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West Nile Virus In Cyprus

NAGAT SALEHBALAMAN
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MEDICAL MICROBIOLOGY AND CLINICAL MICROBIOLOGY
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THESIS APPROVAL

(To be added after thesis defence)

STATEMENT (DECLARATION)

Hereby I declare that this thesis study is my own study, I had no unethical behavior in all stages from planning of the thesis until writing thereof, I obtained all the information in this thesis in academic and ethical rules, I provided reference to all of the information and comments which could not be obtained by this thesis study and took these references into the reference list and had no behavior of breeching patient rights and copyright infringement during the study and writing of this thesis.

Name and Surname of the Student

Nagat Balaman

Signature

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ANPEP gene	Alanyl Aminopeptidase
BBB	Blood-Brain Barrier
°C	The degree Celsius (the centigrade scale)
C 1,2,3	Calibrator 1,2,3
C protein	Capsid protein
C1q	Complement component 1q (protein)
C4	Complement component 4
C6/36 (cell line)	*Aedes albopictus* clone
CCL2	C-C chemokine ligand 2
CCL3	C-C chemokine ligand 3
CCL5	C-C chemokine ligand 5
CCR5 gene	C-C motif chemokine receptor 5
CDC	Centers for Disease Control and Prevention
CD4+	Helper T cells
CD8+	Cytotoxic T cells
CD95	(Fas/APO-1/ TNFRSF6) is a member of tumor necrosis factor receptor family)
CD95-L	CD95 ligand
CNS	Central Nervous System
CPE	Cytopathogenic effect
CR1	Complement receptors 1
CR2	Complement receptors 2
CSF	Cerebrospinal fluid
DC-SIGN	Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non- Integrin receptor
DC-SIGN-R	DC-SIGN- related
DEET	N,N-Diethyl-meta-Toluamide
ECDC	European Centre for Disease Prevention and Control
ELISA	Enzyme-linked immunosorbent assay
E protein	Envelope protein
EU	European Union
FDA	Food and Drug Administration
GAGs	Glycosaminoglycans
HIV	Human immunodeficiency virus
HIA	Hemagglutination Inhibition Assay
ID-NAT	Individual donation nucleic acid amplification-based technique
IFA	Immunofluorescent assay
INFs	Interferons
IRF3	Interferon regulatory factor 3
ISGs	Interferon stimulated genes

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ÖZET

Giriş: Batı Nil virüsü (WNV), kan transfüzyonu yoluyla da bulaşabilen nörotropik bir arbovirüstür. Coğrafi dağılımı genişlemesine rağmen, kuzey Kıbrıs'ta henüz WNV ile ilgili herhangi bir epidemiyolojik veri bulunmamaktadır. Çalışmamızın amacı bağışlanan kan örnekleri kullanarak bu boşluğu doldurmaktır.

Yöntem: Lefkoşa'daki ana devlet hastanesi kan bankasından toplanan örnekler, anti-WNV enzimine bağlı immünosorban analizi (ELISA) (immünoglobulin M [IgM] ve immünoglobulin G [IgG]) ile analiz edildi. Seropozitif örnekler onay için plak indirgeme nötralizasyon testine (PRNT) tabi tutuldu ve ELISA IgG avidite testi ile analiz edildi.

Bulgular: 760 serum örneğinden 2 (% 0.3)'si IgM + ve 31 (% 4.1)'i IgG + idi. Nötralizasyon aktivitesi, IgM + 'olanların hiçbirinde (%0.0) ve IgG + donör numunelerinin 26 (% 83.9) 'sında tespit edilmedi. ELISA IgG avidite testinde 21 (% 67.7) yüksek avidite ve bir (% 3.2) IgG + örnekte düşük avidite bulundu. PRNT ile konfirme anti-WNV IgG + numuneleri sadece sınırda (% 19.2) veya yüksek avidite (% 80.8) değerleri gösterdi.

Sonuç: Kuzey Kıbrıs'ta kan donörleri arasında anti - WNV antikorları tespit edildi. Önleyici tedbirlerin oluşturulması ve Kuzey Kıbrıs'ta WNV'nin coğrafi boyutunun değerlendirilmesi özellikle tavsiye edilir

Anahtar kelimeler: Avidite, ELISA, Flavivirus, IgG, IgM, Kuzey Kıbrıs, PRNT

ABSTRACT

Background: West Nile virus (WNV) is a neurotropic arbovirus that can also betransmitted through blood transfusion. Even though its geographic distribution hasbeen expanding, there has not yet been any epidemiological data on WNV in NorthernCyprus. The aim of our study is to fill this gap by using donated blood samples.

Methods: Samples collected from the main government hospital blood bank inNicosia were analyzed by anti-WNV enzyme-linked immunosorbent assay (ELISA)(immunoglobulin M [IgM] and immunoglobulin G [IgG]). Seropositive sampleswere subjected to plaque reduction neutralization test (PRNT) for confirmation andanalyzed by ELISA IgG avidity test.

Results: Of the 760 sera samples, 2 (0.3%) were IgM+ and 31 (4.1%) were IgG+.Neutralization activity was detected in none (0.0%) of the IgM+ and 26 (83.9%)of IgG+ donor specimens. ELISA IgG avidity test reported high avidity in 21(67.7%) and low avidity in one (3.2%) IgG+ sample. PRNT-confirmed anti-WNV IgG+samples exhibited only borderline (19.2%) or high avidity (80.8%) values.

Conclusion: Anti-WNV antibodies were detected in Northern Cyprus amongblood donors. The establishment of preventive measures and evaluation of thegeographic extent of the WNV in Northern Cyprus are highly recommended.

Keywords: Avidity, ELISA, Flavivirus, IgG, IgM, Northern Cyprus, PRNT

1.INTRODUCTION

West Nile virus (WNV) is an enveloped single stranded RNA arbovirus of Japanese encephalitis virus sero-complex from the *Flavivirus* genus of the *Flaviviridae* family that can infect and replicate in many different hosts including birds, horses, reptiles, mosquitoes, ticks, and humans(OIE, 2020; Chancey et al., 2015; Pisani et al., 2016). Majority of infected human cases (around 80%)are asymptomatic but under certain conditions such as low immunity and chronic medical diseases like diabetes, hypertension, chronic renal failure and cardiovascular disease, can cause severe neurological involvements and may lead to death(CDC, 2016; Colpitts et al., 2012; Horga & Fine, 2001).

The virus was named after the place where it was discovered for the first time, as it was discovered in 1937 in West Nile District of Uganda (Smithburn et al., 2017). During the period between 1950s till 1980s, WNV caused febrile infrequent outbreaksin many countries including Israel, Egypt, France, India andSouth Africa (Chancey et al., 2015). However, the first outbreak of WNV in USA occurred during the nineties in New York City, then the virus during four years was able to cross the whole continent (Hogrefe et al., 2004).The geographic distribution of WNV infection, the severity of the infection and the frequency of the disease were influenced by the effect of global warming on the natural cycle of the climates (Eybpoosh et al., 2019; Platonov et al., 2001; Weinberger et al., 2001).In nature WNV is maintained in a bird-mosquito-bird transmission cycle where birds are considered to be amplifying host, meanwhile, dead end host are humans and other vertebrates like horses(David & Abraham, 2016; Lustig et al., 2018).WNV is an arbovirus so the main way of its transmission is through the bites of the infected mosquitoes such as *Culex*. However, WNV can also be transmittedvertically or through breast milk from an infected mother to her baby and through blood transfusion or organ transplantation.(Colpitts et al., 2012; ECDC, 2018b).

Nowadays in epidemic areas, blood banks and collection agencies,implemented WNV screening in their blood screening programto ensure their safety against WNV(CDC, 2018; Lustig et al., 2018).For diagnosis of WNV infection, different

laboratory methods can be used including direct detection methods through viral genome detection or virus isolation and indirect detection methods. The indirect methods which are based on serology are the most commonly used technique for diagnosis and are based on detection of anti-WNV specific IgM and IgG in serum, CSF or whole blood. Cross -reactivity with other flaviviruses or with their vaccine may occur, and therefore in order to overcome cross reactivity, samples that showed positive serological result should be confirmed by plaque reductionneutralizationtest (PRNT) (ECDC, 2013).

Many Mediterranean countries including Israel, Egypt, Turkey and Greecehave documented outbreaks of WNV in their populations as reported by the Centers for Diseases and Control (CDC) and World Health Organization (WHO)(ECDC, 2018b; Horga & Fine, 2001).

The Turkish Republic of Northern Cyprus (TRNC) captured our attention and made us question about the existence of WNV in the island and about the safety of our blood banks for the following reasons: (1) Cyprus is located in the Mediterranean Sea and surrounded by countries with WNV outbreaks history, and(2) *Culexpipiens s.I*(known WNV vector) was found in the island(Koray Ergunay et al., 2014). Interestingly neuroinvasive WNV case was detected in the Republic of Cyprus (Greek side) in 2016(Paphitou et al., 2017).Yet there is no epidemiological data about WNV in TRNC and safety of blood banks in the country,except for the recent local news in July 2019, that reported the first WNV infection in TRNC (News in Cyprus, 2019).

AIM

The aim of this study is to investigate the presence of WNV in TRNC through using serological screening test with high sensitivity and in relation to this, to evaluate the necessity of the blood bank screening for WNV risk in TRNC. For this purpose, serum samples collected from TRNC Government Blood Bank were screened for the presence of anti-WNV IgM and IgG antibodies by using indirect serological methods, which are the most common laboratory techniques used to screen and diagnose WNV infections. The seropositive samples were then confirmed by the gold standard confirmatory test, PRNT (ECDC, 2018b).

2. GENERAL INFORMATION

2.1West Nile Virus:

2.1.1.History and epidemiology:

WNV was identified for the first time in Africa where it was isolated from native febrile woman in 1937 at Omogo,West Nile distract of Uganda (Smithburn et al., 2017; Pisani et al., 2016). WNV was isolated for the first time in Israel from a febrile child in 1951 during an outbreak that happened near Haifa. On the other hand, the first West Nile Neuroinvasive disease (WNND) case in humanswas notified in 1957. The virus is also thought to beresponsible for similar outbreaks between 1942-1950 (Chancey et al., 2015; Chianese et al., 2019). Since WNV isolation in north of Cairo, Egypt, in 1950, many other WNV outbreaks have been reportedin period between 1950s and 1980s in Egypt, South Africa, France, Israel and India(Chancey et al., 2015). Since the 1990s, the geographic distribution and the frequency of the disease has increased due to the global warming and the changing of naturalclimate cycles. In Europe, the firstWNV outbreak was reported in southern France during 1962-1963 and was responsible for West Nile neuroinvasive disease in human and horses. Nonetheless, the virus was detected for the first time in Europe in 1958, in Albania, besides, the first large outbreak of WNV took place in Romania in 1996 (Pisani et al., 2016). However, WNND human cases were seen in western Ukraine in 1985 prior to the outbreak that happened in Romania (Chancey et al., 2015). While in Italy the first WNV outbreak in horses was documented in 1998 in Tuscany(Pezzotti et al., 2011).

The virus crossed the Atlantic Ocean in 1999 where the first outbreak of WNV was documented in New York City then the virus rapidly crossed the whole continent within4 years(Hogrefe et al., 2004), Whilst, in 2013,WNV was reported and confirmed in human for the first time in China (Chancey et al., 2015). According to an article published by Biçeroğlu et al., 2015,cases of WNV human were reported in 2010 for the first time in Turkey**.** However, in 1970 according to study by Ozkul *et al.*,(Ozkul et al., 2006)the first WNV seropositivity was documented after detection of anti-WNV antibodies by haemagglutination inhibition test. Intrestingly

the virus was reported for the first time in the Turkish Republic of Northern Cyprus (TRNC)and in Republic of Cyprus (Greek side) in July 2019 andin 2016, respectively(Paphitou et al., 2017).

Human infections with WNV in temperate and subtropical zones occur mostly in summer and early fall, while in the tropical zone, the infection mainly occurs during the rainy season. The incidence of West Nile viral infection is equal among all ages and both sexes (males and females)whilst the incidence of West Nile encephalitis increases with age(Campbell., 2002).

Although WNV strains are classified into seven different lineages based on their genetic analysis(Figure 1)(Valiakos et al., 2013), mainly lineage 1 and lineage 2 are the ones responsible for causing outbreaks in humans(Petersen et al., 2013). Lineage1 is the most commonly spread lineage among other WNV lineages isolates, it is isolated from Europe,Africa(northern and central),Israel,Asia,North Americaand Australia(Chowers, 2010; Hayes et al., 2005). This lineage is divided into two clades (sublineages): Lineage 1-a and Lineage 1-b. Lineage 1-a is furtherly subdivided into six evolution clusters and mainly seen in Europe, Middle East, Africa and other western hemisphere countries, while Lineage 1-b is represented by the Australian Kunjin Virus(Valiakos et al., 2013).In early times lineage 5 was considered to be Lineage 1-c which represents the Indian isolates(Chancey et al., 2015). On the other hand, WNV Lineage 2 is found in Sub Saharan Africa, Madagascar and latelywas found in Europe. Furthermore, it was found to be co-circulating with lineage 1 in Central Africa (Valiakos et al., 2013). Sequencing Studies has shown that WNV isolates from Turkey were related in sequence to Lineage 1 isolates of Central Africa, meanwhile different than WNV isolates of the Mediterranean region and Middle East(Ergunay et al., 2015).

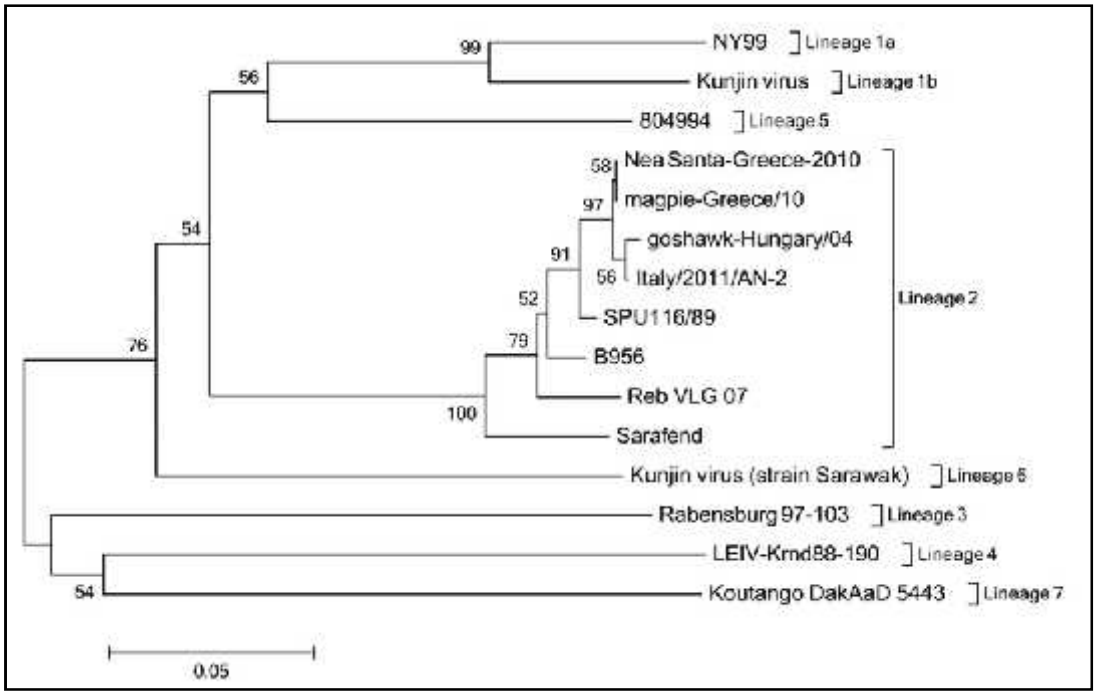

Figure 1: WNV lineages (lineage1- lineage 7) (Valiakos et al., 2013)

2.1.2. Taxonomy: (2018)

WNV taxonomy by the International Committee of Taxonomy of Viruses(Simmonds et al., 2017)was as following: Kingdom: viruses; Realm:Riboviria Family: *Flaviviridae*;Genus: *Falviviruses*/ Japanese encephalitis complex; Species: West Nile Virus.

