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SELECTION OF 2'FY RNA APTAMERS AGAINST CRIMEAN CONGO HAEMORRHAGIC FEVER VIRUS

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

FUNDA ZEKİYE ARDIÇ

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF MASTER OF SCIENCE
IN
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Approval of the thesis:

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submitted by **FUNDA ZEKİYE ARDIÇ** in partial fulfillment of the requirements for the degree of **Master of Science** i**n Molecular Biology and Genetics, Middle East Technical University** by,

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ABSTRACT

SELECTION OF 2'FY RNA APTAMERS AGAINST CRIMEAN CONGO HAEMORRHAGIC FEVER VIRUS

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Crimean-Congo hemorrhagic fever (CCHF) is potentially lethal and mostly infected by ticks. CCHF virus (CCHFV), is a Nairoviridae family member of the Orthonairovirus genus and in general, cases are reported from Asia, Africa, the Middle East, and Eastern Europe. The fatality rate ranges from 30 to 50% worldwide but it is reported as 5% by the Turkish Ministry of Health and it varies between 1.7 and 26.5% for children. ELISA kits used for the detection of infection are time-consuming, expensive, and labor-intensive; and because ELISAs detecting IgG and IgM antibodies are produced against virus, and don't specifically detect virus presence; these techniques may cause some inaccurate diagnosis and also they have no reusability. The diagnosis cost will be a lot higher when considering the cost of extra microbiological tests for infectious diseases (Viral hepatitis, typhoid, leptospirosis and Hantavirus infectious, etc.) that are misdiagnosed with CCHF. As a result, a more effective and faster diagnostic technology is required. Aptamers are tiny nucleic acids which can be selected in vitro and have a high affinity and selectivity for their relevant targets. They can contain ssDNA or RNA backbones, and they fold into their three-dimensional structure naturally. Aptamers can also be

changed to improve their structural stability while maintaining their affinity. Because of these characteristics, they can be used for both therapeutic and diagnostic purposes. CCHFV binding aptamers were successfully selected in this study, and they will be further optimized for CCHF diagnosis.

Keywords: Aptamer, RNA, SELEX, CCHFV, CCHF

ÖZ

KIRIM KONGO KANAMALI ATEŞİ VİRÜSÜNE KARŞI 2'FY RNA APTAMERLERİN SEÇİLİMİ

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Kırım-Kongo kanamalı ateşi (KKKA) potansiyel ölümcül bir hastalıktır ve çoğunlukla kene teması ile bulaşan bir hastalıktır. Etken KKKA virüsü (KKKAV), Nairoviridae ailesinin Orthonairovirus genusuna mensuptur ve vakalar genellikle Asya, Afrika, Doğu Avrupa ve Orta Doğu'dan bildirilmektedir. Dünya genelinde fatalite oranı %30-50 arasında değişmekteyse de Türkiye'de Sağlık Bakanlığı'nca yaklaşık olarak %5 civarında olarak bildirilmiştir ve bu oran çocuklarda %1,7-26,5 arasında değişim göstermektedir. Enfeksiyon tespiti için ELISA kitleri kullanılmaktadır fakat bu kitler oldukça zaman alıcı, pahalı ve yoğun emek gerektiren yöntemler olup, spesifik olarak virüsün tespitinden ziyade hastalık etkeni virüse karşı üretilen IgG ve IgM antikorları hedef aldıklarından hatalı sonuçlara yol açmakla birlikte tekrar kullanıma da uygun değillerdir. Hastalığın sıklıkla karıştırıldığı diğer enfeksiyon hastalıkları (Viral hepatitler, tifo, leprospiroz, Hantavirüs infeksiyonları vb.) için yapılacak mikrobiyolojik test maliyetleri etkili tanı maliyetini daha da yükseltmektedir. Bu sebeplerden dolayı günümüzde KKKA teşhisi için etkin bir hasta başı tanı kitine ihtiyaç duyulmaktadır. Aptamerler, in vitro olarak seçilebilen ve ilgili hedefleri için yüksek afinite ve seçiciliğe sahip olan küçük nükleik asitlerdir. ssDNA veya RNA olarak bulunabilirler ve doğal olarak üç

boyutlu yapılarına katlanırlar. Aptamerler, afinitelerini korurken yapısal kararlılıklarını geliştirmek için de değiştirilebilir. Bu özelliklerinden dolayı hem tedavi amaçlı hem de teşhis amaçlı kullanılabilirler. KKKA virüsüne özgü aptamerler bu çalışmada başarıyla seçilmiştir ve KKKA teşhisi için daha da optimize edilecektir.

Anahtar Kelimeler: Aptamer, RNA, SELEX, KKKAV, KKKA

To my beloved mother…

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INTRODUCTION

For a long time, people have thought that nucleic acids (NAs) were biological compounds that only store genetic information (DNA) that is transferred through generations, and this information is used to synthesize proteins via RNA. However, new functions have been attributed to NAs such as enzymatic catalysis by ribozymes, transcriptional regulation by microRNA, and non-coding RNA species. Exploration of these new functions encouraged scientists to consider other possible functions of NAs. Since life began, NAs may have performed diverse functions and have probably ensured all the catalytic responses according to "RNA world theory" (Gilbert, 1986; Gold et al., 2012).

Studies on "human immunodeficiency virus (HIV)" and adenoviruses in the 1980s demonstrated a new appreciation for the significance of nucleic acid and protein selective interactions. In these studies, it is proven that viruses express small RNA fragments that can bind either viral or cellular proteins with high specificity (Ilgu, Fazlioglu, Ozturk, Ozsurekci, & Nilsen-Hamilton, 2019). The idea is based on the features of single-stranded oligonucleotides that allow specific interactions with target molecules through their unique tertiary structures.

The discovery of oligonucleotides that are known as aptamers can specifically bind different target molecules was a valuable contribution to the multifunctional existence of NAs (Lakhin, Tarantul, & Gening, 2013). Aptamers are obtained by an *in vitro* molecular evolution assay termed "Systematic Evolution of Ligands by Exponential enrichment (SELEX)" (Adachi & Nakamura, 2019; Keefe & Schaub,

2008; Kumar, 2006; Nakamura, Ishiguro, & Miyakawa, 2012). Aptamers are oligonucleotides that are small, single-stranded molecules that can selectively and specifically bind to a particular target. The unique 3-dimensional (3D) structure of single-stranded oligonucleotides provides high binding affinity and specificity. These properties make aptamers analogs of antibodies.

1.1 Aptamer Structure

During SELEX, the selection of aptamer significantly relies on the environmental components. This is because the prevailing structures of oligonucleotides in the pool will directly be affected by the ions surrounding them and the pH of the environment. Nucleic acids being negatively-charged molecules build an "ionic atmosphere," that mimics a willingly connected sheath of negative ions that surround the nucleic acids, for ion-nucleic acid interactions.

