemic inflammatory response syndrome. Patients under 5 years of age should present one of the following: acute upper or lower respiratory disease, dyspnea, breathing anomalies, shortness of breath, tachypnea (polypnea), acute asthma or exacerbation, pneumonia and influenza, acute respiratory failure, acute heart failure, myalgia, altered consciousness, convulsions, febrile convulsions, fever or fever unknown origin or non-specified, cough, gastrointestinal manifestations, sepsis, systemic inflammatory response syndrome being not otherwise specified, and nausea and vomiting. To fulfill the hospitalized patient requirement, the individual should have at least a one-night stay. For inclusion in the study, the patient must report having influenza-like illness (ILI) within the last seven days. ILI case definition included having one systemic (fever, myalgia, malaise, headache) and one respiratory (cough, sore throat, shortness of breath) symptom. Patients above 5 years of age must have their samples collected within 72 hours post-hospitalization. As for patients below 5 years of age, they can have their samples collected within 7 days of being hospitalized. If the patients had been admitted into the hospital within the past 30 days, are institutionalized, or are non-resident i.e. not belonging to the population study base, they were excluded (GIHSN Protocol). A case record form (CRF) - which included information on demographic data, social history, clinical symptoms, etc. - was used to collect the epidemiological and clinical data by physicians at each sentinel site. All patients enrolled in the study had a nasopharyngeal (NP)swab collected. In addition, an oropharyngeal (OP) swab was also obtained from patients ≥ 14 years of age, if they agree to provide one. The

swabs were suspended into vials containing 3ml viral transport media and stored at -20 °C until they were shipped to the Virology laboratory.

C. RNA Extraction, Influenza Screening:

The samples received were aliquoted and stored at -80 °C until further analysis. Two hundred microliters of each swab suspension were subjected to influenza viral nucleic acid extraction using the PureLink RNA Mini Kit (Invitrogen, Van Allen Way, Carlsbad CA) or the High Pure Viral RNA Kit (Roche) following the manufacturer's instructions. Nucleic acids were eluted from the spin column in 50 µl of nuclease free-water and elution buffer respectively and stored at -20°C for further processing. A multiplex real-time PCR assay was used for the detection of influenza A and B viruses using AgPath-ID One-Step RT-PCR kit using primers and probe sets designed by the Center for Disease Control and Prevention (CDC). The primers and probe sets for screening for influenza C were designed by the WHO (WHO, 2017). Four microliters of nucleic acid were used in the PCR reaction of 20 µl total volume per well. The influenza A, B, and C probes were labeled with fluorophores 6-carboxy-fluorescein (FAM), hexachlorofluoroscein (HEX), and sulforhodamine 101 acid chloride (Texas Red) respectively. The cycling conditions included an initial reverse transcriptase step of 10 min at 45°C, followed by an enzyme activation step for 10 min at 95°C and 40 cycles of 15s at 94°C, 60 sec at 55°C. Influenza A or B positive samples were further characterized by RT-PCR assay using WHO primer and probe sets for H1, H3, and HA of influenza A, CDC primer and probe sets for Victoria and Yamagata HA of influenza B.

Patients testing positive in either nasopharyngeal, oropharyngeal, or both were reported positive once for each virus type and subtype.

D. Comparison of Cq Values:

The real-time PCR quantitative cycle Cq value represents the number of cycles needed for the fluorescent signal to exceed the background level or threshold. Cq provides a semiquantitative measure of virus load, whereby its value is inversely proportional to the quantity of the targetThus the lower the Cq, the higher the higher the amount for virus genetic material present in the swab and thus the higher the viral load (Ginzinger, 2002). Cq value has been widely used during the COVID-19 pandemic as an acceptablesurrogate for viral load (Bustin et al., 2021; Yu et al., 2020, Kimball et al., 2020).

E. Statistical Analysis:

SPSS version 19 was used for data analysis. Descriptive analysis was performed, and Chi-square test or Fisher exact test was used to compare medians.

Independent sample T-test or ANOVA was used to compare the Cq values between different independent factors. Mann Whitney test or Kruskal-Wallis test were used to compare variables with a non-normal distribution. The various factors included patients' baseline socio-demographic data (age, gender), BMI, previous vaccination, presence of comorbidities, types of comorbidities, signs and symptoms, length of hospital stay, death, use of antiviral drugs or antibiotics, bacterial co-infection, and the presence of complications including ICU admission, mechanical ventilation, vasopressor use or oxygen requirement, presence of pneumonia, Acute respiratory

distress symptoms (ARDS), sepsis or septic shock. Results were considered significant if the P-value was less than 0.05.

Multivariate analysis using linear regression was carried out to study the outcomes associated with Cq values, considering potential confounding variables that had a P-value <0.20 in bivariate analysis. A stepwise descendent likelihood ratio logistic regression analysis was applied, and the final model that included significant variables (P-value <0.05) was retained. The final model was accepted after ensuring the adequacy of the data using the Hosmer and Lemeshow test.

The sensitivity of NP and OP samples in detecting influenza virus was assessed by considering any positive from either of the specimens as a true positive (Kim et al., 2011).

Paired NP and OP samples simultaneously collected from the same individual were assessed for concordance. The average Cq value for each virus was compared between NP and OP specimens using a 2-tailed Wilcoxon signed-rank test for matched pairs and correlation test (r). The agreement was tested between NP and OP samples using the Kappa statistic. Furthermore, the agreement was assessed by comparing Cq values of NP and OP samples by using Bland Altman test.