2.1.3.Genomeand structure:

Structure:

WNV is an enveloped virus like all other *Flavivirus* family members. The viral particleis a small spherical virus about 50 nm in diameter composed of host derivedlipid bilayer envelop surrounding symmetrical icosahedral nucleocapsid (Figure 2). The virus nucleocapsid is composed of the genome surrounded by a protein capsid which is about 30 nm in diameter and mainly made of C protein(Campbell et al., 2002; Valiakos et al., 2013).

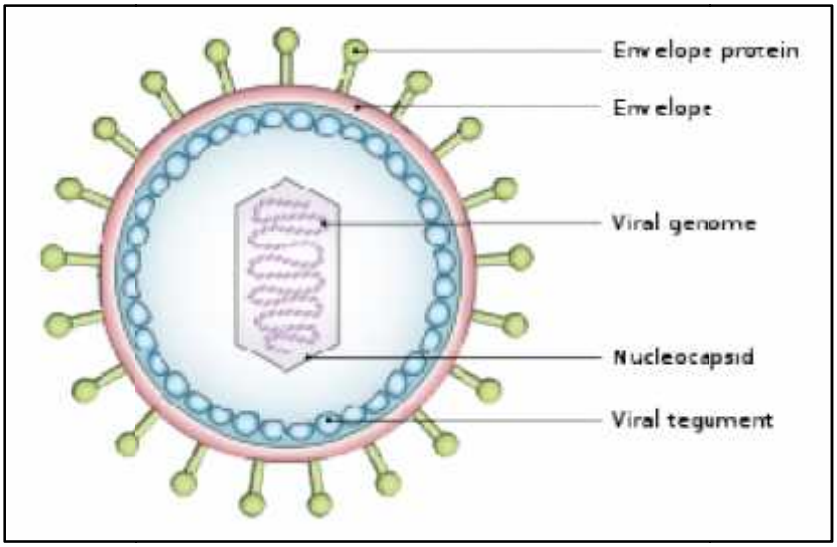

Figure 2: Structure of West Nile virus (Study.com, 2020):

Genome:

WNV genome is a non-segmented positive sense single stranded RNA about 11kb long with two noncoding regions (NCRs) at both ends (5' and 3') and in between those non coding regions there is a single flanked open reading frame that encodes three structural proteins (capsid/C, enveloped /E and premembrane/prM) and seven non-structural proteins (non-structural protein 1,non-structural protein 2A and 2B, non-structural protein 3, non-structural protein 4A and 4B, non-structural protein 5)(Figure 3). Each noncoding region forms stem loop structures which plays an important role in replication, transcription,translation and packing of the virus.Structural proteins (C, E, prM) are mandatory for both entry and fusion of the virus,additionally they also play a role in viral genome encapsidation throughout the process of viral assembly, while the non-structural proteins (NS) are essential for RNA synthesis and virus assembly (Chancey et al., 2015; Colpitts et al., 2012). These are the non-structural proteins:

NS1:there is two forms of this non-structural glycoprotein.The cellular form (intracellular) which is an important viral RNA replication co-factor. The secretory NS1 form which exhibit an immunomodulator action, interfere with complement activation and inhibit Toll-like receptor 3 signal transduction, as well inhibition of STAT1/ STAT 2 activation(Chancey et al., 2015; Colpitts et al., 2012; Martín-

Acebes, 2012). The amount of the secreted form in patient's sera co-relates with the severity of the infection(David & Abraham, 2016). Some of the non-structural proteins are hydrophobic proteins (such as NS2A, NS2B, NS4A, NS4B) and has ability to interfere with innate immune antiviral response, besides that, they are considered to be cofactors for replication complex assembly(Chancey et al., 2015; Colpitts et al., 2012).While NS3 encodes the viral protease (trypsin-like serine protease) which is responsible for cleaving the viral polyprotein once they are activated by NS2B to release structural and non-structural proteins, NS3 not only encodes trypsin like serine protease in its sequence but also encodes other enzymes which have a role in viral replication such as RNA helicase, RNA triphosphatase, nucleoside triphosphatase(Martín-Acebes, 2012).OntheotherhandNS5 encodes methyltransferase and viral RNA-dependent RNA polymerase hence it is important for viral replication also NS5 interfere with host immunity byantagonising interferon signalling(Colpitts et al., 2012; Martín-Acebes, 2012).

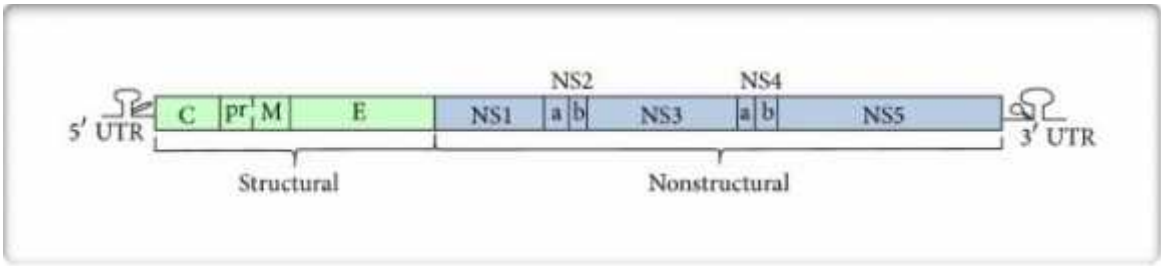

Figure 3: West Nile Virus (WNV) Genome (Chancey et al., 2015).

2.1.4.Replication cycle:

The replication of WNV includes many steps(Figure 4)and starts withrecognition of the target host cell and attachment of the Envelop I viral glycoprotein to host cellular receptors (host surface proteins).In this step the virus attach by its E glycoprotein to different specific receptorondifferent host cell surface receptors such asDendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN), DC-SIGN-related (DC-SIGN-R), Mannose receptor, glycosaminoglycans (GAGs), NKP44, integrin αvb3 ,TAM receptors (Tyro3, Axl and MerTK) Following cellular attachment, WNV enters the cell by clarithrin-mediated endocytosis which precedes fusion of the endocytic membrane with the

WNV lipid membrane forming a fusion pore. This fusion occurs as a consequence of

the activation of the conformational changes in the Envelope protein due to the acidic

PH of the mature endosomal vesicle. The porethen leads to the release of the viral nucleocapsid to the cell cytoplasm.This is followed by the dissociation of viral capsid(uncoating) and release of the positive-sense single stranded RNA genome (+-sense ssRNA) into the cytoplasm. Initiation of translation and polyprotein processing will lead to the release of 3 structural proteins (C, E, prM) and 7 non-structural proteins that is involved in viral replication and assembly.This is followed by formation of the replication complex and initiation of synthesis of the complementary negative RNA strand by RNA dependent RNA polymerase NS5. The negative SS RNA acts as a template for the production of full-length Positive sense ssRNA strand. Assembly of structural proteins will take place on the endoplasmic(ER) membranes followed by encapsidation and the newly formed nucleocapsid is enveloped by a membrane derived from endoplasmic reticulum (ER) forming immature viral particle.The immature viral particles travel through Golgi network and secretary pathway to mature.In this step the viral prM proteinof the immature viral particle are cleaved by host cell furinto produce mature membrane (M) protein.Finally, newly formed mature virions are released by exocytosis(Chancey et al., 2015; Colpitts et al., 2012; David & Abraham, 2016; Martín-Acebes, 2012; Valiakos et al., 2013).

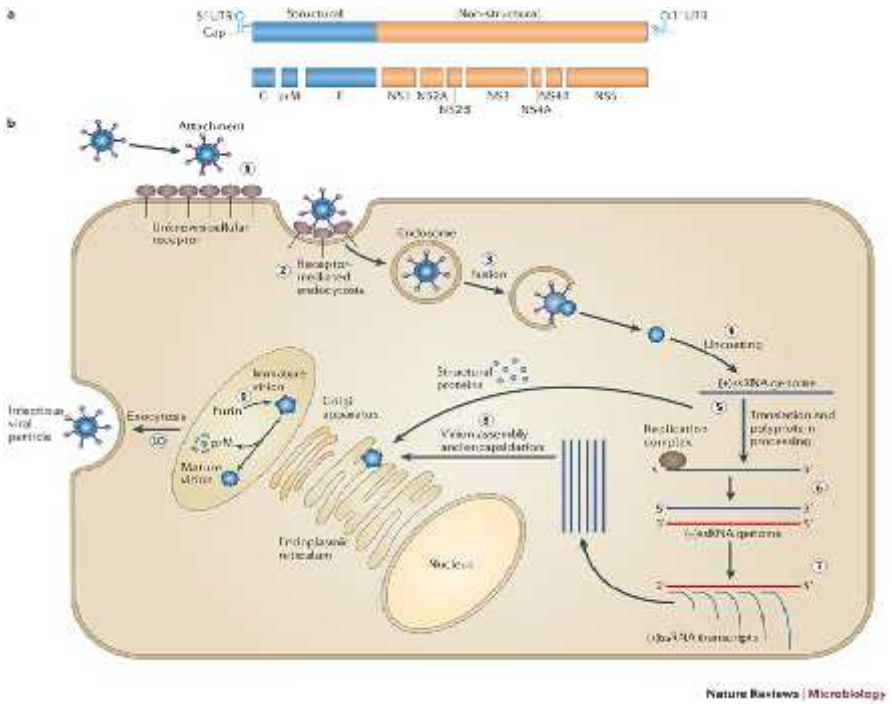

Figure 4: Replication Cycle of West Nile Virus. (Suthar et al., 2013)

2.1.5.Vector:

Mosquitos are considered to be the main vector for WNV transmission. Though there is around 3,500 Mosquito species all over the world(Lam-Phua et al., 2019), not all mosquito species can transmit WNV and not all WN infected mosquito species has ability to transmit and spread the virus, since the transmission is related to the level of viremia within the mosquito and feeding preference of the mosquito(ECDC, 2018b).

Additionally some species were able to transmit WN virus in laboratory setting but not in nature such as *Aedes albopictus and Aedes detritus*(Chancey et al., 2015; Colpitts et al., 2012; ECDC, 2018a; Papa, 2017).Globally the main transmission vector and overwinter reservoir of WNV are mosquitoes of *Culex* genus especiallythose included in the *Culex pipiens* complex(David & Abraham, 2016; Anna Papa, 2017).

2.1.6.Host:

Birds are the main and amplifying host which plays an important role in the life cycle of WNV as they can develop high level of viremia which is sufficient to infect the vector and may remain for one to four days post infection (Campbell et al., 2002; Chancey et al., 2015; Pradier., 2012).Additionally around 30 vertebrates are susceptible for WNV for example , reptiles, amphibians, rodents, bats, and other mammals(Chancey et al., 2015).However, the ability of the birds to acquire,transmit and show clinical symptoms varies among different species; for example birds that belongs to Corvidaefamily such as Crows and blue jays(*Cyanocitta cristata*) may develop fatal infection and die while some infected birds like house sparrows develop high viremia with low mortality rate (Chancey et al., 2015; Colpitts et al., 2012).

Humans and horses are considered to be incidental dead-end hosts because once they get infected by mosquitoes they develop low level of viremia which is not sufficient to infect mosquitoes (vector), hence they do not play any role in WNV life

cycle(Chancey et al., 2015).Also WNV has been isolated from hippoboscid flies, soft and hard ticks however, their role in the transmission cycle of the virus is not proven yet (Campbell et al., 2002).

2.1.7. Mode of the transmission:

West Nile Virus can spread and transmit by different ways. a)-Mosquito to susceptible host to mosquito; this is considered to be the predominant way of transmission as the susceptible host (host which has high viremia) usually gets WNV infection by the bite of an infected mosquito or by consuming an infected mosquito (Chancey et al., 2015). b)-Mosquito to mosquito which occurs through verticaltransmission from an infected female mosquito which has high level viremia to her progeny (David & Abraham, 2016).C)-Bird-to-birds;Susceptible birds can also get WNV infection by direct contact with oral and cloacal fluids of infected birds with high viremia (Chancey et al., 2015). d) Human-to-human; WNV can be transmitted rarely from an infected human to another through substances of human origin (blood transfusion and tissue or organ transplantation); From an infected mother to her fetus during pregnancy or via breastfeeding; throughoccupational and laboratory accidental exposure for example during autopsy or mosquitos collection, etc. (ECDC, 2013).Interestingly, nonviremic transmission between co-feeding mosquitoes has been evinced by many studies (Pradier et al., 2012).

2.1.8.Transmission cycle:

WNV is an arbovirus which can be transmitted in both enzootic and epizootic cycles(Figure 5).However, it is mainly maintained in nature in enzootic cycle between susceptible mosquito (main WNV vector) and amplifying susceptible host which are mainly birds(Chancey et al., 2015; ECDC, 2013).Moreover WNVis maintained in nature through bird to bird transmission and transovarial transmission through specific mosquitoes(Pradier., 2012).

Even though WNV can also be transmitted through an epizootic way, where the infected mosquito (bridge vector) feeds on both infected birds and incidental

mammalian host, such as human and lead to transmission of the virus to humans or other vertebratescausingepidemics(ECDC, 2018b).The epizootic transmission does not play a role in the virus life cycle continuation, as these incidental hosts do not develop sufficient viremia to infect mosquitoes,which is why they are considered to be dead end hosts(Chancey et al., 2015; ECDC, 2013).

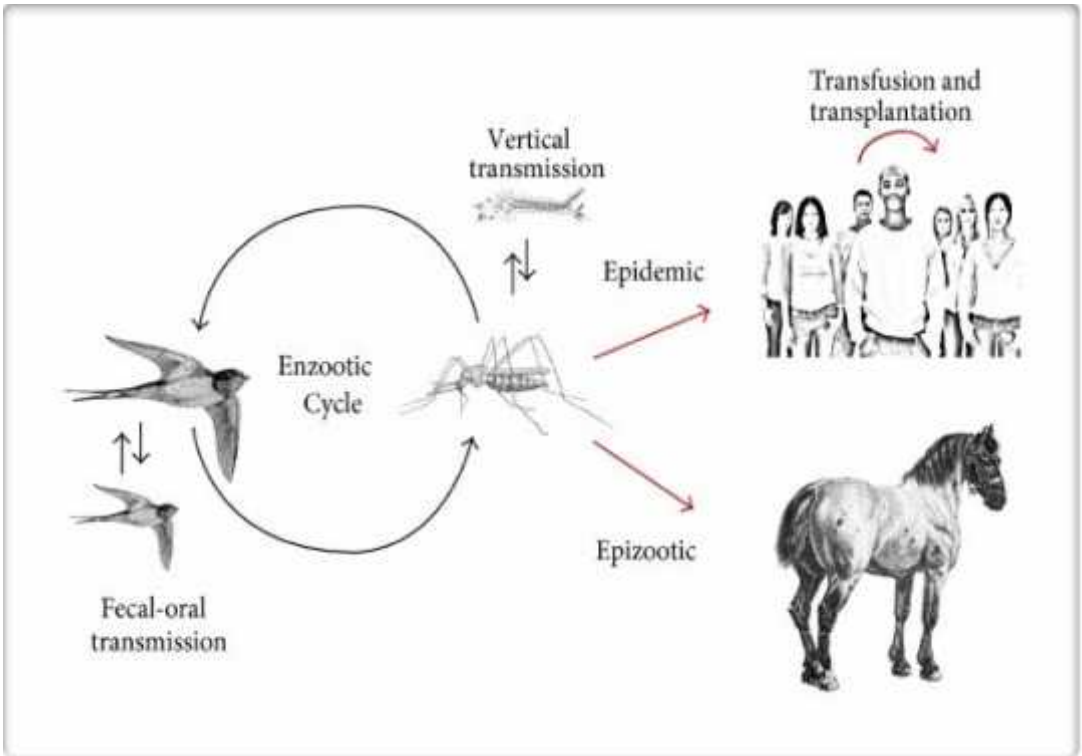

Figure 5: Transmission Cycle of WNV(Chancey et al., 2015).