The aptamer's ability to fold into its correct 3D form is required for particular ligand binding. Charge distribution changes with different ion compositions as their electrostatic interactions influence the NA folding and 3D structure. Therefore, the binding thermodynamics (mainly affinity and stoichiometry) and potential output of aptamers rely on the buffer components used along the selection. To achieve optimum performance from aptamers, the incubation temperature and the choice of specific ions, their concentrations, and the pH need to be critically thought out when developing SELEX protocol (Ilgu et al., 2019).

Other factors in the compounds to be investigated may even have a negative impact on aptamer performance. Aptamers, for example, are sensitive to nucleases found in a wide range of biological materials. That is especially correct for RNA, which has the 2'OH part that has the ability to attack the phosphate backbone electrophilically. In order to catalyze the hydrolysis of RNA, nucleases facilitate this chemical

property that makes RNA chemically more sensitive than DNA at high pH and temperature. Lots of post-selection chemical alterations could be introduced to stabilize nucleic acids and overcome this susceptibility to hydrolysis. However, post-selection aptamer modifications carry a significant risk of altering the 3D structure of the aptamer with a consequent loss or change of affinity for their cognate target. Alternatingly, using chemically substituted nucleotide analogs with nuclease-resistant sugar moieties during SELEX is the less risky method (Ilgu et al., 2019).

Secondary structural motifs observed in the tertiary structures of aptamers include the "stem-loop, hairpin structure, pseudoknot, internal bulge, kissing loop, three-way junction, and the G-quadruplex". X-ray crystallography or nuclear magnetic resonance (NMR) spectroscopy can be used to comprehend these structures in detail. However, these methods are laborious and costly. Computer techniques have been developed to predict the lowest free-energy configurations utilizing sequence-based modeling to make the process easier (Ilgu et al., 2019; Radom, Jurek, Mazurek, Otlewski, & Jeleń, 2013). This allows the secondary structure of oligonucleotides to be easily predicted without much cost. Computational approaches to the acquisition of 3D patterns of nucleic acid structures through primary and secondary structural modeling, however, are still in the development stage, and the findings are not accurate enough for experimental methods (Ilgu et al., 2019)

1.2 Aptamer Selection

1.2.1 Systematic Evolution of Ligands by Exponential enrichment (SELEX)

SELEX is the method for isolating aptamers from a randomized oligonucleotide (oligo) library that is unique to the desired target by mimicking evolution (systematic evolution of ligands). That *in vitro* technique requires several selection rounds (5-

20) alternating with PCR for DNA libraries or RT-PCR followed by *in vitro* transcription for RNA libraries for amplification of the fittest oligonucleotides (exponential enrichment) (Ilgu et al., 2019).

The beginning pool contains around 10^{15} random oligonucleotide sequences in a typical SELEX experiment. The association of intramolecular interactions that involve base pairing, non-canonical interactions, and sugar packing determines the special 3D structures of these sequences in the pool. This structural complexity provides a high likelihood of choosing an oligo from the pool which has the potential to interact with the object of interest avidly and specifically (aptamer). Salt bridges, van der Waals, hydrogen bonds, hydrophobic and electrostatic interactions can enable the specific intermolecular interactivity between the target and the aptamer (Ilgu et al., 2019).

The main steps of a traditional SELEX comprise amplification, binding, elution, and identification. Commonly, to deliver a better result, multiple Multiple SELEX round cycles should be performed due to the limited resolution and effectiveness of available methods. In the first step, to ensure the binding of certain oligonucleotides to the target, which is cultured with a random RNA or DNA library, while the non-binding ones will be removed in the following steps.

Because nucleotide-target compounds and nucleic acids have varying molecular weights, unbound oligonucleotides can be eliminated in the elution step. In this step, either separable nucleic acid or free nucleic acids immobilised on a given substrate interacts with target molecules (Shimada, Fujita, Maeda, & Ishihama, 2005). Sometimes elution of the effectively bound oligos from targets may limit the isolation of the aptamers with extraordinarily high affinity. Therefore, aptamers that have high affinity are generally gained by the help of SELEX with free-form target

particles (Berezovski et al., 2005). It can be difficult to remove unbound oligonucleotides from a free target-oligonucleotide combination in some circumstances. So, for SELEX with free-form target molecules, low-affinity targets are preferable.

The next step after elution is amplification of the bound oligonucleotides with primers via polymerase chain reaction (PCR). For the next round of SELEX, the amplification is expected to produce an enriched oligonucleotide population. Generally, 10–20 amplification cycles are applied during PCR (Kim & Gu, 2014). The nucleotide pool becomes enriched after repeated rounds of selection and amplification, and the affinity between target and oligos becomes higher since oligonucleotides with low to no affinity are expected to be eliminated in previous rounds. It is possible to track the progress of SELEX by quantifying and comparing the percentage of target-bound oligos after each SELEX round (Conrad, Baskerville, & Ellington, 1995).

The selection is done if oligos stick to the target are completely prevalent in the oligonucleotide pool or during multiple SELEX rounds, no additional improvement of target-bound oligos can be seen. The selected oligos are subjected to amplification and generally kept for further analysis.

After completing all SELEX rounds, two different protocols are followed to finalize the SELEX. One way is to clone the last round oligos into a carrier plasmid and sequence the selected clones to determine the sequences of individual oligonucleotides (or aptamer candidates). Optionaly, next-generation sequencing (NGS) could be used to get sequence data after different SELEX rounds for the oligo populations present in different pools (Ilgu et al., 2019). Comparative sequence

analysis facilitates the detection of consensus sites that may be involved in the recognition of targets (Hoinka, Zotenko, Friedman, Sauna, & Przytycka, 2012).

The variety of different aptamer alternatives processed by the SELEX procedure is determined by the rigor of the selection criteria as well as the target characteristics. The most promising aptamer candidates with nanomolar dissociation constants are often found, synthesized, and further characterized. Aptamers with picomolar dissociation constants have also been isolated in several instances. It can take months for *in vitro* selection by conventional SELEX, Thus, Golden et al. suggested a new SELEX technique called photochemical SELEX to reduce the time it takes to select high-affinity aptamers. (PhotoSELEX) (Tombelli, Minunni, & Mascini, 2005). This method involves the creation of a modified DNA pool, capable of forming a covalent photoinduced binding via their targets. The resulting aptamers have high specificity and compared to conventional SELEX methodologies, such aptamers require fewer rounds of selection.

1.2.2 Counter SELEX

Negative SELEX (also known as counter SELEX or subtractive SELEX) is commonly used to improve the specificity of aptamer identification when using only target analytes. Counter SELEX is used to reduce nonspecific binding or inaccurate identification of target analytes with structurally similar compounds, which is especially important when low-abundance target molecules are present in complex matrices, such as tissue or cell lysates. Counter SELEX adds an additional step, where structurally close targets are incubated with oligo pools to discriminate against non-specific oligonucleotides. In order to select more specific aptamers, this process has been significantly extended to other modified SELEX techniques (Zhuo et al., 2017).