Table 2: Primer and probe sequences used for typing and subtyping of influenza virus by multiplex RT-PCR

Target Virus	**Target gene**	**Primers**	**Code**	**Sequence 5' → 3'**
Influenza A	**Matrix M**	Influenza A Forward	FLUAM-7-F	CTT CTA ACC GAG GTC GAA ACG TA
		Influenza A Reverse	FLUAM-161-R	GGT GAC AGG ATT GGT CTT GTC TTT A

		Influenza A Probe	FLUAM-49-P6 (FAM)	(FAM)-TCA GGC CCC CTC AAA GCC GAG-BHQ1
Influenza A/H1N1PDM09	**Hemagglutinin HA**	Influenza A/H1N1PDM09 Forward	H1pdm-169-F	AAA CTA TGC AAA CTA AGA GGG GT
		Influenza A/H1N1PDM09 Reverse	H1pdm-297-R	TGT TTC CAC AAT GTA GGA CCA
		Influenza A/H1N1PDM09 Probe	H1pdm-244-P (FAM)	(FAM)-CCA GAG TGT GAA TCA CTC TCC ACA-BHQ1
Influenza A/H3N2		Influenza A/H3N2 Forward	H3-266-F	ACC CTC AGT GTG ATG GCT TTC AAA
		Influenza A/H3N2 Reverse	H3-373-R	TAA GGG AGG CAT AAT CCG GCA CAT
		Influenza A/H3N2 Probe	H3-315-P (HEX)	(HEX)-ACG AAG CAA AGC CTA CAG CAA CTG TT-BHQ1
Influenza B		Influenza B Forward	FLUBHA-940-F	AAA TAC GGT GGA TTA AAC AAA AGC AA
		Influenza B Reverse	FLUBHA-1109-R	CCA GCA ATA GCT CCG AAG AAA
		Influenza B Probe	FLUBHA-994-P4 (HEX)	(HEX)-CAC CCA TAT TGG GCA ATT TCC TAT GG C-BHQ1

Influenza B/Victoria Influenza B/Yamagata		Influenza B typing Forward	BHA-188F	AGA CCA GAG GGA AAC TAT GCC C
		Influenza B typing Reverse	BHA-270R	TCC GGA TGT AAC AGG TCT GAC TT
		Influenza B VIC Probe	Probe-VIC2 (FAM)	(FAM)-CAG ACC AAA ATG CAC GGG GAA HAT ACC-BHQ1
		Influenza B YAM Probe	Probe-YAM2 (HEX)	(HEX)-CAG RCC AAT GTG TGT GGG GAY CAC ACC-BHQ1
Influenza C	**Nucleoprotein NP**	Influenza C Forward	CNP-1043-F	GCT TTG GAC TTG CTT AT
		Influenza C Reverse	CNP-1141-R	GAC TCT GAA GTT TCC TAT TT
		Influenza C Probe	CNP-1095-P3 (Texas Red)	(Texas Red)-CCC TCT TAA GTT GAG AAA CAG AAT G-BHQ2

CHAPTER III

RESULTS

A. Sample Collection, Positivity, and Seasonality:

During the 2018-2019 and 2019-2020 seasons, a total of 2533 samples were collected from 1654 patients. During the 2018-2019 season, 970 samples (636 NP and 334 OP swabs) were collected from 636 patients, and 1536 samples (1018 NP and 518 OP swabs) were collected from 1018 patients during the 2019-2020 season **(Table 3).**

Table 3: The distribution of specimens across the respective centers during the seasons 2018-2019 and 2019-2020 seasons

Center	**2018-2019 season**	**2019-2020 season**
	Number of samples (number of patients)	**Number of samples (number of patients)**
American University of Beirut Medical Center	357 (207)	374 (231)
Makassed General Hospital	135 (107)	139 (117)
Keserwan Medical Center	35 (19)	78 (50)
Rafik Hariri Hospital	244 (137)	524 (271)
Bekaa Hospital	102 (74)	93 (81)
New Mazloum hospital	97 (92)	328 (268)
Total	970 (636)	1536 (1018)

The patients included 911(55.11%) males and 742 (44.88%) females and one patient's gender was not reported. The samples were screened for influenza A, B and C viruses; 370 patients tested positive for influenza A or B in either the NP (n=256, 69.18%) samples, the OP (n=41, 11.08%) sample, or in both (n=73, 19.72%). Among these, 264 (71.35%) patients tested positive for influenza A and 106 (28.64%) tested positive for influenza B. Only two patients tested positive for influenza C. The distribution of influenza A and B by subtype is displayed in **Table 4**, in addition to influenza C in **Figure 1.**

Table 4: Distribution of influenza A and B-positive patients by subtype during the 2018-2019 and 2019-2020 seasons

Influenza A Virus Subtype	2018-2019 Season Nb of Positive Patients (%)	2019-2020 Season Nb of Positive Patients (%)	Influenza B Virus Subtype	2018-2019 Season Nb of Positive Patients (%)	2019-2020 Season Nb of Positive Patients (%)
A/H1N1pdm09	44(36.06%)	85(59.85%)	**B/Victoria**	1(20%)	95(94.05%)
A/H3N2	55(45.08%)	7(4.9%)	**B/Yamagata**	4(80%)	0
A/Unsubtypable	23(18.85%)	50(35.21%)	**B/Unsubtypable**	0	5(4.95%)
Total	122	142	**Total**	5	101

NP swabs yielded a higher positivity rate than OP samples for influenza A virus (85.98% vs 32.57%, respectively). Similarly, for influenza B virus, NP samples also showed a higher positivity than OP samples (74.52% vs 26.41%, respectively).

During 2018-2019, influenza A/H1N1pdm09 and A/H3N2 were the predominantly circulating strains without a clear peak in activity. During 2019-2020,

influenza circulated between October and March, peaking during January. Influenza A/H1N1pdm09 and B/Victoria were the most commonly detected viruses **(Figure 1)**.