2.1.9.Pathogenesis:

WNV is introduced to human circulation mainly by the bite of an infected mosquito through their saliva while they are having their blood meal. The virus replicates at the site of the primary inoculation (site of the mosquito bite) within keratinocytes, Langerhans cells (LCs) and dendritic cells which resides the epidermis. Then those infected LCs travel to the daring lymph nodes (LNs) and then to the blood circulation causing viremia and subsequent dissemination to spleen and

other organs(Figure 6). The virus may disseminate to the central nervous system (CNS) causing neuropathogenesis (Hayes et al., 2005; Petersen et al., 2013).

According to (Petersen et al., 2013).WNVis capable of infecting the CNS by the following assumed ways: 1)- by infecting directly the endothelium of the brain blood vessels. 2)-by crossing the blood brain barrier as a consequence of increasing the permeability of the BBB and destruction of the tight junction by cytokines. 3)- through the WNV-infected monocytes which carries the virus within and infect the brain cells, (this mechanism is known as Trojan horse). 4)- following infection of the peripheral neurons, the virus may be transmitted to CNS by retrograde axonal transmission(Figure 7).

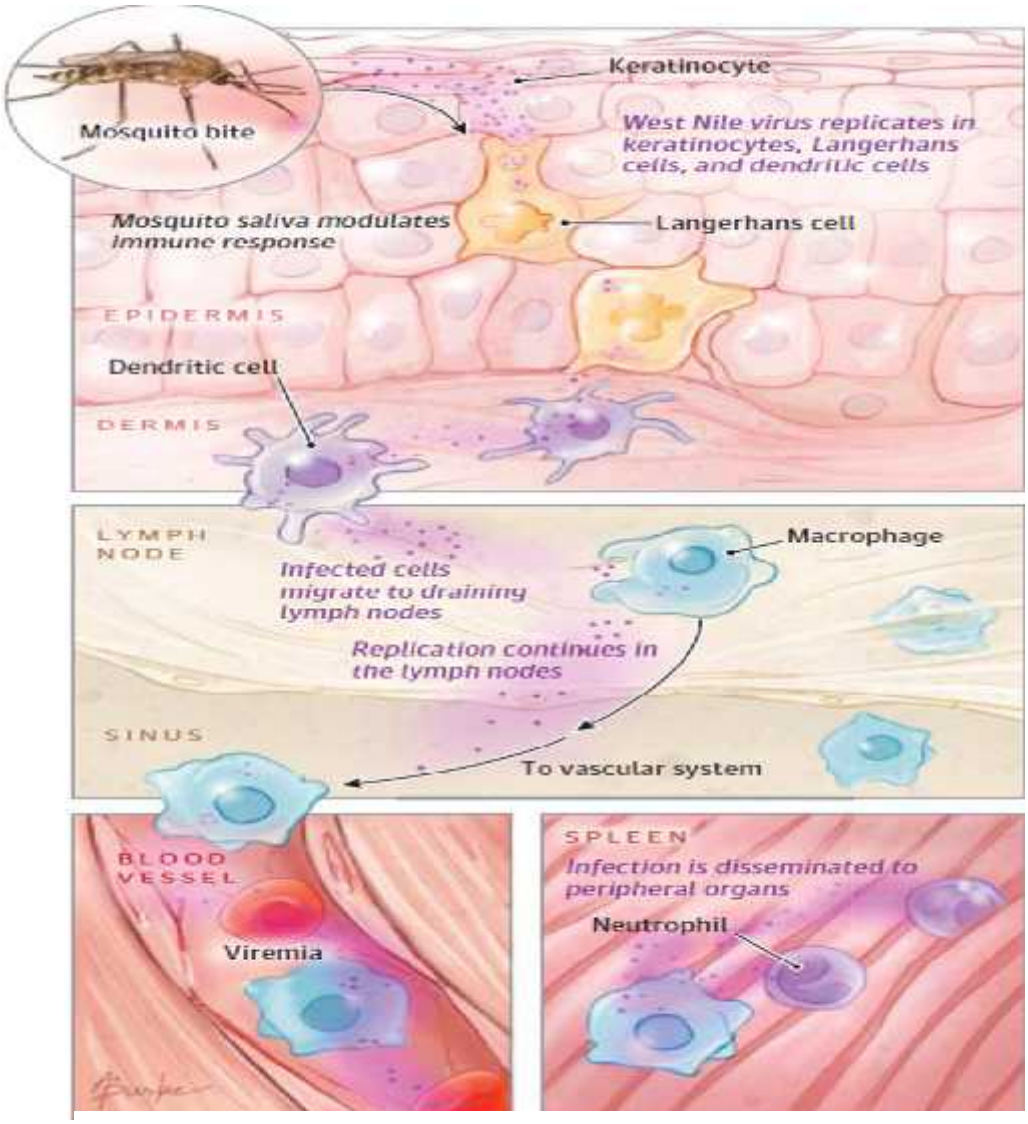

Figure 6: Steps of the WNV pathogenesis in human body (Petersen et al., 2013).

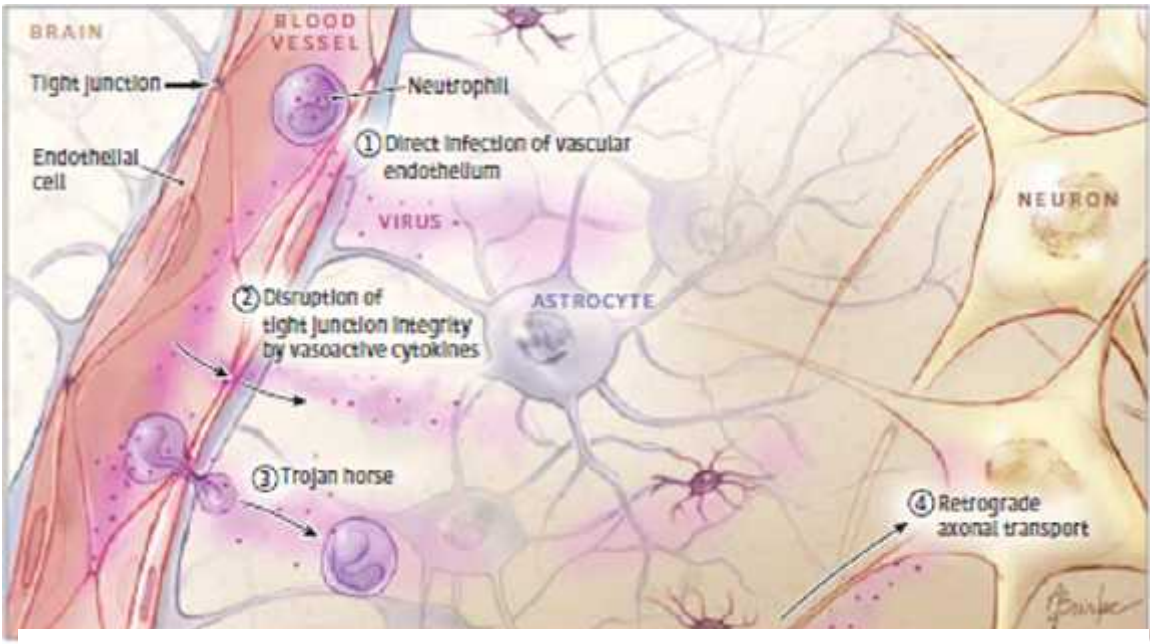

Figure 7: Neuropathogenesis of WNV infection.(Petersen et al.,2013)

2.1.10.Clinical manifestations:

West Nile viral infection in human; around 80% of infected people shows no symptoms while 20% of them shows flu-like symptoms or WN fever (Petersen et al., 2013; V. Sambri et al., 2013).

West Nile Fever (WNF): It is thecommonly seen form of the disease as it can affect all ages. The patient suffers from varying nonspecific symptoms characterized by acute sudden appearance of low-grade fever,nausea,vomiting,diarrhea, generalised body ache, fatigue, eye pain and generalised lymphadenopathy. Additionally, some patients mainly young people may also develop maculopapular nonpruritic rash which is mainly seen on the trunk and extremities but not on the palms and soles (Petersen et al., 2013; Sejvar, 2014)

Even though WNV infection's incubation period is about 2-14 days, it may extend to 21 days in immune suppressed individuals, whereasin WNF cases it is 2-6 days(Campbell et al., 2002; Petersen et al., 2013).

About 1% of infected people with WNV may suffer from neurological involvements, suchasmeningitis, encephalitis, meningoencephalitis and acute

poliomyelitis-like syndrome (Petersen et al., 2013; V. Sambri et al., 2013). These patients will presentand be diagnosed(Table 1) as the following :

West Nile meningitis (WNM) has typical viral meningitis symptoms, including sudden onset of fever, neck rigidity, headache, fatigue, photophobia, nausea, vomiting, diarrhoea, positive Kering's sign and Brudzinski's sign. Also as a consequences of gastrointestinal disturbances the patient my develop dehydration and his systemic illness including headache will get worse (David & Abraham, 2016; Sejvar, 2014).

West Nile encephalitis (WNE) patients will present with typical encephalitis signs ; depressed or altered level of consciousness, personality changes ,lethargy and usually preceded by flue like symptoms (Petersen et al.,2013). The severity of encephalitis may range from mild self-limited to severe encephalopathy which may lead to coma and death. WNE severely affects individuals over the age 55 and immunosuppressed people, including people with HIV infection, organ transplant, chronic debilitating diseases or treated with chemotherapy (Sejvar, 2014). Patients with WNE may developextrapyramidal disorders, features of Parkinsonism, upper extremities coarse tremor and myoclonus of facial muscles and upper extremities. Additionally increased intra cranial pressure, cerebellar ataxia, cerebral oedema rarely may occur (David & Abraham, 2016; Petersen et al., 2013).

Acute poliomyelitis-like syndrome (WNP) occur as a result of viral involvement of lower motor neurons of the spinal cord (David & Abraham, 2016). It is usually characterizedby sudden onset of asymmetric limb weakness (monoplegia) which progress during 48hours, however in case of severe involvement of the spinal cord, symmetric quadriplegia may develop with no sensory loss (David & Abraham, 2016; Petersen et al., 2013). Other symptoms may also present with WNP and has high morbidity and mortalitysuch asneuromuscular respiratory failuredue to diaphragmaticand intercostal muscles paralysis which is a resultof the viral involvement of respiratory muscle innervations. Additionally WNP patients may develop other symptoms related to viral brain stem involvement, for instance, dysarthria, difficulty of swallowing and loss of gag reflex (David & Abraham, 2016; Sejvar, 2014).

Uncommon neurological symptoms that may develop with WN viral infection includes rhombencephalitis, polyradiculitis, myelitis and inflammation of the optic nerve. Additionally, WNV infection can cause extraneurological symptoms in the organs with high viral load, for examples hepatitis,myocarditisand pancreatitis (Campbell et al., 2002).

Table 1: Clinical manifestationsand diagnosis of WNV neurologicaldisorders: meningitis, encephalitis and acute flaccid paralysis (Loeb et al., 2008).

West Nile meningitis
Clinical signs of meningeal inflammation, including nuchal rigidity, Kernig or Brudzinski sign, or photophobia or phonophobia
Additional evidence of acute infection, including ≥1 of the following: fever (temperature >38° C) or hypothermia (temperature <35° C); cerebrospinal fluid pleocytosis (0.005 × 10^9 cells/L); peripheral leukocyte count >10 × 10^9 cells/L; or neuroimaging findings consistent with acute meningeal inflammation
West Nile encephalitis
Encephalopathy (depressed or altered level of consciousness, lethargy, or personality change lasting 24 h)
Additional evidence of central nervous system inflammation, including ≥2 of the following: fever (temperature >38° C) or hypothermia (temperature <35° C); cerebrospinal fluid pleocytosis (0.005 × 10^9 cells/L); peripheral leukocyte count >10 × 10^9 cells/L; neuroimaging findings consistent with acute inflammation (with or without involvement of the meninges) or acute demyelination; presence of focal neurologic deficit; meningismus; electroencephalography findings consistent with encephalitis; or either new-onset seizures or exacerbation of previously controlled seizures
Acute flaccid paralysis
Acute onset of limb weakness with marked progression over 48 h
≥2 of the following: asymmetry to weakness; areflexia or hyporeflexia of affected limb(s); absence of pain, paresthesia, or numbness in affected limb(s); cerebrospinal fluid pleocytosis (0.005 × 10^9 cells/L) and elevated protein levels (4.5 g/L); electrodiagnostic studies consistent with an anterior horn cell process; or abnormal increased signal in the anterior gray matter as documented by spinal cord magnetic resonance imaging

2.1.11. Prognosis:

Prognosis of uncomplicated West Nile fever and meningitis usually good as these patients fully recover but under certain circumstances, such as old age, underlying chronic diseases and immune suppression, the fever may lead to death. However, the prognosis ofWNE mayvary from full recovery to death and not related to the intensity of the infection. Besides, some of the WNE patients may still suffer from physical and cognitive dis-improvement for years after infection(Petersen et al.,

2013). WNP Patients do not recover completely, though variable improvement may be seen in the strength of the affected limb (Hayes et al., 2005).

2.1.12. Determinative genes of the disease:

Several studies have pointed the correlation between specific genes and the severity of WNV disease among infected people,such as OASL gene(2'-5'-Oligoadenylate Synthetase Like), CCR5 gene(C-C motif chemokine receptor 5), OAS-1 gene(2'-5'-Oligoadenylate Synthetase 1), IRF3 gene(Interferon regulatory factor 3),MX-1gene(MX DynaminLikeGTPase1),RFC1 gene(Replication factor C subunit 1), SCN1A gene(Sodium voltage-gated channel alpha subunit 1) and ANPEP gene(Alanyl Aminopeptidase)(Colpitts et al., 2012)(Table 2).

Some of these genes (e.g. OAS-1) were linked to the initiation of WNV infection through single nucleotide polymorphism (SNP). However, SNPs of other genes,such as IRF3,MX-1 and OAS-1 were associated with an elevated risk of symptomatic WNV infection(Bigham et al., 2011; Colpitts et al., 2012). While SNP in the gene OASLwas correlated to increase susceptibility to the infection(Yakub et al., 2005).

Researcher (Bigham et al., 2011) also pointed that the delta 32-bp deletionin CCR5 geneis associated with increased risk of symptomatic West Nile infection. Interestingly other SNPs of certain genes(RFC1,SCN1a, and ANPEP) were linked to increase risk of severe neuroinvasive WNV infection(Colpitts et al., 2012).