1.3 Use of Aptamers

1.3.1 Usage of Aptamers for Diagnosis

The development of new diagnostic tools has become increasingly vital as new public health challenges have arisen. Scientists want to find novel and near-perfect methods to detect biomarkers of interest in order to support precision medicine and early diagnosis, which are critical for the treatment of many diseases, particularly cancer and neurodegenerative diseases (Banerjee & Nilsen-Hamilton, 2013; Kulabhusan, Hussain, & Yüce, 2020; Moulick & Bhattacharjee, 2020). In biological samples, the approach should be sensitive, repeatable, stable, and nonreactive, as well as simple to display, rapid, and inexpensive (Akki & Werth, 2018). For a long time, antibody-based biosensors have been created to address this demand.

Aptamers, rather than antibodies, have become preferred agents due to their reusability, better stability, reduced expense, and the above advantages. Biosensors are diagnostic equipment that produces specific and quantifiable signals as a result of biomolecular interactive relations. A bio-recognition element (bioreceptor) that binds to the target molecule and a convertor that produces a detectable signal as a result of this interaction makes up these tools. The affinity and selectivity of a biosensor's bioreceptor determine its success. In addition, producers prefer cheap and stable bioreceptors. Detection of bacteria, cancer cells, viruses, biomarkers, metabolites, poisons, and medicines is conceivable in addition to these desirable properties (Kulabhusan et al., 2020). For real-time analyte detection, fluorescently tagged aptamers in an optical sensor were incorporated onto solid supports as a recognition element. The majority of these proof-of-concept experiments used thrombin aptamers (TA), which have a highly stable G-quadruplex structure and their target, thrombin, which is detected in serum samples (Avino, Fabrega, Tintore,

& Eritja, 2012). Using less stable aptamers to target less stable particles in other aptasensors, on the other hand, is likely to be difficult (Sun et al., 2018).

Table 1.1 Features of Aptamers and Antibodies Comparison.

Features	**Antibodies**	**Aptamers**
Specificity	High	High
Affinity	High	High
Immunogenicity	High	No humoral response
Production	In vivo	In vitro
Cost	High	Low
Stability	Unstable	Stable
Potential targets	Limited to immunogenic molecules	Wide range
Affinity	High	High
Immunogenicity	High	No humoral response

1.3.2 Usage of Aptamers for Therapeutic Purposes

An aptamer can be employed in therapeutic applications in addition to clinical diagnostics. Aptamers can inhibit target molecules, operate as receptor agonists or antagonists, activate associated pathways, and act as drug delivery vehicles, to name a few therapeutic techniques (Song, Lee, & Ban, 2012). Several aptamer investigations are in the proof-of-concept stage, while others are in the process of clinical trials or are about to do so. Only Macugen (pegaptanib), which targets vascular endothelial growth factor (VEGF), has been licensed by the FDA to use in the treatments of vascular ocular illness in the United States (Song et al., 2012).

Aptamer-based medication delivery systems are one of the most common methods for aptamers. Aptamers are being used as cargo carrier molecules in these systems because they have a higher specificity for a tissue or target cell. The integration of aptamers with appropriate cargo occurs after aptamers bind to the targeted cell receptor. However, only aptamers that identify specific cell surface proteins can be used for this application. Cell-SELEX, a cell-based selection approach, was created to choose aptamers against receptor proteins in their natural state (Ilgu et al., 2019).

1.4 Virus Targeted Aptamers

Aptamers have been used to target a number of viruses. To illustrate, Hepatitis C and B Virus (HCV and HBV) (Gao et al., 2014), Zika Virus (Argondizzo, Silva, & Missailidis, 2020), HIV (Leija-Montoya, Benítez-Hess, & Alvarez-Salas, 2016), Influenza Virus, and Ebola Virus (Shubham et al., 2018) are created for detection or inhibition of the viruses. Different SELEX procedures are used to isolate virus-targeting aptamers. The use of entire viral particles in SELEX has the advantage of producing aptamers which interact with natural viral protein complexes on the particle surface. Then, without knowing anything about the virus's biology, target, or infection mechanism, these aptamers can be exploited in diagnosis and treatments (Leija-Montoya et al., 2016).

The Rous sarcoma virus was the first application to use a full virus strategy (RSV). 19 RNA aptamers were identified in this work, and five of them effectively neutralized virus infection (Pan et al., 1995). This study was a forerunner in the use of aptamers in virology. It leads to the creation of aptamers as therapeutic and, diagnostic agents especially for viral disorders that require rapid diagnosis or result in chronic viral-induced illnesses (Wandtke, Woźniak, & Kopiński, 2015).

1.5 Crimean Congo Hemorrhagic Fever (CCHF)

1.5.1 History of CCHF

A physician from Tadzhikiztan recorded a disease that is now known as CCHF. In those patients, general symptoms were the observation of blood in the gums, urine, vomit, abdominal cavity, rectum and sputum. It was thought to be caused by a tick or louse (Hoogstraal, 1979). CCHF has been named as ‘khungribta’ (meaning: blood taking), ‘khunymunyyy’ (meaning: nose bleeding) or ‘karakhalak’ (meaning: black death) by the local people of Uzbekistan (Hoogstraal, 1979).

Between 1944 and 1945 years, after almost 200 Soviet militarians were infected in Crimea, then the illness was recognized by modern medicine and first identified as a clinical trait (Whitehouse, 2004). The virus etiology has been hypothesized by producing febrile illness in psychiatric patients having pyrogenic treatment following injection with a filterable material from CHF patients’ blood (Whitehouse, 2004). The production of a mild but typical clinical course of 'CHF' in healthy people 48 hours after incubation with filtered solutions of part or full Hyalomma marginatum ticks inside the antibiotics has revealed more evidence of virus etiology and a putative tick-borne route of infection (Spengler, Bergeron, & Rollin, 2016).

In 1967, the first newborn white mouse was used by Chumakov and his colleagues to isolate the CHF virus (Butenko & Chumakov, 1990). Preparation of the CCHFV from a patient was completed and named as the Drosdov strain. Then, this strain has been accepted as the model strain for research in Russia and in many other countries. Now, researchers use the virus in different research studies and it is used to produce necessary reagents such as antibodies or antigens. These reagents have importance for serological surveys or identification and classification of the virus coming from different geographical regions. After research studies, many tick-borne hemorrhagic

fever agents from Kazakhstan and Uzbekistan, as well as other parts of Africa, have been discovered to be inseparable. Experiments showed the 'CHF' virus had been antigenically identical with the Congo virus as a result of this (Whitehouse, 2004) which was produced from patients from both the Uganda and Congo (Whitehouse, 2004). It is realized by scientists that the CHF virus and Congo virus were identical and first called as ‘CHF-Congo’ virus. But, researchers thought that this name was difficult to use and accepted the CCHFV (Crimean-Congo Hemorrhagic fever virus) (Hoogstraal, 1979).

1.5.2 Classification of the CCHFV

CCHFV comes from the Bunyaviridae family's Nairovirus genus. Tospovirus, Phlebovirus, Hantavirus, and Orthobunyavirus are among the other genera in the family. According to the International Committee on Virus Taxonomy, the genus Nairovirus contains 7 identified species and 34 viral strains (Whitehouse, 2004), they are thought to be transferred from either ixodid or argasid ticks, individually.