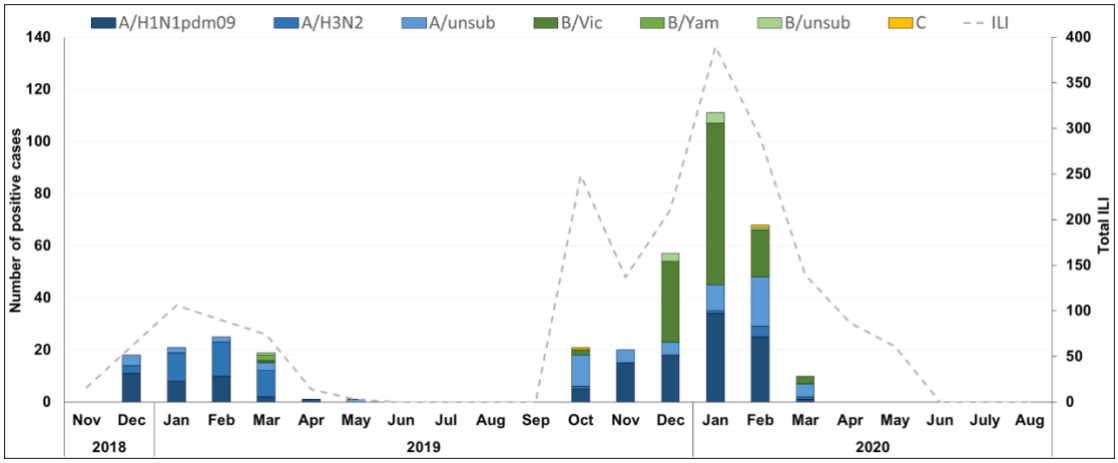

Figure 1: Monthly distribution of ILI and confirmed influenza cases by subtype across during 2018-2020. A/unsub stands for A/Unsubtypable, B/unsub for B/Unsubtypable, B/VIC for B/Victoria, and B/YAM for B/Yamagata

The median age for influenza A and influenza B patients was 19 years. Chronic health comorbidities manifested in 47.16% of patients with influenza A and 34.9% of patients with influenza B. A total of 37 influenza-confirmed patients had diabetes mellitus (DM), 74 had cardiovascular disease (CV), 18 had asthma, and 24 had chronic obstructive pulmonary disease (COPD) **(Table 5)**.

Table 5: Number and percentage of Influenza A and B patients having chronic health comorbidities

Chronic Health Comorbidity	Number of Influenza A Positive Patients (%)	Number of Influenza B Positive Patients (%)
Diabetes Mellitus (DM)	30(11.13%)	7(6.6%)
Cardiovascular Disease (CV)	61(23%)	13(12.26%)

Asthma	15(5.6%)	3(2.8%)
Chronic Obstructive Pulmonary Disease (COPD)	22(8.3%)	2(1.8%)

A total of 224 (84.5%) influenza A patients and all influenza B (100%) were discharged within 7 days of admission. The rest were discharged after 7 days with no recorded mortality among influenza positive patients.

B. Sensitivity and Concordance of NP and OP Samples:

During the study period, 851 samples were paired (i.e., obtained from the same patient) NP and OP samples. Influenza virus was detected in at least one swab (either NP or OP) from 22.32% (n= 190) of the patients with paired samples. Of these, 41.05% (n=78) patients had both NP and OP samples positive for either influenza A and B.

NP samples were more sensitive in detecting influenza A virus compared to OP samples (75.3% vs 60.3%, respectively). Similarly, the NP samples had higher sensitivity in detecting influenza B compared to OP samples (90.9% vs 68.18%, respectively) **(Table 6)**.

Table 6: Sensitivity and Concordance of nasopharyngeal and oropharyngeal swabs

Influenza-positive paired samples (N)	**Number of positive specimens by swab type**		**Kappa**	**P-value**
	NP (%)	OP (%)		
Influenza A (146)	110 (75.3%)	88 (60.3%)	.464	<.001
Influenza B (44)	40 (90.9%)	30 (68.18%)	.732	<.001

Concordance indicates the level of agreement the NP and OP samples. A kappa statistic closer to 1.00 indicates good agreement between the NP and OP samples. Kappa values 0.4-0.59 indicate minor agreement, 0.6-0.79 indicate moderate agreement, and 0.8-0.9 indicate strong agreement (McHugh, 2012). The kappa statistic indicated minor agreement between NP and OP samples for influenza A (kappa=.464, p<.001) but moderate agreement for influenza B (Kappa =.732, p<.001) **(Table 6).**

Figures 3 and 4 show the difference in Cq between paired NP and OP swabs for both influenza A (N=52) and B (N=26). The mean Cq difference between paired NP and OP samples was -2.7±4.79 for influenza A and -4.96±6.49 for influenza B, indicating poor agreement **(Figure 3 and Figure 4)**.