Table 2: Determinative genes of WNV infection (Colpitts et al., 2012).

Gene(s)	SNP(s)	Comparison groups (*n*)	Study results
OASL	rs3213545	WNV^+ cases (33) vs healthy controls (16)	Associated with increased susceptibility to WNV infection
CCR5	Δ32 deletion	WNV^+ cases (395) vs WNV^- (1,463)	Increased risk of symptomatic WNV infection
		WNV^+ cases (224) vs healthy controls (1,318)	Increased risk of symptomatic WNV infection
		WNV^+ cases (634) vs WNV^- (422)	Not a risk factor for WNV initial infection; associated with symptomatic WNV infection
OAS1	rs10774671	WNV^+ cases (501) vs healthy controls (552)	A risk factor for initial infection with WNV
IRF3, MX1, OAS1	rs2304207, rs7280422, rs34137742	Symptomatic cases (422) vs asymptomatic cases (331)	Associated with symptomatic WNV infection
RFC1, SCN1A, ANPEP	rs2066786, rs2298771, rs2565	Severe WNV cases (560) vs mild WNV cases (950)	Associated with neuroinvasive disease in patients infected with WNV

2.1.13.Immune response against WNV:

Antiviral immune response is composed of early nonspecific immune response which is known as innate immune response and late more specific response by the adaptive immune system(Crowe, 2017).

The first protective immune response against WNV is made by the innate immune system, which has ability to sense and detect viruses and viral infected cells by pathogen recognition receptors (PRRs)which can be cytoplasmic PPRssuch as NOD-like receptors(NLRs) andretinoic-acid-inducible gene I- like receptors (RLRs) or membrane-bound PRRs like, Toll-like receptors (TLRs) 3,7. In case of WNV infection, thesePRRs receptors(cytoplasmic and membrane-bound)are activatedleading to enhanced innate immune response with synthesis and release of cytokines, chemokines and interferon stimulated genes (ISGs)that are vital for controlling WNV replication and infection(Bai., 2019; Gack & Diamond, 2016; Rossini., 2013).For instance, it was found that interferons (IFNs)play an important protective role as INF I and INF III are important for the stability of the BBB thus hinder the entrance of WNV virus to the CNS (Luo & Wang, 2018).Moreover, the cytokines and chemokines that are released upon the activation of PRRs links both immune responses together (innate and adaptive)(Gack & Diamond, 2016).In addition the released chemokines help in the clearance of WNV from the Central nervous system by chemotaxis ofimmune cells in the CNS(Wang & Luo, 2018).

Besides these, another protective mechanism against WNV is the complement system which is an important part of the innate immune system(Rus., 2005).Thecomplement system is made of a group of plasma and surface proteins that interact with each other and leading to cascade of enzymatic reaction which helps to protect our body against pathogens such as viruses. This system has ability to recognize viruses or virus infected cell and in turns neutralizes these viruses or kills the viral infected cells (P. Agrawal., 2017; Yancey & Lazarova, 2008).

There are three pathways for complement activation (classical, alternative and lectin pathways). Though each pathway is activated in a different way, all three pathways leads to the formation of membrane attack complex (MAC) which causes

lysis of enveloped flaviviruses including WNV and lysis of the viral infected cells(Bai et al., 2019; Conde.,2017). Interestingly, many clues have suggested the protective role of the complement system against WNV infection. Researchers found that mice with deficiency in complement receptors (CR1, CR2) had lethal WNV infection, high CNS viral load and low Immunoglobulin(Ig) both IgM and IgG(Bai et al., 2019).Moreover, high mortality of WNV infected mice wasassociated with lack of C1q, C4 and factor (B or D). Besides, it was found that any lack in the function of the complement system, affects the response of both T cell and B cell members of adaptive immune system(Bai et al., 2019).For instance,impairment of classical and lectin pathways in mice was found to be associated with lack of T cell response and antibodyproduction, while the impairment of the alternative pathways was linked to deficient CD8+response and normal B cell function(Mehlhop & Diamond, 2006).

As mentioned earlier the released cytokines and chemokines during the innate immune response lead to the activation of the adaptive immune response(Gack & Diamond, 2016). For instance, innate immune cells, such as macrophages and dendritic cells,once encounter WNV or WNV infected cell, they release different cytokines and chemokines, like interferon I, interleukin1 beta, tumor necrosis factor, chemokine ligand 2 (CCL2), CCL3, CCL5, and interleukin 8 , which in turn not only control innate immune response but also plays a role in initiating the response of the adaptive immune system and complement system, therefore helps in controlling viral infection through initiation of T cell response , Tregs (regulator T cells) development and intensifying humoral immune response. Activation of B cell and Tcell which are the major effective cells serving the adaptive immunity, thus playing an important role in clearing the virus from the body and prevention of becoming infected again by WNV (Gack & Diamond, 2016; Suthar., 2013).

One of the most important protective immune response against WNV infection is the humoral immune response which was proved by studies that were applied on mice. These studies have shown that, the mortality rate among mice with deficient B cells or defective IgM production was high, also they noticed that in mice with T cell and B cell dysfunction duet to Rag1 deficiency (Chambers et al., 2008; Diamond., 2003; Diamondet al., 2003). Additionally, studies showed the importance and the role of different T cells subsets in limiting WNV infection. CD8+ T cells deficient

mice had higher replication rate of WNV within theCNS.Besides CD8+, CD4+ T cells also was found to play a protective role against WNV as found to be responsible for initiating WNV-specific IgM,WNV-specific IgG andassisting the response of CD8+ T cells within the central nervous system of mice with deficient CD4+ or lacking MHC molecule (II) or deficient antibodies. Interestingly CD4+ T cells were able throughcytolytic mechanisms by CD95-CD95L and perforin dependent processes to lyse WNV-infected cells(Suthar et al., 2013).Furthermore, the abundance of the regulatory T cells (Tregs) was linked to the severity of the outcome of WNV infection,as lower Treg cell levels were seen in symptomatic WNV infected human and mice(Lanteri et al., 2009).

2.1.14. Treatment:

There is currently no approved specific treatment for WNV infections in humans. Only symptomatic and supportive treatment is given accordingto the clinical manifestations of the infected patient.There is some therapeutic regimen and drugs (such as corticosteroids, ribavirin, interferon and intra venous immunoglobulin) which are still under investigation buttheir efficacy is not fully proven and further studies are needed (ECDC, 2018b; Petersen et al., 2013).

2.1.15. Experimental therapy:

Many medications were being experimented to investigate their effectiveness against West Nile Virus but unfortunately none of them was confirmed or approved to be used yet.

Ribavirinis a purine analogue (with a broad-spectrum antiviral activity against many viruses including *Flaviviridae* familymembers.Though ribavirin was able to inhibit WNV replication within the cell culture, its usage in animal modules was associated with high mortality rate (Campbell et al., 2002; David & Abraham, 2016; Jordan et al., 2000).

Corticosteroids is used to subside cerebral oedema associated with WNED but due to its immunosuppressive effect it may enhance the infection so this risk should be kept in mind and re-evaluated(Campbell et al., 2002).

Interferons are proteins that belongs to cytokines group which are known of its antiviral activity and is used as treatment for many viral infections.Unfortunately though interferons were able to minimise the complications of WNV infection in some infected people,theywere not effective for all infected people(Arnaud, 2002; Rodríguez-Pulido., 2012).

Intravenous Immunoglobulins are Immunoglobulins G(IVIG) derived from plasma of pooled blood donors and given intravenously. They are used as prophylaxis and for treatment of many bacterial and viral infections including some members of *Flaviviridae*family. Many studies and clinical trials tried to test the efficacy of IVIG against WNV infection in animal models.While some of these studies showed a promising results in treating West Nile viral infectionand preventing the consequences of WNV infection of the CNS (David & Abraham, 2016), other studies found no or poor effects on treating or minimising the outcome of WNV infection. However, clinical humans trials are needed to evaluate the effectiveness of IVIG in WNV infected human(A. G. Agrawal & Petersen, 2003; Haley., 2003).

2.1.16 Prevention:

2.1.16.1 Preventive measures:

WNV is an arbovirus so in order to prevent infection in human, arbovirus infection control measures should be followed such as vector control measures, andpersonal protection. Since west Nile Virus can also be transmitted through blood transfusion and organ transplantation, measures to prevent WN virus transmission through substances of human origin (SoHo) should also be considered(Campbell et al., 2002; ECDC, 2013).

-**Vector control measures** : the aim of these measures is to control and reduce the vector (Mosquitoes) which is responsible for transmitting WNV(ECDC, 2018b). These measures include the initiation of local surveillance programs to identify the species of the vector which is present in area and to set an efficient program to eliminate it; elimination and reducingof mosquito breeding sites such as stagnant water sites including any area which label to flooding and can act as breeding site

(underground heating, basement and sewage pipes) by water management, chemical and biological control(Campbell et al., 2002).

-Personal and public protection measures:these measures are based on education of people in the community how to protect themselves against mosquitoes' bites and how to help in reducing the mosquitoes breeding sites at their homes(Williams, 2012). People can protect themselves against the mosquitoes' bite by limiting outdoor activity especially during the active period of the mosquitoes which depends on the species of the mosquito but mainly from dusk to dawn. In addition, people can protect themselves by wearing full cover clothes, spraying exposed parts of the body by mosquitos' insecticides containing N, N-Diethyl-meta-toluamide (DEET) and applying insects screen nets for windows and doors(Campbell et al., 2002). Also, people can participate in reducing and eliminating the vector from the environment around their houses by emptying anything which is filled by stagnant water such as flower pots, birddish, old wasted tires, etc. (ECDC, 2018b) (Figure 8).

Figure 8: WNV prevention ways(coveringkaty.com, 2018).

- Prevention of West Nile virus transmission through substance of human origin (SoHo): according to the European Prevention protocol recommendation; all blood donors coming back from visiting or living in a country which has an active ongoing WNV transmission are forbade from donating their blood for 28 days (deferral period) after coming back, however to avoid shortage in blood storage at the blood bank travelers can be screened by WNV nucleic acid test such as MP-NAT or ID-NAT before donation (ECDC, 2013). Also, organ donors who travelled to an endemic WNV area should be tested for the presence of the virus before donating. Hence, for blood banks to be able to assess the correlation of WN virus infection and travelling history of blood donors, National Authority should provide them with WN maps and ECDC's maps showing active WN virus area.

Incaseif Positive WNV- NAT donor was found during screening,it is recommended to quarantine and dispose of these positive blood samples immediately. Furthermore, post donation data about the positive donor should be collected within 15 days and all blood recipients that received any blood components derived from the blood of the positive donor during 120 days before detecting donor's positivity should be analysed and pathogen inactivation methods should be taken in account (ECDC, 2018b; ECDC, 2013).

Moreover, in EU prevention protocol it is recommended to do epidemiological analysis studies to collect data about both donors and receivers to help authorities to assess the risk of transmission of the virus through SoHo and accordingly to plan an effective strategies to reduce and prevent the infection (ECDC, 2018b).

-Prevention of WN virus transmission among health care and laboratory workers: Raising awareness about the transmission of WNV through SoHo among health and laboratory workers is highly recommended ("Laboratory-acquired West Nile virus infections - United States, 2002," 2002). Standard infection control and laboratory safety measurements should be followed when dealing with suspected WNV infected person or their body samples. Moreover, if any health worker was exposed to blood or other body fluids of an infected person then occupational safety

and health protocols of dealing with blood borne pathogens should be established and followed (OSHA, 2015) .

2.1.16.2 Vaccine:

Till now there is no approved or licensed vaccine for WNV to be used in human but there are candidate vaccines in trials (Martín-Acebes, 2012). Meanwhile there are four approved licensed vaccines used for equines immunization; two of these vaccines are inactivated whole WNV vaccine with adjuvant; one vaccine is a chimeric recombinant (nonreplicating) canary poxvirus vaccine and the remaining vaccineis an inactivated flavivirus chimera vaccine ("West Nile Virus.," 2005).

The process of vaccine development and approval comprise different clinical trial phases and stages which the vaccine should go through and pass before they get approval to be used and manufactured (CDC, 2019). Unfortunately none of the candidate West Nile virus vaccines have passed beyond phase II clinical human trials (Petersen et al., 2013).

Candidate West Nile virus human vaccines that are still in clinical trials until the present time (Table 3)(David & Abraham, 2016), include: Hydovax-001 candidate vaccine, is an inactivated vaccine that is still in phase I human clinical trial and has ability to neutralize antibodies after the second dose in 50% of people; VRC WNV candidate vaccine,is made of circular DNA plasmid that express West Nile virus proteins (premembrane and envelope proteins and this vaccine is still in phase I human clinical trial; HBV-002 candidate vaccine, is a recombinant subunit vaccine which is still in phase I clinical human trial and has demonstrated ability to neutralize antibodies in all people after the third dose; rWN/DEN4Δ30candidate vaccine, is a live attenuated chimeric vaccine that is still in phase I clinical human trial and has succeeded to neutralise antibodies after the second dose in 89% of people; ChimeriVax-WN02 candidate vaccine which is a live attenuated chimeric vaccine composed of yellow fever virus vaccine-17D that serve as a vector backbone expressing WN virus premembrane and envelope proteins,is still in phase II human clinical trial and was able to neutralize antibodies up to 90% after the first dose in older and younger people. Last but not least, Inactivated WNV candidate vaccine

which is still in phase I/II. This vaccine is inactivated by formaldehyde and showed ability to neutralize antibodies after the third dose) (Amanna & Slifka, 2014; Ulbert, 2019).

Table 3: WNV candidate human vaccines (Ulbert, 2019).

Candidate vaccine	Type	Key data to date	Most advanced clinical stage
Hydrovax-001	Inactivated using hydrogen peroxide	Neutralizing antibodies in 50% of individuals after two doses.	I
Inactivated WNV	Inactivated using formaldehyde	Neutralizing antibodies after three doses.	I/II
ChimeriVax-WN02	Recombinant yellow fever vaccine strain expressing the prM/E-fragment of WNV	Neutralizing antibodies (>90%) in younger and older age groups after one dose	II
rWN/DEN4Δ30	Recombinant attenuated DENV expressing the prM/E-fragment of WNV	Neutralizing antibodies in 89% of individuals after two doses.	I
HBV-002	Recombinant truncated E-protein	Neutralizing antibodies in all individuals after three doses	I
VRC WNV	DNA plasmid expressing the prM/E fragment	Neutralizing antibodies (>90%) in younger and older age groups after three doses	I

2.2.Diagnosis:

WNV infection laboratory diagnosis is not an easy to be made because of certain issues that will be faced while dealing with WNV infected cases. Among these issues arelow viral load, short viremia, cross-reactivity with other flavivirus members or with their vaccine and the ongoing seasonal viral mutation (David & Abraham, 2016).WNV infection can be diagnosed in laboratory by direct methods and indirect methods. Though both direct and indirect methods are used for diagnosis, The indirect methods which is based on serological tests are the most commonly used tests (ECDC, 2013).

2.2.1.Direct methods:

These methods, include virus isolation, molecular methods and immunohistochemistry

Virus isolation test:

This test is not commonly used for WNV infection detection as it requires laboratory with level 3 biosafety and it is time consuming because it takes about 5-7 days to see the cytopathic effect (CPE) (ECDC, 2018b; Vittorio Sambri et al., 2013). In order observe the CPE, it may requires passage into many (more than one) susceptible cell culture which can be a mammalian derived or mosquito derived cell line, such as pig kidney cells, Rabbit kidney, African green monkey, or C6/36 (Chowers, 2010; David & Abraham, 2016).