The Nairobi sheep disease (NSD) and CCHF groups were the most important serogroups. The former group contains CCHFV and Hazara virus, of which Hazara has been shown to be not dangerous for humans. The latter group contains Dugbe and NSD viruses. Among all, just 3 members, CCHF, Dugbe, and NSD viruses, are known to cause human infections. In humans, the Dugbe viral infection results in a moderate fever and thrombocytopenia (Burt, Spencer, Leman, Patterson, & Swanepoel, 1996). NSDV is mostly a sheep and goat disease causing high fever, hemorrhagic gastroenteritis, abortion, and close to 90 % mortality in small ruminants (Krasteva, Jara, Frias-De-Diego, & Machado, 2020)

1.5.3 Structure and molecular biology of the virus

The structure of CCHFV has not been studied extensively. The structure of CCHFV inside the brain of infected neonatal mouse was studied by scientists and they noticed similarities to Bunyaviridae members. In fact, in the way of their replication cycle, physicochemical features, morphogenesis and basic structure, nairoviruses and CCHFV, generally, are similar to other parts of the Bunyaviridae family (Donets, Chumakov, Korolev, & Rubin, 1977). The main phases of the replication for Bunyaviridae viruses are like those for lots of other enveloped viruses. Virions are spherical, measuring almost 100 nm in diameter, and have a 5–7 nm thick lipid bilayer envelope formed from the host cell, through which protrude glycoprotein spikes about 8–10 nm in length emerge (Whitehouse, 2004).

CCHFV seems to be unique from other Bunyaviridae viruses when studied under negative stain electron microscopy. They consist of small morphologic covering units and without any center holes that are arranged in an arbitrary manner. Bunyaviridae virions contain three structural proteins:an approximately 200 kDa protein called large polypeptide (L) that is the virion-associated RNA-dependent RNA polymerase (RdRp, or RNA replicase), a nucleocapsid protein (N), and two envelope glycoproteins (Gn and Gc) (Whitehouse, 2004).

The entire nucleotide sequence of the L segment was disclosed (Honig, Osborne, & Nichol, 2004). It is roughly 60% similar to the L portion of Dugbe virus, its only known fully sequenced Nairovirus genome, the only other Nairovirus genome to be fully sequenced, both at the nucleotide and amino acid levels, with the most highly conserved region coding the corresponding core catalytic domains of the RdRp. Further examination of the L segment sequences from Dugbe and CCHFV virus showed the presence of a zinc-finger domain and a leucine zipper motif, implying that nairovirus L segments have characteristics of viral helicases (i.e., having both

helicase and polymerase activity from a single polyprotein), which are commonly shown in positive-strand RNA virus replicates. Moreover, high-sequence homology was observed with a superfamily of anticipated cysteine proteases known as ovarian tumor (OTU)-like proteases, which was also suggested by the Dugbe virus L segment sequence (Makarova, Aravind, & Koonin, 2000). Based on those findings, the OTU-like protease might act in the Nairovirus L protein by autoproteolytically splitting the polyprotein to generate a polymerase and a helicase (Whitehouse, 2004). Deubiquitination activities for the viral L3 23 K proteinase are further proposed functions of the OTU-like proteases (Balakirev, Jaquinod, Haas, & Chroboczek, 2002).

Clearly, more research will be needed to fully understand the complicated structure of the protein outputs encoded by the CCHFV L part. The genome is made of S, M, and L segments that are negative-strand RNA segments, which encode the N nucleocapsid, Gn and Gc glycoproteins, and the L polymerase, and is like those of other members of the family. Individual L, S, and M nucleocapsids are formed by combining the RNA segments with N to form loosely helical or circular nucleocapsids (Whitehouse, 2004).

The M part of nairoviruses is 30 to 50 percent bigger than other M parts of Bunyaviridae genera, with a theoretical coding capability of up to 240 kDa protein (Elliott, 1990). For infectivity, a virion must have minimum one of each of the L, S, M and ribonucleocapsids; but, the same amounts of nucleocapsids might not be stuck in mature virions all the time (Whitehouse, 2004). Without native RNA parts, N protein is focused onto the perinuclear area of infected cells, and that targeting is actin filament dependant (Andersson et al., 2004). The initial 8 to 13 nucleotides at the 3' termini of all three RNA segments get a sequence that is conserved across the genus (3-AGAGUUUCU...), with a corresponding consensus sequence at the 5' termini (Whitehouse, 2004). Base pairing of the terminal nucleotides is thought to

produce permanent panhandle structures and non- covalently closed circular RNAs, both of these properties have been confirmed by electron microscopy of RNA extracted from virions of another bunyavirus, Uukuniemi. (Hewlett, Pettersson, & Baltimore, 1977).

The identification of receptor sites on subject cells is accomplished by viral glycoproteins. Viruses which bind to receptors on vulnerable cells are internalised via endocytosis and replicate in the host cytoplasm. In the Golgi, virions mature by budding into cytoplasmic vesicles that are thought to merge with the plasma membrane to exhaust the virus (R & CA, 2005). The molecular characterization of CCHFV glycoproteins has received a lot of attention recently. The mature Gn (37-kDa) and Gc (75-kDa) proteins represent the major structural glycoprotein constituents of the virus during CCHFV infection (Sanchez, Vincent, & Nichol, 2002). They also discovered that the mRNA region of CCHFV encodes a polyprotein that is proteolytically processed into a 140 kDa Gn and an 85 kDa Gc precursor protein. As demonstrated for the Lassa virus, Arenavirus (Lenz, Meulen, Klenk, Seidah, & Garten, 2001), for the principal glycoprotein precursor cleavage processes, CCHFV uses the subtilaze SKI-1 and perhaps similar cellular proteases, at least in part (Whitehouse, 2004).

A CCHFV reverse genetics system was recently developed (Flick & Pettersson, 2001) and The approach was based on RNA polymerase I transcription that was newly utilized to develop a reverse genetics system for the Uukuniemi virus (Flick & Pettersson, 2001). The generation of a reverse genetics system for CCHFV was a significant step forward in the study of its biology. The creation of an infectious clon for CCHFV will enable further in-depth research into its biology and pathology, potentially leading to improved therapeutic and preventative strategies for CCHFV infections.

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1.5.4 Strain variation and phylogenetic relationships

Early investigations based on serological testing concluded that there are only minor variations between CCHFV strains. Studies using modified agar gel diffusion precipitation, neutralization, cell culture interference, and complement fixation assays, for example, found no antigenic differences across strains from various geographical areas in the former Soviet Union and Africa (Whitehouse, 2004). Recent analyses based on nucleic acid sequences, on the other hand, have revealed a great deal of genetic variation. The S RNA segment (which encodes the viral nucleoprotein) of the Chinese sheep isolate C68031 was the first CCHFV sequence data to be published. Several more S segment sequences from CCHFV isolates from other parts of the world have been reported since then (Whitehouse, 2004).