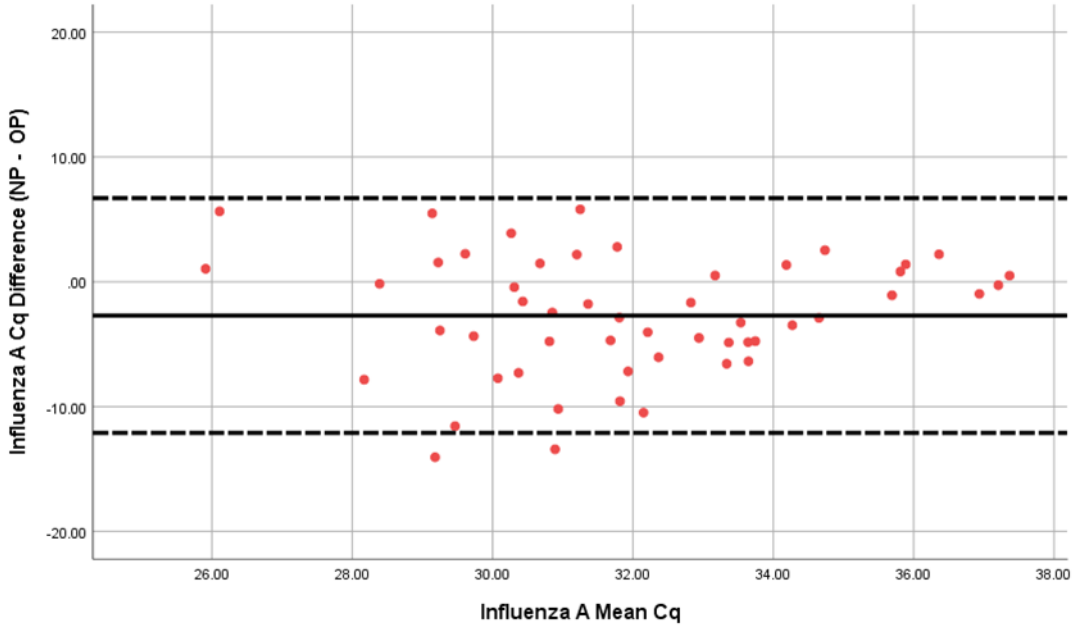

Figure 3: Graph showing Cq difference between influenza A positive paired NP and OP swabs. The solid line represents the mean Cq difference between paired NP and OP samples and the dotted lines represents the standard deviation. Points above the solid

line indicate greater sensitivity for OP samples. Those below the solid line indicate greater sensitivity for NP samples.

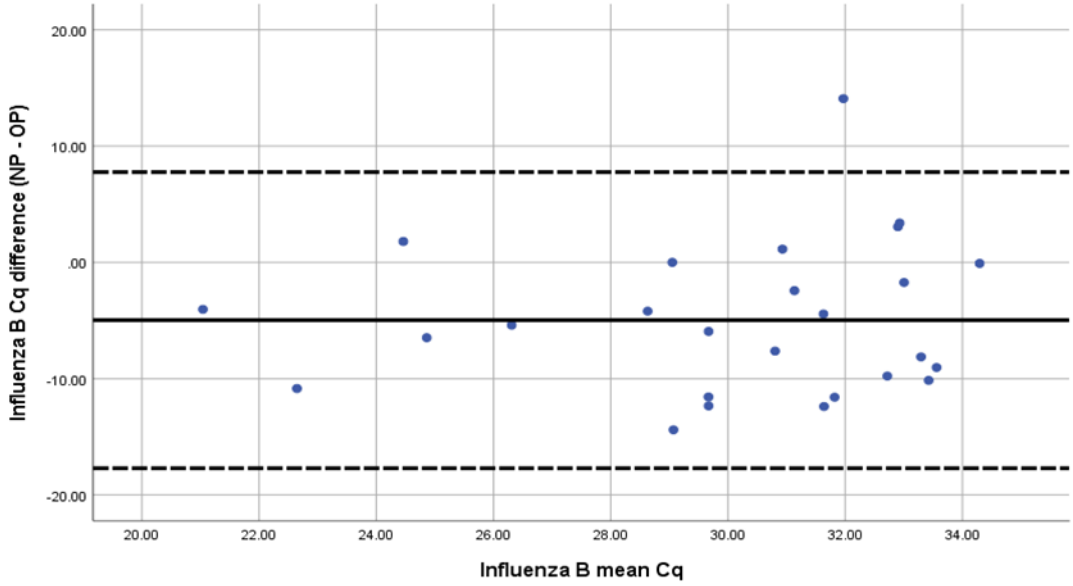

Figure 4: Graph showing Cq difference between influenza B positive paired NP and OP swabs. The solid line represents the mean Cq difference between paired NP and OP samples and the dotted lines represents the standard deviation. Points, representing a positive pair, above the solid line indicate greater sensitivity to OP samples. Those below the solid line indicate greater sensitivity to NP samples.

The viral loads detected in paired NP and OP were then compared. For influenza A positive pairs (N=52), the average Cq was significantly higher in OP samples (mean Cq=33.3 +/- 3.42) compared to NP samples (mean Cq=30.6 ±3.71) (p=.001), indicating a lower viral load in the former. Similarly, influenza B positive paired OP samples (N=26) presented a significantly lower mean viral load (mean Cq=32.5±4.55) than NP samples (Cq=27.6 ±5.03) (p=.001) **(Figure 2)**.

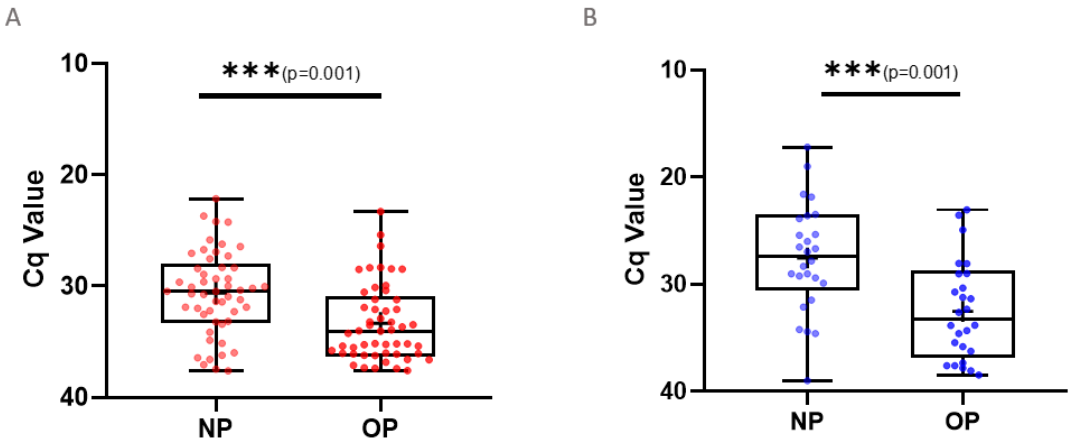

Figure 2: Average mean viral load in paired NP and OP samples for (A) influenza A (N=52) and (B) B (N=26). Upper and lower bars indicate min and max VL values. Horizontal bars indicate median VL values. P values from Wilcoxen model.