Molecular methods:

These methods are based on the detection of West Nile virus nucleic acid (RNA). Many samples can be used to detect WN viral RNA, such as urine, serum, cerebrospinal fluid, plasma and whole blood. Among these samples, the whole blood considered to be the best because of its high sensitivity (Chowers, 2010). Usually West Nile virus RNA genome can be detected in plasma and blood, 2-18 days post infection and until 5 days after the onset of the symptoms, however in rare cases the detection period may extend to 35 days after the appearance of symptoms (ECDC, 2018b; Vittorio Sambri et al., 2013). Moreover, viremia can be detected in asymptomatic WNV-infected people and also in infected symptomatic people before the appearance of the symptoms(Villanueva, 2012).Unfortunately, due to low and short-lived viremia, the usage of molecular methods is limited to be used only for: 1)- Screening of blood and organs for donations and transplantations in areas where WNV is endemic. 2)-Testing samples obtained from patients presenting with suggestive symptoms of WNV infection (Vittorio Sambri et al., 2013). Furthermore, to be able to detect such low level of viremia in human cases, molecular methods based on amplification of the viral nucleic acid such as Polymerase Chain Reaction (PCR) can be used (Campbell et al., 2002). Different types of PCR have been used in diagnosis of WNV, including Real-Time PCR (rt PCR), Reverse Transcriptase (RT-PCR), Nested RT-PCR, Real-Time RT-PCR and others (Chowers, 2010; Vittorio Sambri et al., 2013).

Immunohistochemistry:

This method is mainly used to detect WNV antigen present in brain tissues of WNV fatal encephalitis cases. It is noteworthy that, this method is not used in diagnostic laboratory routine tests for WNV diagnosis because the result of this test depends on the amount of the virus present in the sampled tissue and the quality of the sampled tissue , thus the test consider to has low sensitivity results (Campbell et al., 2002; Vittorio Sambri et al., 2013).

2.2.2. Indirect Methods:

These methods are based on the detection of virus-specific Immunoglobulin M (IgM) and virus-specific Immunoglobulin G (IgG) or the detection of the interaction between the virus and the antibody by serological tests, such as Enzyme-linked immunosorbent assays (ELISA), Immunofluroscence assay (IFA), Neutralization test (plaque reduction neutralization test; PRNT) and Haemagglutination-inhibition assay (HIA) (ECDE, 2013; Organisation, 2007; Vittorio Sambri et al., 2013).

ELISA (Anti-West Nile Virus ELISA IgM, IgG, IgG avidity):

The Enzyme-linked immunosorbent assay is one of the common commercial serological kit that is used to detect IgM and IgG antibodies against WNV. ELISA usually is used for screening because of its quick results, high sensitivity and affordable cost (Sanchini et al., 2013). Hence it is used to detect the presence of both IgM and IgG antibodies which will help to differentiate acute recent infection with IgM-positive ELISA from an old WNV infection with positive-IgG ELISA. The raise of IgM antibody levels in the serum usually occurs within 3 to 8 days after the beginning of the symptoms, while IgG antibodies raises on the 8th day after the appearance of WNV symptoms and continue to be present for several years; therefore its presence indicates past WNV infection (ECDC, 2018b). Additionally, it was found that IgM may persist in the serum and continue to be present for prolonged period up to 500 days or even several years(Papa et al., 2015). As a result of this prolonged presence of WNV-IgM, recent WNV infection diagnosis cannot be made only by the detecting specific WNV-IgM antibodies in the serum. However, the presence of IgM antibodies in cerebrospinal fluid (CSF) is confirmatory for WNV infection of the CNS because normally IgM antibodies cannot cross blood brain barrier (BBB) (ECDC, 2018b; Levett et al., 2005; Petersen et al., 2013).

Commonly to overcome the IgM prolonged persistent issue, IgG avidity test is used and considered more reliable to distinguish recent WNV infection from an old WNV infection (David & Abraham, 2016). On the other hand, IgG Avidity test is usually done to test the strength of the binding between the antigen and antibody in another way we are testing the specificity of this binding (Levett et al., 2005). If the antigen-antibody binding is strong and is not cleaved by urea or any other inhibitor, it means the antibody specificity is high and represents an old infection. While low avidity represents the weak non-specific binding and acute recent infection (Levett et al., 2005).

Indeed, ELISA (IgM, IgG, IgG avidity) is the gold standard laboratory test used for screening and detection of WNV infected cases, however because of its low specificity it may cross-react with other flaviviruses antigens due to recent infection with any other member of flavivirus or recent vaccination with any of flavivirus vaccines, thus gives false positive results. In order to exclude false positive cases due to cross-reactivity, neutralisation test and other tests are required for confirmation (ECDC, 2018b; Petersen et al., 2013).

Immunofluroscence assay (IFA):

This test is used to detect WNV specific IgM and IgG antibodies in serum or CSF (ECDC, 2013)**.**IFA test is rapid, easy and no need for a specific biosafety level laboratory; it can be done in normal laboratory (David & Abraham, 2016). On the other hand this test has some disadvantages, such ascross-reactivity, needs specific microscope and short life of the used solutions(David & Abraham, 2016)**.** To exclude cross-reacting samples and confirm diagnosis of IFA test, PRNT test is highly recommended to be done (ECDC, 2013)

Plaque reduction neutralization test:

This test is used to confirm WNV infection in people with positive ELISA or IIFA tests' results and it is considered to be the gold standard serological test for flavivirus infection diagnosis (ECDC, 2018b; Hobson-Peters, 2012). The advantage of PRNT test over other WNV serological tests is that it excludes cross-reactivity

with other flavivirus and has high specificity. However such test takes time to be performed and needs level 3 biosafety laboratory as well as experienced lab workers to ensure safety while dealing with live virus (ECDC, 2018b; Sanchini et al., 2013; Roehrig, J. T et al., 2008)**.** End point PRNT50 assay is suggested to detect infected cases with sub clinical symptoms and has low titer antibodies, while end point PRNT90 is used to detect symptomatic cases with clinical evident viremia (David & Abraham, 2016; Vittorio Sambri et al., 2013).

Haemagglutination-inhibition assay (HIA):

The HIA test is used to detect and quantify antibodies against WNV within the sera of the suspected infected person. Since the cross-reactivity with other flavivirus members is high and since fresh RBCs are needed to achieve good results, this test is not commonly used anymore (Vittorio Sambri et al., 2013; Southam & Greene, 1958).

2.3. Blood Transfusion and West Nile Virus Transmission:

Blood transfusion is considered to be one of the rare and most important route of West Nile virus transmission in humans. It is considered to be an important route because of the fatal consequences of the infection in people who will receive an infected blood with WNV and eventually may lead to their death.

Many pathogens can be transmitted to human through blood transfusion and may lead to serious infections. So in order to protect blood recipients, many blood screening programs had been developed by health organisations and health authorities (Safety, 2012; WHO, 2010). Those blood screening programs are aimed to detect within the donors' bloods the presence of transfusion-transmissible infections (TTI); microorganisms that can be transmitted through blood components and may lead to high morbidity or mortality in those who receives the infected blood. For a microorganism to be able to cause transfusion-transmissible infection, it should possess some of the following characteristics: **a)-** able to remain stable in blood, when it is stored at 4 ℃ or less than. **b)**- has ability to stay for longer duration in the

blood. **c)-** has Prolonged incubation period. **d)**-has asymptomatic phase or mild symptoms that may not be noticed during blood donation process (WHO, 2010). World Health Organization stated clearly in their recommendations to establish efficient blood screening program, that each country should have their own screening program to ensure the safety of their blood banks and this program should constitute of: a)-mandatory screening for certain infections, such as HIV (1 and 2), Hepatitis (B and C) and syphilis; b)- screening for certain infections depending on the epidemiology of these infection within an area (e.g. *Plasmodium*, Human T-lymophotropic Virus, *Trypanosoma Cruzi*, etc). Screening of donated blood for TTI diagnosis, is usually made based on, the detection of antibodies against the focused on pathogen; detection of the pathogen's antigen or their genomic material (e.g. deoxyribonucleic acid or ribonucleic acid) (U.S. Food & Drug Administration, 2018; WHO, 2010).

Turkish Republic of Northern Cyprus is ruled by Turkey; hence, TRNC follow the screening blood program of Turkey. All donated blood in Turkey are screened for the presence of human immune deficiency virus (1 and 2), Hepatitis virus (B and C) and syphilis. Besides these, they also used to screen all donated blood for the presence of Malaria. However, Malaria screening was ceased in 1998 ("WHO/Europe | Blood safety - Country profile: Blood services in Turkey," 2008). The Turkish blood screening program has passed through many important milestones until today, to improve the safety of blood transfusion among the Turkish population. Prior to 1970s, the only TTI was screened for among blood donors in Turkey, was syphilis. However, in 1983, screening for Hepatitis B by searching for hepatitis surface antigens (HBsAg) using an enzyme immunoassay was added to Turkish screening program. In addition, screening for HIV was added in 1985 and hepatitis C in 1996 (Kocak et al., 2004).

The Turkish Ministry of Health in 1997 obligated all blood collection agencies including blood banks and Turkish Red Crescent (TRC) to not only screen for the presence of Hepatitis B antigen (HBsAg); antibodies against HIV (1 and 2); antibodies against HCV and antibodies against *Treponema pallidum* (syphilis), but also donor evaluation questionnaires should be used for all blood donors to ensure better safety. In addition to screening for the presence of TTI among donated blood,

each blood sample should be tested for blood typing (ABO and Rh) and for legal purpose all samples should be archived up to one year (Kocak et al., 2004;WHO, 2020).

The CDC in the year 2002 received the first notification about WNV infection transmitted through blood transfusion. This notification and other notifications regarding blood transfusion and organ transplantation associated WNV infections were noted after the advice and the alert made by FDA and CDC regarding the safety of blood banks and donated blood against WNV infection (Pealer et al., 2003).

Furthermore ,in 2003, blood collection agencies including blood banks were asked by CDC and FDA to use nucleic acid amplification test (NAT) as screening test to screen for WNV presence in donated blood (CDC, 2004)and since then many blood transfusion safety protocols like EU blood safety recommendations were established to ensure the safety of the donated blood against WNV. Moreover, many countries including North Cyprus started to question their blood bank safety and whether their community is safe against WNV infection.

3. MATERIALS ANDMETHODS

3.1. Ethical Approval:

Acceptance and approval by Ethical Committee of Near East University (24.02.2017) and Ministry of Health was granted before starting collecting and processing the samples.

3.2. Sampling:

Seven hundred sixty sera samples were randomly collected from blood donors at the main Government Blood Bank in Nicosia from October 2017 till March 2018. Collected samples were briefly centrifuged at 2500 rpm for 15 min, then sera were collected and stored in cryotubes at -80°C freezer.

3.3. Laboratory Tests:

All sera samples collected from blood bank donors were tested by using Anti-west Nile Virus ELISA IgG and IgM kits (Euroimmun,Germany); then positive-IgG samples were tested using adapted ELISA IgG avidity test. Besides that, PRNT test was performed to confirm the positive results of the previous serological tests.

3.3.1. Anti-West Nile Virus ELISA IgG, IgM:

All sera were screened for the presence of IgM and IgG antibodies against West Nile virus among the blood bank donors. Euroimmun semiquantitative analysis pipetting protocol and manual test performance and instructions were followed.

- Calculations and interpretation of the Anti-west Nile Virus ELISA (IgG and IgM): Semiquantitative analysis test results for both IgG and IgM were evaluated by calculating the ratio.

Ratio= Extinction of the patient's sample or control/ Extinction of calibrator 2.
If the ratio:

- Was less than 0.8, it means the sample is negative.
- Was equal to 0.8 and less than 1.1, it means the sample is a borderline.
- Was equal or more than 1.1, it means the sample is positive.

3.3.2. Adapted ELISA IgG avidity test:

The samples that showed positive Anti-WNV ELISA IgG were tested using adapted ELISA avidity test, where Euroimmun Anti-west Nile Virus ELISA IgG were adapted by treating it with 7M Urea for 10 minutes as suggested by other researchers (Levett et al., 2005).

-**Adapted ELISA IgG avidity Test (Used Protocol):**

All positive-IgG sera were diluted with normal buffer (1: 100) and mixed well, besides the pipetting protocol of Anti-West Nile Virus ELISA IgG kit was followed with minor modification.

The test was started by pipetting 100µl of the calibrators (C1, C2, C3), positive control, negative control and normal buffer (blank) into separate wells of the IgG WNV ELISA kit plate (96 well plate). Then about 100 µl of each diluted serum was piped twice into two separate wells as shown in the experiment plate planner (Table 4). After completing pipetting all diluted samples into the plate, the plate was incubated for 60 minutes at 37℃. After incubation, normal buffer was added to one of the duplicated wells for 10 minutes, meanwhile 7M urea buffer was added to the other wells (avidity test wells side of the plate) for the same duration. Once the 10 minutes are over, the wells were emptied and washed 2 times with 300µl normal buffer for 30-60 seconds. Next, 100µl conjugate was added to all wells and incubate for 30 minutes at Room temperature. Again,the wells were emptied and washed 3 times with 300µl normal washing buffer for 30-60 seconds. Then 100µl of Substrate was added to all wells and the plate kept protected from light for 15 minutes at room temperature. At the end the reaction was stopped by adding 100µl of Stop solution to all wells. Once the reaction is stopped, the plate was scanned and read immediately with wave length 450-650 nm.

- Calculations and interpretation of the Adapted ELISA IgG avidity test:

The relative avidity index (RAI) was calculated as percentage as the following:

OD of the well washed with 7M urea / OD of the corresponding well washed with normal control buffer) X 100.

- The samples with RAI less than 40% are considered to have low avidity and it means recent infection.
- The samples with RAI more than 60% were considered to have high avidity and it means old infection, meanwhile the samples with RAI in-between 40% to 60 % are considered to be in the grey zone and were considered equivocal.

Table 4: Thesis experiment plate plannerfor adaptedavidity ELISA technique.

Adapted Avidity test (Positive IgG pt/well)

Date: 15/2/2019 Experiment/Plate #: Adapted IgG avidity test

C1	61	315	453	613				61	315	453	613
C2	127	320	466	614				127	320	466	614
C3	191	325	480	635				191	325	480	635
POS	201	341	542	707				201	341	542	707
NEG	216	346	543	732				216	346	543	732
BL	274	367	565	733				274	367	565	733
	295	386	593	753				295	386	593	753
	303	411	611	------				303	411	611	-----

normal ELISA avidity wells

3.3.3 Plaque neutralization test: all seropositive ELISA samples were furthertested using PRNT to exclude cross-reacting samples (false positive ELISA) and for confirmation of all ELISA positive samples n=33 (both IgM and IgG positive samples) were tested using PRNT 90. The strain NY99-4132 (WNV) and Vero cell cultures was used in PRNT test that we performed and followed the test procedure which was described previously (Ergunay., 2014).

Test procedure:

Each collected serum was diluted (1:10) then inactivated by heat (56°C) for half an hour. Then for production of 100 PFU (plaque forming units) of the virus / 0.2ml, a mixture of an equal amount of both diluted inactivated serum and Dulbecco's modified Eagle's medium which contain 5% fetal calf serum was prepared and incubated for an hour at 37°C. In a plate (24-well) with an overlay media which consist of 3.2%carboxymethyl cellulose and 2X Dulbecco's modified Eagle's medium, around 0.2mL of the prepared mixture (virus-antibodies) was inoculated on to a monolayer of Vero cell culture. This is followed by incubation at 37°C for four days; On the fourth day, the cells were fixed with (30%) formaldehyde, then Crystal violet stain was used for staining. The sera which scored 90% neutralization was considered positive.