These investigations have begun to shed light on the mRNA segment's genetic variability. Although a complete M RNA sequence of the reference strain IbAr 10200 was previously deposited in GenBank, the first reported characterization of the CCHFV M RNA segment was of the Chinese strains BA66019 and BA8402, obtained in 1965 and 1984 years from a CCHF patient and Hyalomma spp. ticks, individually. The coding nucleotide sequences of that two Chinese strains differed by a mean of 22% from the Nigerian strain IbAr 10200, demonstrating the degree of genetic variety of all these viruses. Sequence study of mRNA segments from CCHFV samples from Central Asia (Tajikistan) and Russia has revealed that they each belong to phylogenetically separate groupings (Whitehouse, 2004).

1.5.5 Ecology and epidemiology of CCHFV

1.5.5.1 Vertebrate reservoir hosts

In an enzootic tick–vertebrate–tick cycle, CCHFV, as other tick-borne zoonotic pathogens, spread in nature unrecognized. Sheep, Cattle, hedgehog shares, goats, mice, and even domestic dogs have all been shown to be carriers of CCHFV. Antibodies to CCHFV have been found in domestic pigs, cattle, sheep, donkeys, horses, and goats from different parts of Asia, Africa, and Europe, and seroepidemiological searches have discovered antibodies to CCHFV in domesticated animals, pigs, donkeys, horses, goats, and sheep from various parts of Europe, Africa, and Asia (Whitehouse, 2004). However, most of the studies show that, by delivering virus-infected ticks, ground-feeding birds may play a significant role in the ecology and epizootiology of "CCHF". But, the involvement of the birds themselves, if any, is unclear, and further research is needed in this area to settle these difficulties. In conclusion, while vertebrates are important sources of blood for vector ticks, and the number of vertebrate species linked mostly in natural history of "CCHF" is large, the specific importance of vertebrates in the virus's transmission and maintenance remains unclear.

1.5.5.2 Tick vectors

At least one biting midge and 31 tick species have been shown to carry CCHFV (Whitehouse, 2004). Two species of Argasidae (soft ticks) and 7 genera of Ixodidae were used to isolate viruses from ticks (hard ticks). The presence of a virus in a tick species does not, however, make it a carrier. In many circumstances, there is no conclusive proof that these arthropods can serve as virus vectors; instead, virus could be found inside a current blood meal from just a viremic host. In one case, a virus was obtained from a biting midge caught in a light trap beside a cattle shed in Nigeria, and the midge may have carried undigested blood (Whitehouse, 2004). Similarly, it

appears unlikely that argasids can transmit CCHFV because the virus could not replicate after intracoelomic inoculation in 3 species of soft ticks, and the same was shown for the soft tick Ornithodoros sonrai) (Whitehouse, 2004). Ticks of the genus Hyalomma are far more essential to the epidemiology and ecology of "CCHF". Based on a link between reported trials and tick bites, Hyalomma spp. ticks were included in the ecology of "CCHF" as early as 1944. In fact, a healthy volunteer who was subcutaneously injected with a solution of 370 larvae H. marginatum ticks exhibited mild "CCHF" the following year. The virus was first isolated from adult Hyalomma, as well as numerous other tick species, in the late 1960s, which not only served to verify the viral etiology of the disease, but also identified Hyalomma spp. ticks as potential vectors. The known incidence of CCHFV in Europe, Africa and Asia roughly corresponds to the global distribution of Hyalomma ticks (R & CA, 2005).

Dickson and Turell investigated the replication of the "CCHF" virus and its tissue tropism through Hyalomma truncatum ticks. Virus was recovered in greatest titers from reproductive tissues and salivary glands in ticks that were experimentally infected with CCHFV, and blood feeding was positively linked with virus recovery. Virus was also found in muscle, Malpighian tubules, midgut, and nerve tissues of nearly every tick tested; but, viral titers were consistently low in these tissues. Although Hyalomma spp. ticks are thought to become the most significant in the epidemiology of CCHF, the virus has already been obtained from ticks of other species, which could explain its vast geographical distribution. Ticks have a vital biological role in nature, serving not just as virus vectors but also as virus reservoirs (Whitehouse, 2004).

1.5.6 Clinical features

Humans seems to be the hosts of CCHFV that can cause illness. Infection in humans with CCHFV often leads in severe hemorrhagic illness, in contrast to infection in most of the other vertebrate hosts. Hoogstraal examined the historical descriptions of sickness linked to CCHF in great detail (1979). Some writers have described the normal course of CCHF as consisting of four separate phases: hemorrhagic, incubation, convalescence, and prehemorrhagic (Hoogstraal, 1979); but, the duration and concomitant symptoms of these phases might vary substantially. The incubation time following a tick bite might be as little as 1–3 days, but it can also be much more, depending on various factors, including the route of exposure. Different hosts are thought to produce phenotypic variations in CCHFV strains which alter viral pathogenicity (Gonzalez, Wilson, Cornet, & Camicas, 1995).

It's uncertain whether the differences in incubation lengths and, as a result, disease outcomes are attributable to this phenomena or other factors like viral dosage. The prehemorrhagic stage begins with a fast onset of cold, fever, disorientation, severe headache, abdominal and back aches, and photophobia following the incubation period. Additional signs and symptoms include diarrhea, a loss of appetite vomiting, and nausea. Fever is frequently ultrahigh (39 to 41 °C) and might last for 5–12 days or be biphasic. Neuropsychiatric alterations have been described in certain CCHF patients, which is intriguing. Sharp mood swings, feelings of perplexity and hostility, and even aggressive behavior have all been reported (Whitehouse, 2004). Cardiovascular abnormalities, such as bradycardia and hypotension, might also be noted. This isn't always the case; during a CCHF crisis inside the UAE from 1994 to 1995, neither of the 11 victims showed indications of cardiovascular problems, albeit eight (72.7 percent) eventually died (Schwarz, Nsanze, & Ameen, 1997). Hemorrhagic signs appear 3–6 days after the commencement of the disease in severe patients. Petechiae to extensive regions of ecchymosis might occur on the skin, and mucosal membranes particularly on the upper torso and/or extremities. By day 4 or

5, melena, bleeding as in form of epistaxis, and hematemesis is prevalent, with dark "coffee grounds" vomitus and tar-like stools arising from intestinal hemorrhages. Other types of bleeding have been documented, including gingival bleeding, vaginal bleeding, and, in its most severe cases, brain hemorrhage (Swanepoel et al., 1989). In severe cases, brain bleeding and significant liver necrosis are unsurprisingly linked to a bad prognosis. Nosocomial infections have substantially greater mortality rates than illnesses got naturally through tick bites. The specific cause of this occurrence is unknown, but it could be related to viral dosage (Whitehouse, 2004).