Given the higher sensitivity and higher viral load in NP samples compared to OP samples, the former where used in the subsequent analysis.

C. Viral Load and Sociodemographic Factors:

Viral load was assessed for both gender and age **(Table 7)**. For influenza A and B, gender was not associated with viral load (p=.686 and p=.221, respectively). When age was investigated, viral load did not significantly differ among the different age groups for either virus types (p=.406 and p=.344, respectively). This was further confirmed with a correlation test performed on age as a continuous variable (p=.226 and p=.237 for influenza A and B, respectively) **(Table 11)**.

Table 7: Patient demographics and viral load

Variable	Influenza A Mean Cq (SD)	p-value	Influenza B Mean Cq (SD)	p-value

Gender	Male	31.24 (4.68)	.686	28.66	.221
	Female	31.48 (4.19)		28.83	
Age	<5years	31.07 (4.67)	.211	29.83 (4.36)	.344
	5-<18 years	31.18 (4.31)		29.42 (5.73)	
	18-<50 years	31.02 (4.26)		29.16(4.52)	
	50-<64 years	32.96 (3.63)		26.37(5.27)	
	>64 years	31.41 (4.44)		25.71(.43)	

D. Viral Load and Respiratory Symptoms

Patients with runny nose had a higher influenza A viral load (p=.002) **(Figure 5A and Table 8).** Runny nose was also associated with higher viral load in A/H1N1pdm09 patients (p=.033) **(Figure 5B).** This was not the case for A/H3N2 **(Figure 5C).** However, the viral load for influenza A and B did not significantly differ between patients with other respiratory symptoms **(Table 8**).

Table 8: Association between respiratory symptoms and viral load

	Variable	**Influenza A Mean Cq (SD)**	**p-value**	**Influenza B Mean Cq (SD)**	**p-value**
Cough	Yes	31.23(4.45)	.3	29.29(4.74)	.692
	No	32.14(4.53)		28.31(7.71)	
Sore Throat	Yes	30.93(4.11)	.172	29.76(4.77)	.193
	No	31.78(4.69)		28.35(5.37)	
Runny Nose	Yes	30.58(4.48)	**.002**	29.55(4.29)	.403
	No	32.46(4.2)		28.51(6.04)	

Shortness of Breath	Yes	31.18(4.15)	.651	29.65(4.89)	.412
	No	31.45(4.73)		28.85(4.80)	

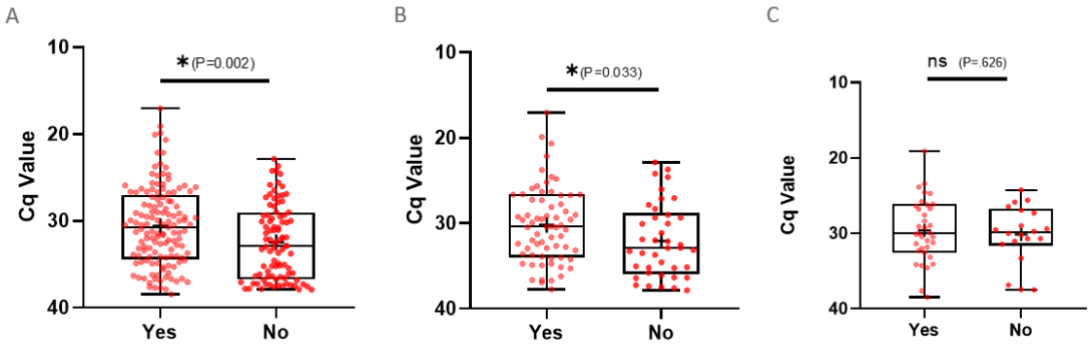

Figure 5: Correlation between viral load and runny nose for (A) influenza A (B) A/H1N1pdm09, and (C) A/H3N2. Upper and lower bars indicate min and max VL values. Horizontal bars indicate median VL values. P values from bivariate analysis model.

E. Viral Load and Patient Health Characteristics

Patient health characteristics and their respective mean viral loads for each virus type were assessed (**Table 9)**. There was no association between viral load and vaccination status for influenza A and B (p=.861 and p=.99, respectively). Given that vaccination would have expected to reduce viral load, we examined the difference in time of sample collection with respect to onset of symptoms among vaccinated and unvaccinated patients. The mean difference in days between sample collection and onset of symptoms for vaccinated and unvaccinated patients was 1.88 vs 2.67 respectively for influenza A (p=.09). For influenza B, patients who were vaccinated presented a mean difference of 2.66 days between symptom onset and sample collection, almost identical to those who were not vaccinated (mean difference 2.73)(p=.92). Therefore, the timing of sample collection could not explain the lack of correlation between viral load and vaccination status.

We found that chronic diseases (CV, COPD, DM) were not correlated with viral load of either influenza A and B. However, viral load was positively correlated with asthma for influenza B and B/Victoria (p=.018) **(Figure 6),** not influenza A (p=.771). All the asthmatic patients were influenza B/Victoria positive. There were not enough samples positive for B/Yamagata to perform the analysis.