-Calculations and interpretation of PRNT

For test evaluation we count formed plaques in each well and we calculate the mean plaque value of each serum dilutions, then we calculate the inhibition percentage based on the following formula:

100- plaques (n) / number of plaques given x 100

4. FINDINGS:

Seven hundred sixty sera samples were collected from the main Government Blood Bank in Nicosia and screened for the presence of both IgM and IgG antibodies using anti-WNV ELISA kits.The results of anti-WNV ELISA were; 2 donors (0.3 %) were IgM-positive while 758 donors (99.7%) were IgM-negative. IgG antibodies were positive only in the sera of 31 (4%) donors, none of the sera were positive for both IgM and IgG (Table 5).

Positive IgG samples (n=31) were further tested by ELISA avidity test using previously described technique(Levett et al., 2005).The avidity test revealed the following: 21 (67.7%) had high avidity as they showed RAI more than 60%; 1 (3.2%) had low avidity because their RAI was less than 40%; 6 (19.4%) showed borderline values (equivocal values) as their RAI was between 40%-60%;and 3 (9.7%) were not applicable (Table 6).

Additionally, all seropositive ELISA samples (n=33) were tested by PRNT for confirmation. Among these samplesonly 26 (78.8%) sera samples showed positivity for both PRNT andanti-WNV ELISA (IgG) tests. Whilst all positive-IgM sera, tested negative for PRNT test (Table7).

Table 5: WNV ELISA IgG, IgM results of 760 blood donors' samples collected from the main Government Blood Bank in Nicosia,TRNC, October 2017-March 2018.

Result	**WNV ELISA**	
	IgM n (%)	**IgG n (%)**
Positive	2 (0.3)	31 (4)
Negative	758 (99.7)	729 (95.9)
Total	760 (100)	760 (100)

Table 6:ELISA IgG avidity (adapted) test results of the blood donors that showed positive IgG results (n=31).

IgG Avidity	RAI	n	Percentage
Low	less than 40%	1	3.2%
Borderline	between 40-60%	6	19.4%
High	more than 60%	21	67.7%
NA*	-	3	9.7 %
Total	-	31	100%

*NA: Not applicable. These samples were found IgG positive by WNV ELISA IgG test then became negative when tested by adapted ELISA IgG avidity test.

Table 7: WNV PRNT results of seropositive (IgM and IgG) serum samples (n=33).

Result		WNV ELISA n (%)		
		IgM Positive	IgG Positive	Total
PRNT n (%)	**Positive**	0 (0.0%)	26 (83.9%)	26 (78.8%)
	Negative	2 (100%)	5 (16.1%)	7 (21.2%)
	Total	2 (100%)	31 (100%)	33 (100%)

Table 8: Comparison of PRNT and ELISA IgG avidity testresults of IgG positive serum samples (n=31).

Sample no	PRNT	ELISA IgG avidity			ELISA IgG	
		RAI %	Untreated OD	Urea treated OD	Ratio	OD
753	Positive	94	0.990	0.935	2.058	1.696
565	Positive	93	1.035	0.967	2.333	1.923
386	Positive	91	1.285	1.170	1.349	1.09
732	Positive	88	1.565	1.374	2.080	1.714
635	Positive	85	1.595	1.356	2.941	2.424
346	Positive	85	1.711	1.449	2.710	2.19
480	Positive	84	1.619	1.361	2.318	1.873
61	Positive	82	1.845	1.520	3.086	1.861
295	Positive	82	1.649	1.353	2.145	1.294
216	Positive	81	1.933	1.562	2.540	1.532
542	Positive	81	1.742	1.419	2.883	2.376
367	Positive	78	1.086	0.847	2.000	1.616
127	Positive	77	1.952	1.506	3.044	1.836
411	Positive	75	1.041	0.785	1.365	1.103
274	Positive	75	1.977	1.492	2.686	1.620
303	Positive	72	1.556	1.125	2.386	1.439
325	Positive	72	1.485	1.066	2.368	1.914
191	Positive	71	1.143	0.806	2.504	1.510
320	Positive	71	1.675	1.196	2.471	1.997
201	Positive	64	1.364	0.871	1.933	1.166
315	Positive	63	1.874	1.186	2.724	2.201
466	Positive	57	1.223	0.696	1.555	1.257
341	Positive	56	0.719	0.402	1.585	1.281
453	Positive	56	1.287	0.723	1.518	1.227
614	Positive	53	0.722	0.382	1.324	1.091
613	Positive	50	1.111	0.552	1.223	1.008
611	Negative	59	0.658	0.391	1.804	1.487
543	Negative	20	0.794	0.157	2.705	2.229
593	Negative	NA	0.478	0.230	1.471	0.824
707	Negative	NA	0.501	0.210	1.378	0.772
733	Negative	NA	0.037	0.009	1.828	1.507

OD: Optic density, OD values were calculated as the arithmetic mean of three repeats.

ELISA IgG avidity

Untreated: The wells that were not treated with urea; Urea treated: The wells that were treated and incubated with urea.

For all samples C1= 1.998, C2= 0.505, C3= 0.063, PO= 1.252, NE= 0.092, BL=0.000

RAI: Relative index. Calculated as (OD of the well washed with 7M urea / OD of the corresponding well washed with normal control buffer) X 100. RAI < 40 =low avidity (shown as red), > 40 to < 60 =borderline (shown as grey), > 60 = high avidity.

NA: Not applicable. These samples were found to be IgG positive by WNV ELISA IgG test, but then became negative when evaluated by the adapted ELISA IgG avidity test (shown as yellow

ELISA IgG

Ratio for ELISA: Extinction of the samples/Extinction of the calibrator 2. Interpreting results as follows: Ratio < 0.8 =negative, > 0.8 to < 1.1 =borderline, > 1.1 = positive

- For samples 61, 127, 191, 201, 216, 274, 295, 303: C1=2.153, C2=0.603, C3=0.065, PO=1.361, NE=0.100, BL=0.000
- For samples 315, 320, 325, 341, 346, 367, 386, 411, 453, 466, 480: C1= 2.344, C2= 0.808, C3= 0.093, PO= 1.467, NE= 0.120, BL=0.000
- For samples: 542, 565, 613, 614, 635, 732, 753, 543, 611, 733: C1= 2.165, C2= 0.824, C3= 0.095, PO= 1.684, NE= 0.124, BL=0.000
- For samples: 593, 707: C1= 1.866, C2= 0.560, C3= 0.065, PO= 1.291, NE= 0.109, BL=0.000

Table 9:WNV ELISA-IgG, PRNT, rRT-PCR tests' results of the ELISA-IgM-positive serum samples (n=2)

IgM-Positive sera sample No	PRNT	ELISA						
		IgG	Details of IgM					
			Samples OD	Samples Ratio	Positive Control	Negative Control	Calibrator	Blank
9	Negative	Negative	0.648	2.07	0.895	0.021	0.313	0.003
351	Negative	Negative	0.974	2.26	1.095	0.031	0.431	0.001

5. DISCUSSION:

WNV, is a single stranded RNA neurotropic Flavivirus, which under certain conditions can progress causing neurological disease and may lead to death (Vittorio Sambri et al., 2013; Sanchini et al., 2013). Humans are considered to be dead-end host as they do not play role in continuation of the natural cycle of the virus of the virus. Though the main route of the transmission of the virus to human is through mosquitoes' bites, the virus can also be transmitted to human through other routes, such as blood transfusion, organ transplantation, accidentally during laboratory work, and from an infected mother to her fetus through breast milk or vertically during pregnancy (Colpitts et al., 2012; ECDC, 2018b).

WNV is an arbovirus which causes vector-borne disease (VBD) and interestingly the Mediterranean area including Turkey and TRNC is a hot spot for emerging of VBD (Negev et al., 2015; Paz, 2015). The Mediterranean zone is a transitional zone that constantly liable to changes in the climate because of its location between the Atlantic Ocean and Asia. In addition to that, it lies between different climates zone including the rainy climate of Europe (central) and the arid climate of North Africa (Gualdi et al., 2013).Over the past years the Mediterranean countries' climates have changed; their climates became more warm with a change in the patterns of rainfalls as a result of increasing heat waves in this area and decreasing in precipitation rate. Moreover, heat waves became more intensified, more frequent and with longer duration (Negev et al., 2015).

Meteorological (climate related) factors, such as humidity, temperature, wind and rainfall pattern has influenced insects (vectors). This in turn affects the epidemiology of VBD which explains how the climatic changes that took place in Mediterranean countries led to emerging of VBD and increased their outbreaks (IPCC, 2013; Negev et al., 2015).

Many researches (Negev et al., 2015; Zachariadis, 2012) have discussed and proved the impact of climate changes on the mosquito vectors and how this will

influence the epidemiology and the ecology of the VBD like WNV, Dengue virus and Malaria. One of the most important climatic factors is temperature. Researchers have found that increased in temperature will lead to an increase in mosquito's multiplication rate, hence increasing their density within the area; change in the feeding behaviour of the mosquitoes, therefore, increasing in the frequency of blood meals; increase in the virus expansion rate as a result of shortening of the infectivity period of the mosquito. In addition to temperature, humidity precipitation was found to be another important climactic factor as high humidity increases the density of the mosquitoes, thus the out breaks of WNV and other VBD increases (Kilpatrick., 2008; Landesman., 2007; Reisen., 2006). However, studies found that the effect of rainfall on the vector depends on their species. For example, increasing in rainfall leads to an increase in the stagnant water within the area, thus increasing development and growth of the larval form of some mosquito species. Meanwhile, decreased rainfall and drought may increase density of some mosquito species and frequent outbreaks as a result of affected aquatic food web interactions (Chase & Knight, 2003; Chevalier., 2013).

As we mentioned earlier the Mediterranean zone climates was subjected to many climatic changes. Therefore, the climate in this area became warmer because of the intensified hot waves with long hot summer periods and reduced rainfall which resulted in increasing VBD within the Mediterranean countries (Negev et al., 2015). As a matter of fact, the climatic changes are not the only reason that VBD has increased in the Mediterranean countries, other reasons also have contributed to this increase, For instance, an increase in the Mediterranean population density, Social outdoor activities and habits of the Mediterranean people (e.g. outdoor restaurants, weddings, etc.) can also be influential(Reiter, 2001).

Cyprus is considered to be one of the largest islands within the Mediterranean Sea, in fact it is the third in size after two Italian islands (Sardinia and Sicily), which makes it a hot spot for climatic changes. For instance, the weather in Cyprus became hotter with reduced rainfalls and frequent droughts, which makes it more prone to emerging of VBD such as WNV(Kassinis & Mammides, 2016; Zachariadis, 2012). In addition, Cyprus is an important stop off point of many migratory birds (amplifying host of WNV) and considered to be a major Mediterranean route for bird

migration that connect the Middle East with Europe and Africa (Kassinis & Mammides, 2016).

In TRNC, during 2011 to 2013, the presence of WNV vector (*Cx. Pipiens s.l*) in the island was proved by a study which investigated the presence of WNV vector in fifteen Turkish provinces including TRNC (Koray Ergunay et al., 2014). This together with other factors, such as climate changes, emerging of VBD, changes in the epidemiology of the vector and the presence of the amplifying host within the island, suggest the presence of WNV in the island and makes it an ideal place for harboring the vector of WNV. Nevertheless up until today there is no scientific evidence and proper epidemiologic study that approves the existence of WNV in TRNC, except the recent newspapers' reports (*First report of West Nile virus infection - Cyprus Mail*,2019).

As a consequence of the geographical expansion of West Nile Virus and the increasing epidemics of it among humans, the virus became the focus of attention and many studies were carried out to document the presence of the virus among population. For instance, in many Mediterranean countries, the presence of antibodies against WNV was detected and reported as follows: Egypt(1-61%),Libya (2.3%), Tunisia (4.3-31.1%), Morocco (0-18.8%).(Eybpoosh et al., 2019).

The expansion of WNV infection has pushed CDC and FDA to recommended the necessity of screening of all donated blood for the presence of WNV, hence USA in 2002 was able to detect WNV for the first time among their blood donors. Since then, many countries, such as India, UAE, Nebraska and many others had documented WNV seropositivity among their blood donors (David & Abraham, 2016; Pealer et al., 2003). In Turkey many studies were conducted to document WNV among the Turkish population or to determine the seroprevalence of WNV infection among blood donors. For instance, a study was carried out in central Anatolia, Turkey in 2009 showed that the seroprevalence of anti-WNV IgG was 1.6% among 1200 blood donors. Meanwhile, another study that was undertaken in Izmir in 2015 showed 2.5% WNV IgG seropositivity among 438 blood donors (Biçeroğlu et al., 2015). As shown by a Greek study done in 2013, WNV seropositivity was about 2.1%among their population and in addition, this study

highlighted that WNV seropositivity may differ among different geographical area even within the same country (Hadjichristodoulou et al., 2015).Comparing the seropositivity of these different areas or countries shows divergencyin their seropositivity prevalence among their population. These disagreementcan be related to different fundamentals, for example species of the vector, the density of these vectors and the population of humans in the understudy geographic area (Schweitzer et al., 2006).

WNV can be detected by direct and indirect methods. However, the indirect methods specifically the serological tests are the golden standard and most commonly used (ECDE, 2013). For this reason, in this studyall serum samples which were collected from blood donors were screened by anti-WNV ELISA (both IgM and IgG) kits to detect and discriminate recent infections from past infections with WNV (CDC, 2018a; ECDC, 2018b). Additionally, all IgG positive (n=31) samples were subjected to ELISA IgG avidity test. The reason behind this is to be able to distinguish recent acute WNV infection from past infection with persistent IgM antibodies; as in some cases IgM antibodies can be unreliable due to the fact that IgM may persist in serum for years and give false positive results (Levett et al., 2005; Lustig et al., 2018; Pisani et al., 2016).

This study revealed that among all tested 760 sera, 2 (0.3%) of these samples were IgM-positive and IgG-negative; therefore, these donors was concluded to have recent WNV infection. While 31(4%) of tested blood donors showed positive IgG results with negative IgM results, suggesting past WNV infection. To distinguish recently infected samples from old infection, ELISA IgG avidity test was performed on the IgG-positive samples (n=31) and according to RAI which was calculated as percentage by using this formula (optic density of the wells containing urea divided by the optic density of the normal buffer washed corresponding well) multiply by 100, samples of this study were categorized as high avidity which represent an old infection and low IgG avidity which represent recent infection. Among all 31 IgG-positive samples; 21(67.7%) of them displayed high avidity, which means that those donors had past WNV infection and they are safe to donate their blood; One (3.2%) sample showed low avidity result and this blood donor considered recently infected. Such blood donors according to CDC guidelines cannot donate their blood

immediately instead a period of 120 days should be waited before blood donation(CDC, 2018b) ; Six(19.4%) samples had borderline avidity and needs to be investigated more. Interestingly when ELISA IgG avidity test was preformed, three (9.7%) samples displayed negative results, hence considered not applicable (NA). Those samples though they gave positive results when they were tested by normal anti-West Nile Virus ELISA IgG kit, they gave negative results when they were tested by adapted ELISA IgG avidity test. The reason behind these conflicting results could be the extra 10 minutes incubation of the wells containing diluted patients' sera with washing buffer while preforming the adapted technique of ELISA IgG avidity test. It is possible that the extra 10 minutes incubation made the cross-reacting antibody to dislodge from the antigen causing negative result which means that those not applicable samples gave false positive IgG ELISA result. Therefore, the adapted technique of the ELISA IgG avidity test likely can help to exclude some of cross reacted cases and give more precise result comparing to the normal IgG avidity test.However, further investigations and comparing both normal ELISA IgG avidity and adapted ELISA IgG avidity tests should be done for further confirmation.