1.5.7 Pathogenesis

CCHF's etiology is not fully understood. The capacity of haemorrhagic fever viruses to block the host immune reaction by targeting and influencing the cells which trigger the antiviral reaction is a popular pathogenic trait (Geisbert & Jahrling, 2004). The virus replicates aggressively, causing dysregulation of the circulatory system and lymphoid tissues (Feldmann, Jones, Klenk, & Schnittler, 2003). The pathophysiology of CCHF is complicated by endothelial infection (Ergonul, Mirazimi, & Dimitrov, 2007). The endothelium can be attacked in 2 ways: through viral components, indirectly or virus-mediated host-derived factors causing endothelial dysfunction and activation, or through virus infection and reproduction in endothelial cells (Feldmann et al., 2003). Endothelial injury causes haemostatic failure by causing blood clotting and degranulation, which activates the intrinsic clotting cascade. Indeed, at an early stage of illness, fatal CCHF cases exhibit grossly aberrant indicators of disseminated intravascular clotting, and clotting system function is identified as early and significant aspect of the disease.

1.5.8 Diagnosis

Early diagnosis is critical for the patient's outcome as well as the prevention of disease transmission due to the risk of nosocomial infections. The initial signs of

CCHF are clinical indications and patient records, particularly travelling to endemic regions and a history of tick bites or contact to blood or tissues from animals or human patients. Rickettsiosis, leptospirosis, and borreliosis should all be considered in the differential diagnosis (relapsing fever). Other diseases that cause hemorrhagic disease, like meningococcal infection, malaria, hantavirus, Kyasanur Forest disease, dengue, Omsk hemorrhagic fever, hemorrhagic fever, and yellow fever must also be regarded. Lassa fever, as well as infection with the filoviruses Marburg and Ebola should be observed in derivative diagnosis.

Before 1980, serologic assays like immunodiffusion, complement fixation, and hemagglutination inhibition were employed to explore and diagnose CCHFV infection, but they lacked sensitivity and repeatability (Hoogstraal, 1979). In CCHF infections, the neutralization of antibody response is also modest and hard to show. With the invention of the indirect IFA and the invention of enzyme-linked immunoassays for detection of IgM and IgG antibodies, these issues were essentially resolved. IFA detects the IgM and IgG antibodies about 7 days after the onset of sickness, and they are represent in the serum of survivors on the ninth day. (Whitehouse, 2004). IgM antibody levels drop to low or indiscernible even by fourth month from infection, and IgG antibody titer may start to drop at this time as well, but they remain detectable for at least five years. Seroconversion, or a four- fold or more increase in antibody titer in matched serum samples, or IgM antibodies in a single sample, are used to confirm recent or ongoing infection (Burt et al., 1996). In fatal instances, an antibody response becomes infrequently evident, and the virus is usually isolated from serum or liver biopsy samples to confirm the diagnosis. New immunological tests including recombinant CCHFV nucleoprotein has recently been developed and employed in an IFA or an ELISA to identify blood antibodies from infected patients (Whitehouse, 2004).

Molecular-based diagnostic techniques, like the RT-PCR (reverse transcription-polymerase chain reaction), are increasingly often used in the diagnosis of CCHF and other viral hemorrhagic fevers as a supplement to serodiagnosis (Drosten, Kümmerer, Schmitz, & Günther, 2003). There are numerous advantages to employing such tests. Since RT-PCR finds the virus's genetic substance and can be tailored to be extremely precise, an initial diagnosis of CCHF can be made without the requirement to grow the virus that would necessitate the use of specialist biocontainment lab facilities. So, because of RT-high PCR's sensitivity, positive results can frequently be gained from materials without culture (Whitehouse, 2004). Furthermore, the assay could be used to analyze blood samples that have been preserved. In one study, viral RNA was identified in samples up to the 16th day of sickness, but infective virus was gradually removed from the serum after one week (Burt, Leman, Smith, & Swanepoel, 1998).

Another advantage of molecular diagnostic assays over virus culture is their speed, which allows for a preliminary diagnosis to be made within 8 hours of taking the first material (Burt et al., 1998). The creation of automatic real-time assays is a step forward from the traditional RT-PCR assay. The real-time PCR assay supplies a number of advantages over traditional RT-PCR procedures, including a lower incidence of contamination, improved specificity and sensitivity, and the ability to get findings in minutes rather than hours. Several researchers have reported using real-time PCR tests to detect Ebola, Rift Valley disease, and dengue viruses, which are all viral origins of hemorrhagic fevers. Scientists employed primers to the nucleoprotein gene to build one-step RT-PCR test for detecting CCHFV; but, they have used DNA-intercalating dye SybrGreen I to detect the PCR output since no consistent binding site for a 5′-nuclease probes could be detected. Then, created a RT-PCR assay employing a TaqMan-minor groove binding protein (MGB) probe, that allowed for higher precision with a shorter probe length, which partially alleviated this problem (Whitehouse, 2004).

1.5.9 Treatment

Ribavirin is the antiviral drug of choice for infected patients, even though exact mechanism is unknown (Watts, Ussery, Nash, & Peters, 1989). Ribavirin was demonstrated to decrease viral activity in an in-vitro research, and many CCHF virus strains seemed to be more responsive. Ribavirin therapy significantly reduced neonatal mouse fatality and delayed the meantime to death in an experimental trial in mice (Tignor & Hanham, 1993). It should be mentioned that there is no proof for its use of ribavirin to treat CCHF based on randomized clinical trials; its efficacy was only documented in observational studies (Khan et al., 1995).

Ribavirin is available in both oral and injectable versions in several countries. Patients are treated for ten days (30 mg/kg as a loading dosage, then 15 mg/kg each six hours for four days, then 75 mg/kg every eight hours for six days) ("Crimean Congo Haemorrhagic Fever | WHO | Regional Office for Africa," n.d.). Paragas and colleagues (Paragas, Whitehouse, Endy, & Bray, 2004) tested drugs for antiviral activity against the CCHF virus and discovered that ribavirin inhibited CCHF virus replication, ribamidine had antiviral reaction that was 45- to eightfold lower than ribavirin, and 3 other drugs (tiazofurin selenazofurin, and 6-azauridine) seemed to have no antiviral reaction at all. When present intracellularly, MxA, a newly discovered molecule that belongs to the interferon-induced GTPases, which is a member of the dynamin superfamily, inhibited the replication of "CCHF" viral RNA and the output of new infective virus particles by interacting with a part of the nucleocapsid (Ergonul et al., 2007).

One study proposed using passive immunotherapy to treat infected patients by transferring plasma from recovering individuals to infected patients (Ergonul et al., 2007). The study, however, lacked control groups and also was confined to only 7 patients.

CHAPTER 2

MATERIALS AND METHODS

2.1 Inactivation of the CCHF Virus

A heat-inactivated local isolate (Ank-1) of the CCHF virus was obtained from the bio-bank of the Virology Department, Faculty of Veterinary Medicine, Ankara University. The virus CCHFV-Ank1 is a BSL-4 class pathogen and is cultivated in a BSL-3(+) containment facility of the above-given department. Briefly, the virus was incubated in a water bath at 60°C for 60 minutes. The subsequent confirmation of inactivation was performed by three blind passages of the heat-treated virus in the Vero cell line. In each passage, the virus-specific cytopathic effect was monitored by invert microscopy for 4 days. In case of the absence of cytopathic effect after the third passage, the virus was accepted as heat-inactivated.