Table 9: Health characteristics of patients with influenza infection by type and viral load

	Variable	**Influenza A Mean Cq (SD)**	**p-value**	**Influenza B Mean Cq (SD)**	**p-value**
Vaccination	Yes	31.15(4.54)	.861	29.24(5.83)	.99
	No	31.33(4.44)		29.26(4.74)	
COPD	Yes	32.12(3.62)	.517	25.82(12.16)	.754
	No	21.32(4.52)		29.32(4.71)	
Asthma	Yes	30.29(5.36)	.771	22.89(4.48)	**.018**
	No	31.44(4.41)		29.45(4.73)	
DM	Yes	31.3(3.99)	.492	28.66(3.85)	.759
	No	31.99(4.52)		29.92(4.91)	
CV disease	Yes	31.53(4.69)	.771	29.82(4.41)	.7
	No	31.32(4.41)		29.19(4.9)	

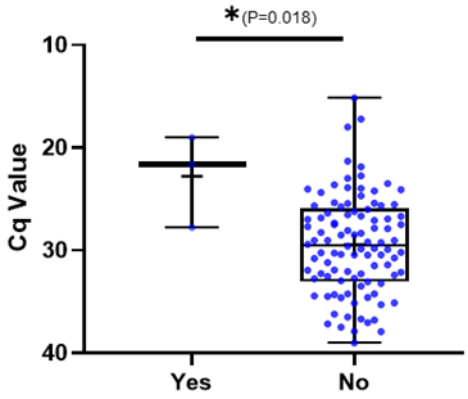

Figure 6: Correlation between viral load and asthma for B/Victoria. Upper and lower bars indicate min and max VL values. Horizontal bars indicate median VL values. P values from bivariate analysis model.

F. Viral Load and Clinical Outcomes:

Antibiotic administration and antiviral treatment were not associated with the viral load of influenza A and B **(Table 10).** No association was found between having a bacterial co-infection and viral load of influenza B (p=.38) **(Figure 7A)**. Influenza A patients with a bacterial co-infection or ARDS had a significantly lower viral load (p=.048 and p=.003, respectively) **(Figure 7B)**. Mortality, ICU admission, oxygen supplementation, mechanical ventilation, vasopressor use, and the presence of pneumonia were not correlated with influenza A and B viral loads **(Table 10)**. However, when considering subtype, A/H1N1pdm09 patients on oxygen supplementation had significantly lower viral load (p=.025) **(Figure 7C)**.

Table 10: Correlation between viral load and patient outcomes

	Variable	Influenza A Mean Cq (SD)	p-value	Influenza B Mean Cq (SD)	p-value
Antibiotic use	Yes	31.6(4.6)	.242	28.13(4.42)	.067

	No	30.88(4.33)		29.94(5.09)	
Bacterial co-infection	Yes	32.66(4.49)	**.048**	27.7(5.79)	.38
	No	31.01(4.43)		29.28(4.8)	
Antiviral use	Yes	31.66(4.66)	.433	29.61(4.93)	.66
	No	31.15(4.38)		29.19(4.7)	
ICU admission	Yes	31.78(4.17)	.727	27.03(3.08)	.361
	No	31.3(4.49)		29.3(4.88)	
Ventilation	Yes	29.85(5.44)	.383	-	-
	No	31.34 (4.43)		29.21(4.84)	
Oxygen Supplementation	Yes	32.61(4.22)	.127	28.62(2.17)	.782
	No	31.16(4.48)		29.24(4.94)	
Vasopressor use	Yes	32.46(6.18)	.537	30.19(4.42)	.571
	No	31.32(4.42)		29.18(4.88)	
Pneumonia	Yes	31.41 (4.21)	.838	27.01(5.22)	.094
	No	31.31(4.6)		29.5(4.74)	
ARDS	Yes	34.84(1.17)	**.003**	-	-
	No	31.28(4.52)		29.21(4.83)	

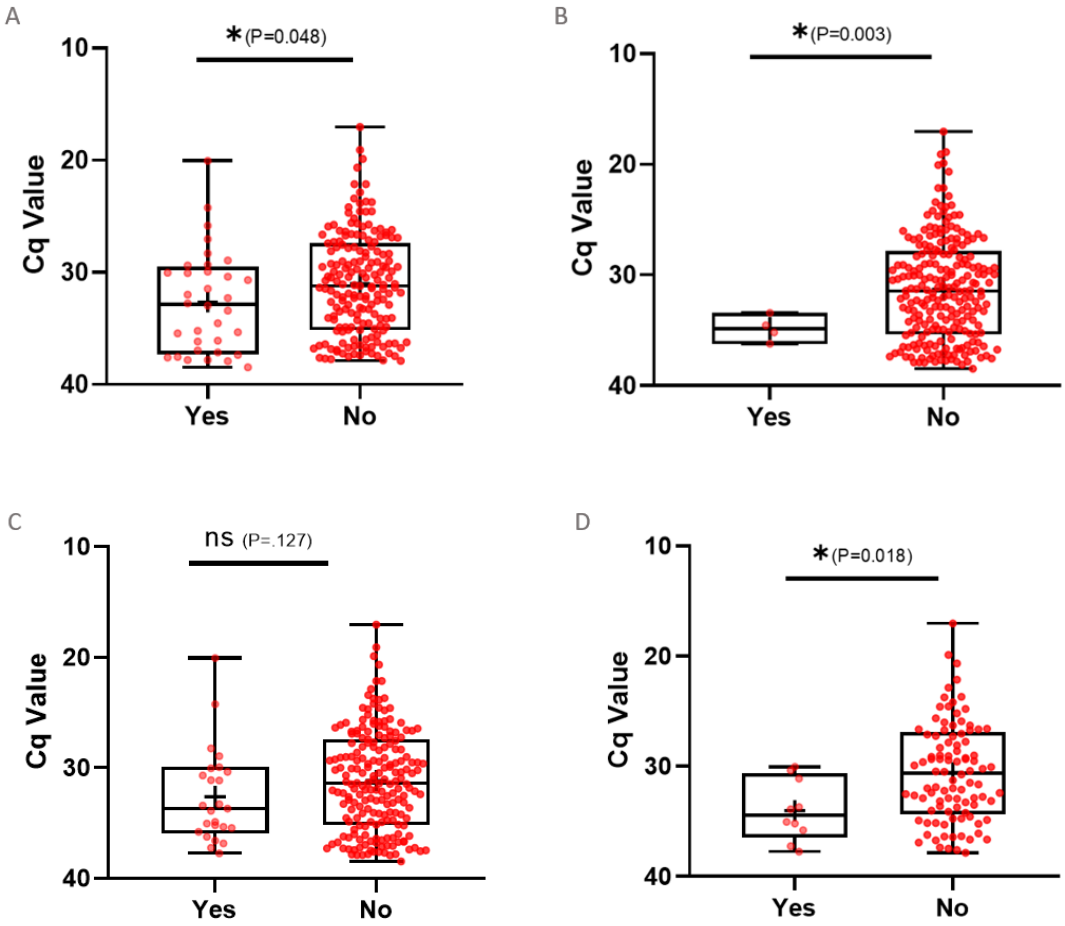

Figure 7: Correlation between viral load and patient outcomes: (A) bacterial co-infection and influenza A, (B) ARDS and influenza A, (C) oxygen supplementation and influenza A, and (D) oxygen supplementation and A/H1N1pdm09. P values from bivariate analysis model.