Aside from that, all seropositive sera (both IgM and IgG) were subjected to PRNT test which was negative for all IgM-positive ELISA samples (n=2), which means that those blood donors had false positive IgM results. On the other hand, 26 (83.9%) samples out of all analysed IgG positive serum samples (n=31) showed positive PRNT test and only 5 (16.1%) samples tested negative for PRNT. This drop in the proportion of seropositivity results between PRNT results and ELISAresults of positive IgG samples, could be because of the fact thatthose ELISAseropositive samples were cross-reacting with other *Flavivirus*family members and displayed false ELISAseropositive results, as suggested by Algerian study (Hachid et al., 2019).

Indeed, this study was able to prove the serological evidence of the presence of WNV infection among blood donors. However, there is some major statistical andepidemiological data collection limitations, which is needed to evaluate the extent of the distribution of WNV among population and the percentage of WNV transmission through blood transfusion, hence further epidemiological studies are required.

6. CONCLUSION:

As a conclusion; the presence of WNV antibodies was detected among blood donors in TRNC as revealed by this study. For confirmation purpose, all seropositive samples were tested by PRNT test and positive PRNT reaction was noticed in some seropositive samples, hence the presence of WNV specific antibodies was confirmed. Unfortunately, since there is no approved therapeutic regime for treating WNV in human and no approved human vaccine yet, we highly recommend and stress on the finding ways to eliminate or reduce WNV vectors and prevent WNV infection in the island. In order to succeed, further studies should be conducted in the both sides of the island (Greek and Turkish sides) to collect valuable epidemiological data, including species and density of the vector in the island; causes of higher vector density within certain areas; details of both blood donors and receivers. These data will be useful to develop preventive measures suitable for the island. Additionally, spreading awareness among locals about the personal and public protective measures that can be used to minimize the probability of getting infected, is also recommended. Last but not least, adding WNV to the TTI blood screening tests of TRNC screening program is highly suggested.

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8. ENCLOSURES:

Adapted ELISA avidity test

Preparation of 50ml of 7M urea:

The formula weight of urea that was used is 60.06 g/mol.
Next, you need to figure out how much you would add if you were making 1 L of it. Remember we want our solution to be 7M.
60.06 g/mol x 7 mol/L = 420.42 g/L
Now we need to calculate the amount needed for the correct volume (100 mL rather than 1 L). To do this we can set up our calculation for cross multiplication. Specifically, we know that we need 420.42 grams if we were making 1 L, but we need to know how many grams we need if we are making 50 ML. Mathematically, this can be set up as follows:

$$\frac{420.42\ g}{1\ L} = \frac{X\ g.}{0.05 L}$$ Note:50ml= 0.05 L

We can cross multiple to get (1 L)(X g) = (420.42 g)(0.05 L), which can be re-arranged to:

X g = (420.42 g x 0.05 L) / (1 L)

X = 21.021 grams of urea

Note:-When we are preparing 7M urea we Should dissolve it in washing buffer.

- Add washing buffer to urea slowly not the whole amount until you reach the desired amount of the urea buffer (ml).

Steps of adapted ELISA avidity test:

1)- Dilute patients' sera with normal buffer (1: 100) and mixed well.

2)- Transfer 100 ul of the calibrators, positive control, negative control, and duplicate the diluted patients' sera (each patients' sample /2 wells). Incubate for 60 minutes at 37 ℃.

3)- Add 7M urea (buffer) to avidity test wells (one of the duplicated wells) for 10 minutes. At the same time, we should add normal buffer to the other wells (wells will be tested for normal IgG ELISA)

4)- After incubation for 10 minutes with urea, Empty the wells and wash it 2 times more with normal washing buffer

Note: each washing step in Elisa is composed of 3 times and 300ULx 30-60 seconds.

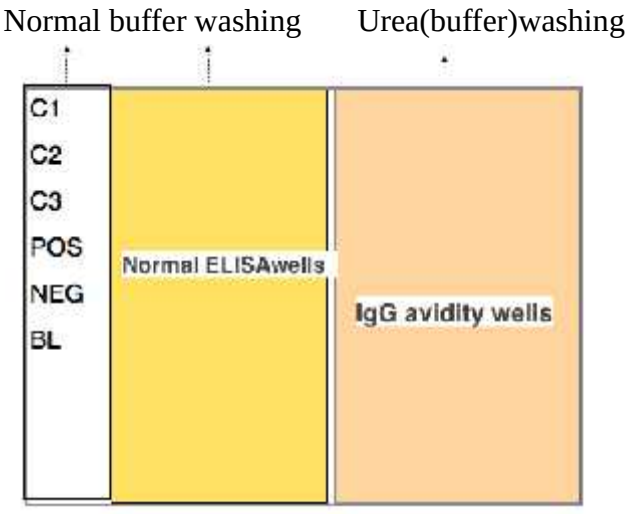
5) Add 100 UL conjugate and incubate for 30 minutes - Room temperature.

6)- Empty and re-wash (normal washing, no urea used) 3 times and 300ULx 30-60 seconds.

7)- Add 100UL Substrate x 15 minutes-room temp- protect from light.

8)- Add 100UL Stop solution.

9)- Read immediately at wave length 450-650 nm. (normal reading / like normal ELISA reading).



Additional charts and Tables

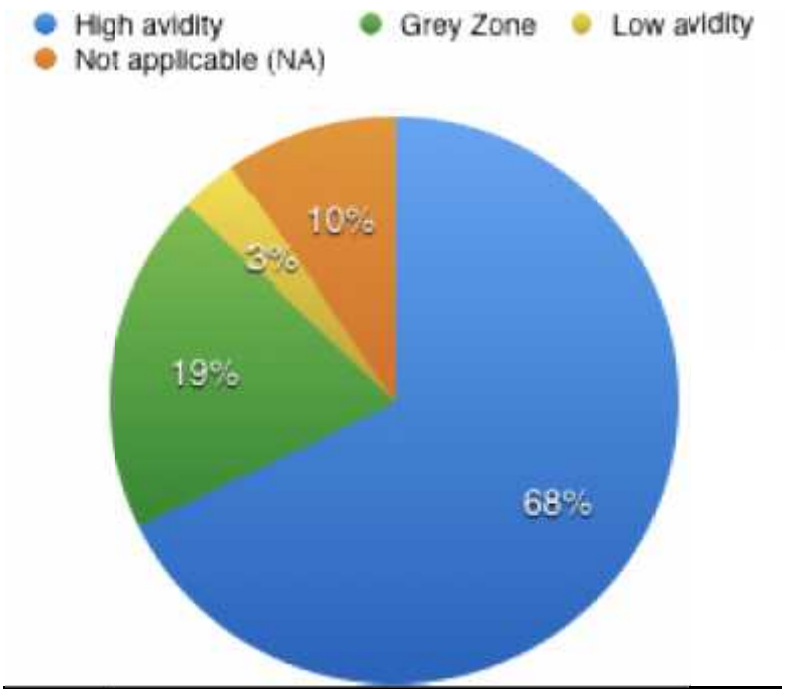


Figure 9: ELISA IgG avidity test results of all IgG-positive donors' samples (n=31).

Table 10: West Nile Virus ELISA IgG avidity test and PRNT tests' results among IgG-positive (n=31) serum samples

	RAI*	**PRNT n (%)**		
		Positive	**Negative**	**Total**
IgG Avidity (RAI) n (%)	**Low avidity (less than 40%)**	-	1	1(3.2)
	Borderline avidity (between 40-60%)	5	1	6 (19.4)
	High avidity (more than 60%)	21	-	21(67.7)
	NA	-	3	3(9.7)
	Total	**26 (83.9)**	**5 (6.1)**	**31(100)**

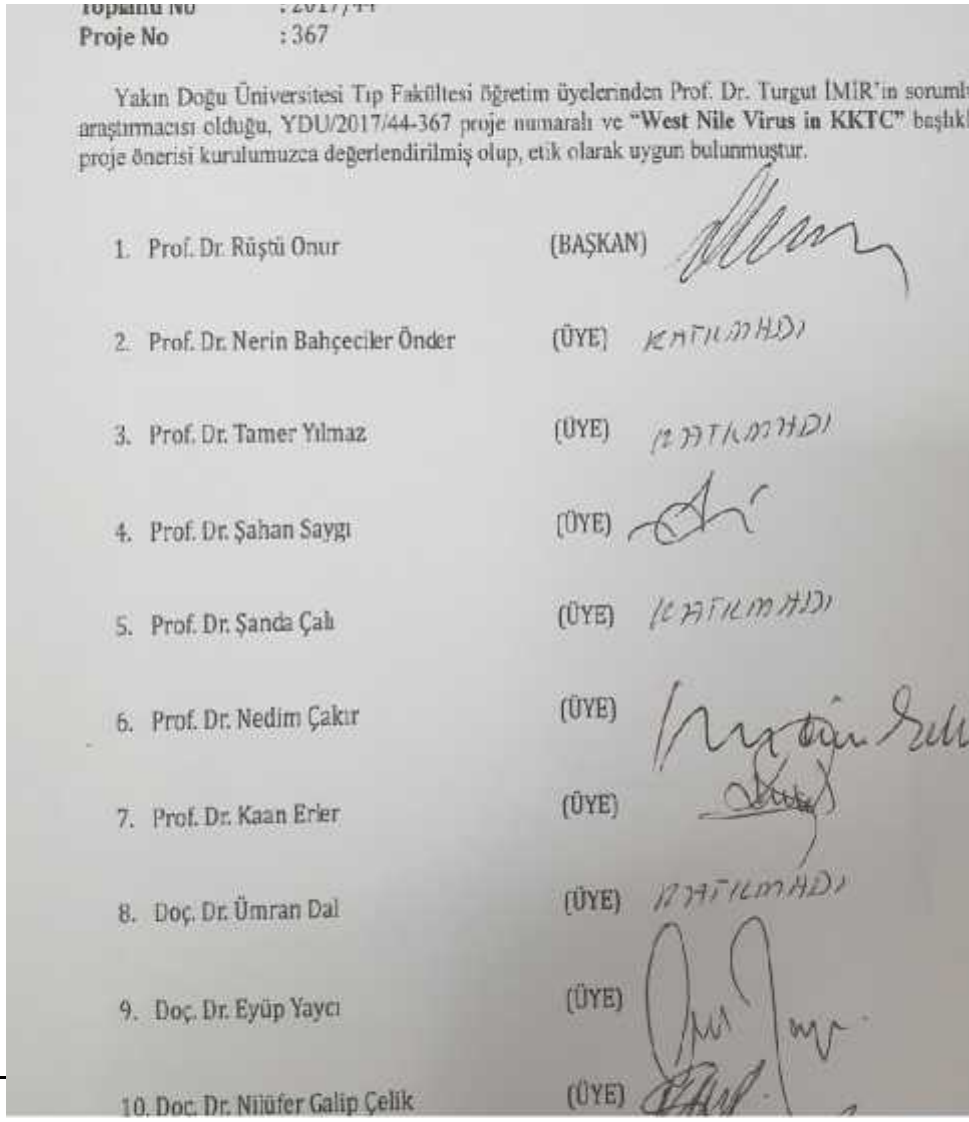
Table 11: Comparing old and new results of Elisa IgG +Avidity+PRNT results

Sample no	Old IgG Elisa test (ratio)	IgG ELISA test. (15/02/19)(ratio)	ELISA Old IgG test OD	IgG ELISA test. (15/02/19) OD	IgG ELISA avidity test. (15.02/19) OD	PRNT	Avidity reading /normal reading	Avidity%	Avidity/Result
61	3.086	3.653	1.861	2A (1.845)	9A (1.520)	Positive	0.82	82.30	High/ Old infection
127	3.044	3.865	1.836	2B (1.952)	9B (1.506)	Positive	0.77	77.10	High/ Old infection
191	2.504	2.263	1.51	2C (1.143)	9C (0.806)	Positive	0.71	70.50	High/ Old infection
201	1.933	2.700	1.166	2D (1.364)	9D (0.871)	Positive	0.64	63.80	High/ Old infection
216	2.540	3.827	1.532	2E (1.933)	9E (1.562)	Positive	0.81	80.80	High/ Old infection
274	2.686	3.914	1.62	2F (1.977)	9F (1.492)	Positive	0.75	75.40	High/ Old infection
295	2.145	3.265	1.294	2G (1.649)	9G (1.353)	Positive	0.82	82.00	High/ Old infection
303	2.386	3.081	1.439	2H (1.556)	9H (1.125)	Positive	0.72	72.30	High/ Old infection
315	2.724	3.710	2.201	3A (1.874)	10A (1.186)	Positive	0.63	63.20	High/ Old infection
320	2.471	3.316	1.997	3B (1.675)	10B (1.196)	Positive	0.71	71.40	High/ Old infection
325	2.368	2.940	1.914	3C (1.485)	10C (1.066)	Positive	0.72	71.70	High/ Old infection
341	1.585	1.423	1.281	3D (0.719)	10D (0.402)	Positive	0.56	55.90	Borderline
346	2.710	3.388	2.19	3E (1.711)	10E (1.449)	Positive	0.85	84.60	High/ Old infection
367	2.000	2.150	1.616	3F (1.086)	10F (0.847)	Positive	0.78	77.90	High/ Old infection
386	1.349	2.544	1.09	3G (1.285)	10G (1.170)	Positive	0.91	91.00	High/ Old infection
411	1.365	2.061	1.103	3H (1.041)	10H (0.785)	Positive	0.75	75.40	High/ Old infection
453	1.518	2.548	1.227	4A (1.287)	11A (0.723)	Positive	0.56	56.10	Borderline
466	1.555	2.421	1.257	4B (1.223)	11B (0.696)	Positive	0.57	56.90	Borderline
480	2.318	3.205	1.873	4C (1.619)	11C (1.361)	Positive	0.84	84.00	High/ Old infection
542	2.883	3.449	2.376	4D (1.742)	11D (1.419)	Positive	0.81	81.40	High/ Old infection
565	2.333	2.049	1.923	4F (1.035)	11F (0.967)	Positive	0.93	93.40	High/ Old infection
613	1.223	2.200	1.008	5A (1.111)	12A (0.552)	Positive	0.50	49.60	Borderline
614	1.324	1.429	1.091	5B (0.722)	12B (0.382)	Positive	0.53	52.90	Borderline
635	2.941	3.158	2.424	5C (1.595)	12C (1.356)	Positive	0.85	85.00	High/ Old infection
732	2.080	3.099	1.714	5E (1.565)	12E (1.374)	Positive	0.88	87.70	High/ Old infection
753	2.058	1.960	1.696	5G (0.990)	12G (0.935)	Positive	0.94	94.40	High/ Old infection
543	2.705	1.572	2.229	4E (0.794)	11E (0.157)	Negative	0.20	20.00	Low/Recent infection
611	1.804	1.302	1.487	4H (0.658)	11H (0.391)	Negative	0.59	59.40	Borderline
593	1.471	0.946	0.824	4G (0.478)	11G (0,230)	Negative	0.48	48.00	NA
707	1.378	0.992	0.772	5D (0.501)	12D (0.210)	Negative	0.42	42.00	NA
733	1.828	0.073	1.507	5F (0.037)	12F (0.009)	Negative	0.24	24.00	NA

Optic density of all calibrators, positive control, negative control of all IgG seropositive samples:

Samples: 61 /127/ 191 /201 /216 /274 /295 /303 (same reading plate) C1=2.153 C2=0.603 C3=0.065 PO=1.361 NE=0.100 BL=0.000
Samples: 315/ 320/ 325/ 341/ 346/ 367/ 386/ 411/ 453/ 466/ 480 (same reading plate) C1= 2.344 C2= 0.808 C3= 0.093 PO= 1.467 NE= 0.120 BL=0.000
Samples: 542/ 565/ 613/ 614/ 635/ 732/ 753/ 543/ 611/ 733 (same reading plate) C1= 2.165 C2= 0.824 C3= 0.095 PO= 1.684 NE= 0.124 BL=0.000
Samples: 593 / 707 (same reading plate): C1= 1.866 C2= 0.560 C3= 0.065 PO= 1.291 NE= 0.109 BL=0.000
For IgG ELISA & Avidity that was done on 15.02.2019. C1= 1.998 C2= 0.505 C3= 0.063 PO= 1.252 NE= 0.092 BL=0.000 All previously positive samples were tested together (same reading plate)

Project documents



Toplantı No : 2017/44
Proje No : 367

Yakın Doğu Üniversitesi Tıp Fakültesi öğretim üyelerinden Prof. Dr. Turgut İMİR'in sorumlu
araştırmacısı olduğu, YDU/2017/44-367 proje numaralı ve **"West Nile Virus in KKTC"** başlıklı
proje önerisi kurulumuzca değerlendirilmiş olup, etik olarak uygun bulunmuştur.