2.2 Design of DNA Library and Primer for SELEX

Integrated DNA Technologies (IDT; Coralville, IA, USA) produced the oligonucleotide pool (or oligo library) and Oligomer (Ankara, Turkey) synthesized the other oligos in the library. The ssDNA library has the GCC TGT TGT GAG CCT CCT GTC GAA (53 N) TTG AGC GTT TAT TCT TGT CTC CC sequence. N describes A, C, G, and T that each was added as an equimolar mixture during synthesis. To create dsDNA template, the primers 5'-**TAA TAC GAC TCA CTA TA**G GGA GAC AAG AAT AAA CGC TCA A-3' (oligo484) and 5'-GCC TGT TGT GAG CCT CCT GTC GAA-3' (oligo485) were used for PCR. The red bolded sequence is the T7 polymerase promoter region, necessary for 2′FY-RNA pool generation during *in vitro* transcription (IVT) reactions as previously explained (Shubham et al., 2018).

2.3 Synthesis of 2'FY RNA Pool

The extension reaction with oligo484 (65 °C for 15 minutes) was used to commence the synthesis of the dsDNA template for 2'FY-RNA pool (Shubham et al. 2018). WizPureTM Taq DNA Polymerase Kit was used to perform the reaction (Wizbiosolutions, Seongnam, South Korea). The thermocycler was used to incubate the solution at 94 °C for 5 minutes (denaturation), then at 65 °C for 15 minutes (annealing) and then at 72 °C for 99 minutes (synthesis) (Thermo Scientific, Arktik Thermal Cycler). The dsDNA was examined on an agarose gel (2%) in 1X TAE Buffer (50X TAE Buffer: 242 g of Tris-base, 100 mL of EDTA (0.5 M, pH 8.0), 57.1 mL of Glacial acetic acid, distilled water up to 1000 mL). Finally, dsDNA was purified and its concentration was measured using a UV/Vis spectrophotometer (BioDrop, Nanodrop Technologies).

IVT (Beckert & Masquida, 2011) used the Apt-Get T7 transcription kit to make 2′FY-RNA (Roboklon, Berlin, Germany). 2 nmoles of dsDNA were mixed with dNTP Mix (5 mM ATP, 5 mM 2′F-UTP, 5 mM 2′F-CTP and 5 mM GTP), T7 RNA polymerase, and 10X reaction buffer in a tube (total volume is 25 µL for each tube). The mixture was incubated for 4 hours at 42 °C. After that, the dsDNA template was digested using 0.5 µl of DNAse I (GeneAll® Biotechnology, Seoul, South Korea) at 37 °C incubation for 30 minutes.

2.4 RNA Precipitation

The following protocol was used to make the IVT product. Final concentration of 0.143M Sodium Acetate was used to elute samples at a 1:10 ratio (Sample:0.143M C2H3NaO2). The solution was vortexed, and then 100% isopropanol was added in a 1:1 ratio. After vortexing again, the solution was incubated for an overnight period at -80°C. The materials were then centrifuged at 13,000 g for 0.5 hours at 4 °C to collect the precipitate. The pellet was then washed with 70% ethanol and centrifuged

at 13,000 g for 15 minutes at 4 °C. Pellet was resuspended in 50 µl of RNase-free water to complete elution.

2.5 SELEX

To refold 2'FY-RNA pool, 1X PBS was used as a refolding solution (0.0018 M KH2PO4, 0.137 M NaCl, 0.01 M Na2HPO4, 0.0027 M KCl,). 5 µM of 2'FY-RNA was refolded in 1X PBS at the beginning of the SELEX by heating to 95 °C for two minutes and slowly cooling to room temperature for an hour. The pool was then incubated in an Eppendorf tube at room temperature for 15 minutes with $2.5x10^5$ virus particles kept in DMEM without FBS. The incubation solution (which included 2'FY-RNA with virus particles) was sucked onto a nitrocellulose membrane that had been pre-wetted with PBS. To eliminate unbound oligos, the nitrocellulose membrane was washed with 15 mL 1X PBS under vacuum. By heating 1M Urea to 55 °C for 15 minutes, the bound 2'FY-RNA oligos were eluted from the membrane and precipitated by following the RNA precipitation method explained previously.

2.5.1 *In vitro* Transcription (IVT) and Reverse Transcription PCR (RT-PCR)

80 µl of RNase-free water was used to resuspend the precipitate obtained after. Then, using reverse transcription-polymerase chain reaction (RT-PCR), the eluted 2'FY-RNAs were amplified (Bachman, 2013). The T7 polymerase sequence was annealed to the 2'FY-RNA sequence in the reverse transcription stage by heating at 65 °C for 5 minutes and cooling at 4 °C for 5 minutes. The reverse transcription procedure was then initiated using a thermocycler (heating samples to 65 °C for 1 hour, then heating to 85 °C for 5 minutes). The results were separated on a 1 percent agarose gel in 1X TAE Buffer after RT samples were amplified by PCR (Denaturation: at 93°C for 3 min; Annealing: 93°C for 30 sec., 65 °C for 1 min, 72°C for 1 min.; Extension: 72 °C for 10 min). The PCR products were cleaned with a PCR Purification kit

(QIAGen, Germantown, MD, USA), and in vitro transcription was proceeded with a Roboklon Apt-Get 2'F T7 Transcription Kit (Roboklon GmbH, Berlin, Germany) incubation solution at 42°C for 8 hours.

To improve the selection stringency on oligos, the amount of 2'FY-RNA and virus particles were reduced by about 20% for each cycle of SELEX. In addition, depending on the PCR products shown on the agarose gels, PCR cycles were adjusted between 8 and 13. A counter selection was used in the fourth round to eliminate membrane binding oligos. During the SELEX, a total of seven rounds were applied.

2.6 Competent Cell Preparation

A single colony of the *E. coli* BL21 cell line was inoculated into 3 mL Luria Broth (LB) medium (10g Bacto tryptone, 5 g yeast extract and 10g NaCl in 1L water and the pH was adjusted to 7.2) and incubated overnight at 37 °C. The culture was then used to inoculate 200 mL LB medium and incubated at 37 °C until the OD600 became 0.4-0.6. The culture was incubated on ice for 15 minutes and the cells were collected by centrifuging for 5 minutes at 4°C at 3500 rpm.

The supernatant was discarded and the pellet was resuspended in 20 mL ice-cold Buffer1 (30mM CH3CO2K, 15% Glycerol, 100 mM RbCl, 10 mM CaCl2 and the pH is adjusted to 5.8 with dilute acetic acid and filter sterilized). The resuspended cells were then kept on ice for 15 more minutes before centrifugation for 5 minutes at 4°C at 3500 rpm. The pellet was gently resuspended in 8 mL of ice-cold Buffer2 (75mM CaCl2, 15% Glycerol, 10mM MOPS, 10mM RuCl, pH is adjusted to 6.5 with 0.2 M KOH and filter sterilized). Then, for the next 30 minutes, 100 µL aliquots were incubated on ice. After incubation, aliquots were stored at -80 °C (Hanahan, 1983).