G. Continuous Variables:

Viral loads of influenza A and B did not correlate with any of the continuous variables such as age, BMI, time since onset of symptoms **(Figure 8A and 8B)**, duration of hospital stay, and time since hospital admission **(Table 11)**.

Table 11: Pearson's Correlation between viral load and continuous variables

Variable	Influenza A Pearson Correlation	p-value	Influenza B Pearson Correlation	p-value
Age	.102	.241	-.156	.237

BMI	.016	.817	-.155	.139
Duration of hospital stay	.017	.803	-.115	.239
Time since onset of symptoms (days)	.119	.13	.18	.069
Time since hospital admission (days)	.076	.252	.015	.887

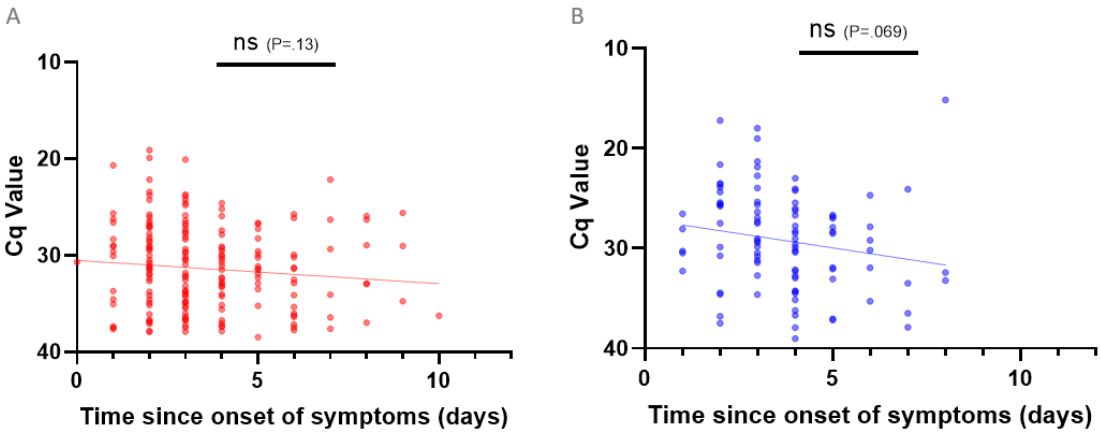

Figure 8: Correlation between viral load and time since onset of symptoms for (A) influenza A and (B) influenza B.

CHAPTER IV

DISCUSSION

Influenza A and B viruses are major causes of acute respiratory illness in humans, leading to significant morbidity and mortality rates (Moghadami, 2017). In this study, we characterized the association between influenza A and B viral loads and patients' characteristics and outcomes during the seasons 2018–2020. Similar to what was previously reported, influenza was prevalent during the winter season between December and February, peaking during January (Assaf-Casals et al., 2020).

In this study, NP and OP samples were used to screen for influenza viruses. Similar to other studies, our results showed higher sensitivity for NP swabs for influenza A and B (Lieberman et al., 2009; Covalciuc et al., 1999; Robinson et al., 2008). Nevertheless, a study by Kim et al., conducted on eight respiratory viruses indicated that OP swabs were more sensitive for influenza A (91.2% vs. 70.4%) while NP was more sensitive for influenza B (83.3% vs. 61.5%) (Kim et al., 2011). NP samples in our study had a significantly higher mean viral load than OP samples for both influenza A and B. A previous study by Hernes et al. also showed that NP swabbing was superior to OP swabbing in terms of diagnostic yield by RT-PCR (Hernes et al., 2013).

Kappa statistics indicated minor agreement between NP and OP samples for influenza A, but moderate agreement for influenza B. Kim et al., however, found that the NP and OP samples had good, albeit not statistically supported, agreement for influenza A (kappa=.712, p=.07), and a lower yet statistically significant agreement between NP and OP samples (kappa=.61, p=.02) for influenza B (Kim et al., 2011). The Bland Altman plot revealed that the mean Cq difference between NP and OP samples

being well below zero favoring higher sensitivity for NP samples. The graph also displayed a wide standard deviation (SD) skewing towards the NP samples being more sensitive. Therefore, NP and OP swabs can complement each other when resources allow. Based on our data, when resources are limited, NP swab is preferable due to better sensitivity. Our study showed no association between viral load and age when measured as a continuous variable and upon dividing it into age groups for both influenza A and B viruses. This was supported by a study conducted by Alves et al. that found that there was no significant difference in viral load in patients of different age groups infected with influenza B for both lineages Victoria and Yamagata (Alves et al, 2019). In contrast, a study also conducted by Alves et al. showed that the age group 5-11 years of age had higher viral load of A/H1N1pdm09 than other age groups (Alves et al., 2020). Similarly, a study by Lee et al. in 2011 demonstrated that younger age groups had significantly higher viral loads for A/H1N1pdm09 (Lee et. Al., 2011). In a study conducted by Granados et al., for influenza A/H3N2 and influenza B, children ages 1-4 and adults over the age of 65 had the highest viral load amongst the 5 age groups analyzed (Granados et al., 2017).