1. Prof. Dr. Rüştü Onur (BAŞKAN)
2. Prof. Dr. Nerin Bahçeciler Önder (ÜYE) KATILMADI
3. Prof. Dr. Tamer Yılmaz (ÜYE) KATILMADI
4. Prof. Dr. Şahan Saygı (ÜYE)
5. Prof. Dr. Şanda Çalı (ÜYE) KATILMADI
6. Prof. Dr. Nedim Çakır (ÜYE)
7. Prof. Dr. Kaan Erler (ÜYE)
8. Doç. Dr. Ümran Dal (ÜYE) KATILMADI
9. Doç. Dr. Eyüp Yaycı (ÜYE)
10. Doç. Dr. Nilüfer Galip Çelik (ÜYE)

Tarih: 21/12/2017

Sayı: 2017/92

Konu: Ara Rapor

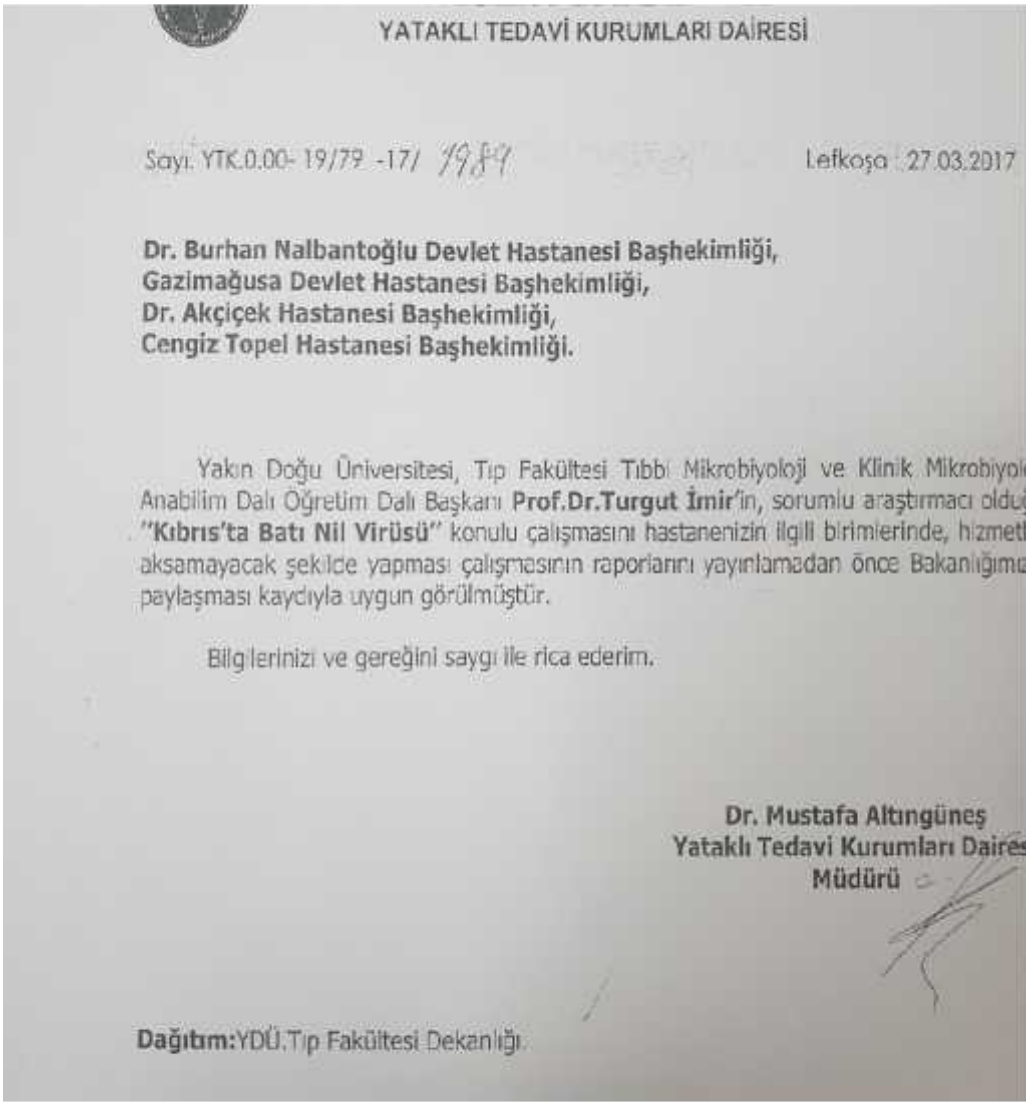
Sayın Prof. Dr. Turgut İmir

Yakın Doğu Üniversitesi Bilimsel Araştırma Projeleri Koordinasyon Birimi tarafından onaylan
FEN-2016-2-006 nolu ve **"West Nile Virus in Cyprus"** başlıklı proje kapsamında 04/12/2017 tarih
1. Ara Rapor'unuz incelenerek kabul edilmiştir.

Bilgilerinize rica ederim.

Prof. Dr. H. Seda Vatansever

BAP Birim Koordinatörü



YATAKLI TEDAVİ KURUMLARI DAİRESİ

Sayı: YTK.0.00- 19/79 -17/ 1984 Lefkoşa 27.03.2017

Dr. Burhan Nalbantoğlu Devlet Hastanesi Başhekimliği,
Gazimağusa Devlet Hastanesi Başhekimliği,
Dr. Akçiçek Hastanesi Başhekimliği,
Cengiz Topel Hastanesi Başhekimliği.

Yakın Doğu Üniversitesi, Tıp Fakültesi Tıbbi Mikrobiyoloji ve Klinik Mikrobiyo
Anabilim Dalı Öğretim Dalı Başkanı **Prof.Dr.Turgut İmir**'in, sorumlu araştırmacı oldu
"Kıbrıs'ta Batı Nil Virüsü" konulu çalışmasını hastanenizin ilgili birimlerinde, hizmet
aksamayacak şekilde yapması çalışmasının raporlarını yayınlamadan önce Bakanlığım
paylaşması kaydıyla uygun görülmüştür.

Bilgilerinizi ve gereğini saygı ile rica ederim.

Dr. Mustafa Altıngüneş
Yataklı Tedavi Kurumları Daire
Müdürü

Dağıtım: YDÜ.Tıp Fakültesi Dekanlığı

9. CURRICULUM VITAE:

Personal Information

Name: Nagat BALAMAN

Nationality: Libyan

Date of Birth: 19/7/1984

Place of Birth: Madrid, Spain

Sex: Female

Contact Details

Address: Cyprus (TRNC) – Lapta

Mob: 05428896239

Email: nsw_29@yahoo.com

Characteristics

I am self-motivated, ambitious and eager to learn. I am a responsible individual with strong communication skills and work ethics besides being creative, focused and highly determined. I am willing to take responsibility and work independently. At the same time, I can work well in teams.
Looking for both personal and professional growth makes me capable of working confidently under pressure.

Education

- Bachelor of Medicine and Bachelor of Surgery (MBBS) September 2008 fromGulf Medical University (GMU), United Arab Emirates.
- Master of Dermatology 2011-2014: Alexandria Medical University, Egypt, I have done three years of the program then changed and moved to Near East University (I did not finish the whole five years duration of the master).
- PhD of Medical Microbiology and Clinical Microbiology started on 2015-2016 and passed my PhD qualification exam on September 2018, besides I am currently working atthe Medical and Clinical Microbiology Department, Near East University.

Achievements

- Certificate of participating, **Free Health Camp held in GMC hospital & Research Center** (Ajman – United Arab Emirates) on 17th Oct 2003.
- Graduation **Project done in Oral Contraceptive pills**, Community Medicine Department, Gulf Medical University (2004-2005).
- Certificate of Participating, **Free Health Camp held in GMC Hospital & Research Center** (Ajman – United Arab Emirates) on 14th January 2005.
- Certificate of appreciation, for my contribution as Student Presenter **at 1st UAE (United Arab Emirates) Medical Student conference** held in Dubai on 14 and 15th March 2006.
- Certificate of attending Continuing Medical Education Program **on Prevention and Management of Common Cardiovascular Diseases in PHC** (Sharjah-UAE) on 10th March 2007.
- Certificate of attending **one-year attachment at Sheikh Khalifa Hospital (Ajman), Qassimi Hospital, & Kuwaiti Hospital in Sharjah(UAE)** between 22ndJanuary 2007 to 29th January 2008.
- Certificate of attending **Dermatology& Andrology Scientific Day** held by Department of Dermatology, Venereology & Andrology, Alexandria Medical University (Egypt) on 15th December 2011.
- Certificate of satisfactory completion of Rotary Club of Alexandria**Scientific Session on Skin Bacterial Infection (**Egypt) on 5th April 2012**.**
- Certificate of participating in the activities of the **8th Annual Conference of Alexandria Society of Dermatology, Venereology & Andrology (Egypt)** on 14-15th March 2013.
- Certificate of participating in the activities of the **5th Alexandria Dermatology & Andrology Scientific Day** held by Department of Dermatology, Venereology & Andrology, Alexandria Medical University (Egypt) on 31st October 2013.

- Certificate of attending **Training Course of Dermatohistopathology** held in the Department of Dermatology, Venereology & Andrology, Alexandria Medical University (Egypt) on 4th-5th March 2014.
- Certificate of attending **Workshop onPeeling, Dermaroller & Dermapen** held during the pre-congress workshops of the 11th Biennial Conference of the Department of Dermatology, Venereology & Andrology (Alexandria Medical University-Egypt)on 10-14th May 2014.
- Certificate of attending **Workshop on Mesotherapy Hair Lipolysis** held during the pre-congress workshops of the 11th Biennial Conference of the Department of Dermatology, Venereology & Andrology (Alexandria Medical University-Egypt)
  On 10-14th May 2014.
- Certificate of attending the **11th Biennial Conference of the Department of Dermatology, Venereology & Andrology** (Alexandria Medical University-Egypt) held on 15-17th of May 2014.
- Certificate of attending the Scientific meeting organized by the Department of Dermatology, Venereology & Andrology (Alexandria Medical University-Egypt) On 30th October 2014.
- Certificate of attending of the Scientific meeting organized by the Department of Dermatology, Venereology & Andrology (Alexandria Medical University-Egypt)
  On 4th of December 2014.
- Certificate of attending thescientific meeting organized by the Department of Dermatology, Venereology & Andrology (Alexandria Medical University-Egypt) On 25th December 2014.
- Certificate of attending the **2nd International Clinical Mycology Master Class: Challenges in Diagnosis and Management of Invasive Fungal Diseases** On 23-25 November 2017,Izmir, Turkey.
- Certificate of attending **theKKTC Yükseköğretim Stratejik Planlama Çalıştayı** by YÖDAK on 6th July 2018.

- Certificate of attending the **9th National & 2nd International Congress of Hydatidology 15-17 November 2018** atNear East University, Nicosia , TRNC.
- Certificate of attending the **1st AIDS Awareness Conference** 2018 at Near East Hospital on December 2018, Nicosia, TRNC.
- Certificate of attending the **Drug Development and Vaccine Production Symposium** on 6th of December 2019 at Near East University, Nicosia, TRNC.
- Certificate of attending the **Mathematical Modeling Course in Health Science**. On 20th of December 2019 at Near East University, Nicosia, TRNC.
- Certificate of attending the '**II. Bioinformatics Winter School: Computer Methods in Molecular Sciences** ' 16-18 February 2020 at Near East University, Nicosia, TRNC.
- Certificate of attending the **Rare Disease Day Symposium** on 28th February 2020 at Near East University Hospital, Nicosia, TRNC.

Publications

- Mustapha, A., Abba Disa H., **Balaman N.** (2017). Anti-bacterial activity of *Salvadora Persica* (chewing stick) on *Streptococcus mutans* isolate from patients attending University of Maiduguri Teaching Hospital Dental Unit. *PIJR*: Vol: 6, Issue: 4 April 2017 doi: 10.15373/22501991.
- **Balaman, N.,** Gazi, U., Imir, T., Sanlidag, T., Ruh, E., Tosun, O., Ozkul, A., Taylan-Ozkan, A. (2020). Serological screening of West Nile virus among blood donors in Northern Cyprus. *Journal of Medical Virology*, jmv.25669. https://doi.org/10.1002/jmv.25669

Work Experience

- Gulf Medical University Hospital (Ajman-UAE) Medical Trainee from Sep 2003 tell Jul 2004.
- Hammad Hospital (Qatar) Medical Trainee (Internal Medicine Department) for 3 weeks summer training on Aug 2004.
- Iranian Hospital (Dubai) Medical Trainee from Sep 2004 tell July 2005.
- Al Mafraq Hospital (Abu Dhabi) Medical Trainee from Sep 2005 tell July 2006.
- Al Qassimi Hospital (Sharjah), Sh. khalifa Hospital (Ajman), Kuwaiti Hospital (Sharjah), and Al-Raffa Primary Health center (Sharjah) Internship on 2007-2008.
- Alexandria Faculty of Medicine, Department of Dermatology,Venereology and Andrology Dermatology Trainee 2011-2014.
- Near East University, Medical Faculty, Department of Medical and Clinical Microbiology, Research Assistant 2016- 2020 (continue).
- Uludag University / Faculty of Medicine, Immunology Department Trainee for 4 weeks July 2017.

Additional Information

- Languages (spoken & written):
  -Arabic: Fluent (Mother tongue).
  -English: Excellent.
- Excellent written communication skills.
- Excellent time management skills.
- Advanced Microsoft Office user.
- All my medical degrees are in English.
- I Scored 7 in IELTS exam (Australia-Sydney) on 2010.
- I received a certificate of enrolling in an intensive English course at Kaplan International College Sydney City from 19th oct 2009 tell 17th December 2010, (Australia).
- Passed Near East University English Proficiency Exam with high score on March 2018.
- Passed Medical and Clinical Microbiology Qualification Exam on September 2018.

References available upon request