Upon analyzing the viral load of influenza A and B patients in relation to respiratory symptoms, no association was found between viral load and cough, sore throat, and shortness of breath for both influenza A and B. Tsou et al., found no correlation between viral load and either cough and sore throat for influenza A (Tsou et al., 2012). However, a study conducted by Hijano et al. in 2019 found that viral loads were significantly higher in patients with cough for influenza A and B (Hijano et al., 2019). Considering that the above studies had a different population than ours, being only children, this could explain the discrepancy between our results. Having a runny

nose in our study was significantly associated with an increased viral load for influenza A. This could be attributed to the presence of mucus that traps respiratory pathogens including viruses (review by Zanin et al., 2016).

Jackson et al., found that prior vaccination was associated with reduced influenza B viral shedding but not influenza A (Jackson et al., 2020). Consistently, Lalueza et al. found that viral load was higher in unvaccinated individuals with influenza A (Lalueza et al., 2019). Nonetheless, in this study, there was an unexpected lack of association between viral load and vaccination status for both influenza A and B. This is consistent with the findings of Tsou et al., who found that there was no association between viral load and vaccination status in children diagnosed with influenza B (Tsou et al., 2012). We did not find a significant difference between in the timing of sample collection with respect to onset of symptoms that would explain the lack of correlation between viral load and vaccination status.

Several chronic conditions were evaluated in relation to viral load. COPD, DM, and CV disease were not correlated with viral load for both influenza A and B. Similarly, the study conducted by Lalueza et al found no association between either COPD or CV and influenza A (Lalueza et al., 2019). Asthma was not associated with viral load for influenza A. However, it was associated with having a higher viral load in influenza B/Victoria. The study conducted by Oshansky et al., showed no association between peak in viral shedding and severity of disease in patients with asthma (Oshansky et al., 2014). The small number of asthmatic patients in our study limits our analysis and warrants further studies that recruit more asthmatic patients.

The results revealed no association between viral load and most patient outcomes **(Table 10),** such as receiving antibacterial and antiviral treatment for

influenza A and B. Only bacterial co-infections for influenza A, ARDS for influenza A, and oxygen requirement for influenza A/H1N1pdm09 showed a significant association with decreased viral load. The study by Ip et al in 2016 showed that viral levels of influenza A peaked within the first two days after onset of symptoms and gradually decreased to undetectable levels matching closely with the dynamics of clinical illness (Ip et al., 2016). Patients infected with influenza become most susceptible to secondary bacterial infections 4 to 14 days post-influenza symptom onset (Boyd et al., 2006). In our study, the average difference in days of sample collection since symptom onset was 3.62 days for influenza A patients presenting with bacterial co-infection (data not shown), which is just around the timing when a secondary bacterial infection might develop. This might explain, the low viral load accompanying bacterial infection observed in our study (Beadling & Slifka, 2004). In our study, being both subject to oxygen supplementation on admission and ARDS were associated with having a lower viral load for A/H1N1pdm09 and influenza A respectively. Oxygen requirement is a marker of severity. Most patients with ARDS must be supplied with oxygen (Hasleton PS, 1996). In our study 13/17 of the patients with ARDS received oxygen supplementation. ARDS was previously shown to not be associated with viral load of influenza A (Lalueza et al., 2019). Oxygen supplementation and ARDS occur towards the end of the viral phase when patients are transitioning into the inflammatory phase of disease (Kalil & Thomas, 2019), which could explain the lower viral load in these patients

Being admitted into the ICU was not associated with viral load for influenza A and B. Similarly, the study by Hijano et al., found no association between viral load and ICU admission for influenza B (Hijano et al., 2019). However, the study conducted by

Granados et al., found that higher viral loads were inversely associated with being admitted into the ICU for influenza A/H3N2. For influenza B though, a higher viral load was associated with ICU admission (Granados et al., 2017). It was speculated that the lack of difference in viral load between patients in the wards and ICU was due to the lower respiratory tract being more important in determining severity of illness than the nasopharynx (Granados et al., 2017). However, the date of admission into the ICU was not recorded, and hence the patients could have had different clinical presentations and severity of disease at the time of admission.

In contrast to data reported by other authors (Lu et al., 2010; Li et al., 2010), we found no significant association between viral load and pneumonia, similar to what was found in a study by Meschi et al. (Meschi et al., 2011). This could also be explained by the samples being collected from the nasopharynx rather than the lower respiratory tract (Granados et al., 2017).

In our study, there was no significant correlation between days since the onset of symptoms and viral loads for both influenza A and B. Influenza levels peak 1-2 days after onset of symptoms before gradually decreasing to undetectable levels, usually 7 days post-symptom onset (Ip et al., 2016). Other studies also found a negative correlation between viral load of A/H3N2 and A/H1N1pdm09, and the day of onset of symptoms (Lee et al., 2009; Li et al., 2010).

The study has some limitations one of which is the missing dates for some variables such as antiviral and antibiotic drug administration, ICU admission and mechanical ventilation. We were also not able to accurately compare the viral loads of patients due to the variable time of onset of symptoms of the collected samples. In this study, Cq value was used as a surrogate virus load which is a semiquantitative method

and does not provide absolute quantification of the virus gene copy number. However, Cq has been widely accepted as a surrogate of viral load during the COVID-19 pandemic (Bustin et al., 2021; Yu et al., 2020, Kimball et al., 2020).

In conclusion, our study adds to the scant epidemiological data on influenza in Lebanon. This study reflects the high burden of influenza virus in the hospitalized patients in Lebanon. We demonstrated that asthma and runny nose were significantly associated with a higher viral load. On the other hand, bacterial co-infections, ARDS, and oxygen supplementation were associated with a lower viral load. Further studies are needed to explore the relationship between viral load and various variables for immunocompromised patients as well as to compare viral loads between hospitalized and outpatients with influenza virus in the Lebanese community.

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