2.7 PCR Cloning

CloneJET PCR Cloning Kit (Thermo Scientific; #K1232) was used for PCR cloning, and the protocol supplied with the kit was followed. In the blunting reaction set up, a SELEX 7th round PCR product was used as a PCR product. After a 5-minute incubation period at 70°C, the samples were cooled on ice. A ligation reaction containing the ampicillin resistance gene and pJET1.2/blunt Cloning Vector (50 ng/μL) was set up and mixed with the blunting reaction mixture. After 5 minutes of incubation at 22 °C, the ligation reaction mixture was ready for transformation.

2.8 Transformation

Chemically competent *E. coli* BL21 cells were chosen for transformation. On ice, 120 μL of competent cells were thawed. Then 20 μL of ligation reaction was added and the tube was gently tapped for mixing. After 40 minutes on ice, cells were incubated for 45 seconds at 42°C before being placed on ice for another 10 minutes of incubation. Following the heat shock, 880 μL sterile LB was added to the mixture and incubated for 1.5 hours at 37 °C in a 210 rpm shaker (Hanahan 1983). Eventually, using the spread plate method, 250 μL of cultured cells were inoculated into LB Agar plates with 100 μg/mL ampicillin (total of 4 plates).

2.9 Plasmid Isolation

100 clones were chosen for plasmid isolation using the Miniprep Plasmid isolation kit (Machery-Negel Company, Catalog#740588.50). The concentrations of isolated plasmids were measured using BioDrop and prepared for Sanger sequencing (Sanger, Nicklen, & Coulson, 1977).

2.10 Sanger Sequencing

INTERGEN company used reverse primer 5’AAGAACATCGATTTTCCATGGCAG-3’ to perform Sanger sequencing (Sanger et al. 1977) (ABI, 3130XL Genetic Analyzer).

2.11 Secondary Structure Prediction of ProtoAptamers and Family Analysis

For further study, proto-aptamer sequences were aligned using their full sequences. CLUSTAL 0(1.2.4) multiple sequence alignment (Sievers et al., 2011) was used to align the sequences, and ClustalW2 was used to create the phylogenetic tree (Larkin et al., 2007). After utilizing the RNAfold website (Hofacker, 2003) to predict the 2D structure of oligos without modifying the default parameters, eight of the oligos were chosen as aptamer candidates based on their structures and sequence similarities.

2.12 Aptamer Screening

The oligos were synthesized, then annealed to generate dsDNAs, which were then transformed to 2'FY RNA by using IVT process. The RNA precipitation technique was used to precipitate IVT products. Then, at room temperature in an Eppendorf tube, 0.5 M of RNAs were treated for 10 minutes with $1x10^5$ virus particles kept in DMEM without FBS, according to the SELEX methodology. Following the collection of bound oligos, a 15-PCR cycle RT-PCR procedure was used. In 1X TAE Buffer, PCR products were separated on a 2 % agarose gel.

2.13 CCHFV Binding Assay

In order to see the virus binding, the same protocol was done after better binding oligos were determined. The oligos were incubaterd with DMEM with 10^5 number

of virus particles. The same SELEX and RT-PCR protocols were done. After SELEX and RT-PCR, PCR products were separated 2% agarose gel with 1X TAE Buffer.

CHAPTER 3

RESULTS & DISCUSSION

3.1 2’FY RNA Aptamer Selection Against CCHF particle

3.1.1 Primer and Oligonucletide Library Design

A 2'Fluoropyrimidine (FY)-RNA pool was created using a ssDNA library with constant primer binding regions and a core region with random sequences in the center (Shubham et al., 2018). One of the most important aspects in successfully selecting aptamers against the target of interest is pool design. Since PCR is so important in aptamer selection, the primer design for the initial library is critical. The number of bases in forward and reverse primers should be 16-28, with melting temperatures (Tm) ranging from 50°C to 62°C and being within 5°C of one another (Wallace et al., 1979). If the difference is greater, amplification will be less efficient or may not work at all because the primer with the higher Tm will misprime at a lower than optimal annealing temperature, and the primer with the lower Tm will only bind in low concentrations at a higher than optimal annealing temperature (Apte & Daniel, 2009).

Another factor to consider is the G and C content of the primer, as these bases form strong hydrogen bonds with one another, increasing the primer's stability and resulting in a greater PCR yield. Repeating G or C bases, on the other hand, can result in the formation of primer-dimers. As a result, the primer's GC concentration should be between 40% and 60%, also repeated G and C bases should be minimized (Chuang, Cheng, & Yang, 2013). Furthermore, a balanced ratio of AT-rich and GC-rich domains is essential to prevent the production of self-dimers/hairpins or primer-

dimers and to improve PCR efficiency (Wallace et al., 1979). The library and primers used to synthesize the 2'FY-RNA pool for aptamer selection against CCHF particles were designed with these concerns.

3.1.2 Synthesis of the 2'FY RNA Pool

In vitro studies show that RNA aptamers have a short half-life in plasma, compared to DNA aptamers, which have a half-life up to 1 hour (E. Wang, Wu, Niu, & Cai, 2011). Since RNA molecules have 2'-hydroxyl group (2'-OH) on their structure, they have shorter half life. Furthermore, endoribonucleases that play a role in RNA catalysis, such as RNase-A, target the hydroxyls. As a result, modifications of the pyrimidine at 2' position of RNA aptamers are common (Patra et al., 2012).

To increase the stability of the RNA aptamer these modifications can be applied during both SELEX protocol or designing of the library. Modified RNA aptamers might be parallel or can even outperform DNA aptamers in terms of stability. Affinity changes may occur if aptamers are modified after SELEX (Lakhin et al., 2013). As a result, we created the first pool as 2'FY-RNA from a dsDNA pool in order to select aptamers that would not have stability issues in their final usage.

3.1.3 SELEX

In our study, chemically inactivated CCHF particles were used to select aptamers. The goal of using complete viral particles was to select aptamers against virus glycoproteins in their naturaal forms, that increased aptamer use success rates in vivo and in vitro (Ohuchi, 2012). Another important parameter of the increasing the success is content of the selection buffer. Selection buffer has a direct impact on aptamer structure and thus affinity (Ilgu & Nilsen-Hamilton, 2016). Therefore we selection buffer used in the study is similar to human serum, to maket he selected aptemers usable in the human body for therapeutic purposes or in human serum outside of the body for diagnosis. The effect of

temperature on aptamer folding is also significant for aptamers (Ilgu & Nilsen-Hamilton, 2016). In order to use the selected aptamers in vitro, the incubation temperature was set to room temperature.

5 nmol 2'FY-RNA was incubated with $2.5x10^5$ chemically inactivated CCHF particles in the first step. Then, after each cycle of incubation, the oligo and viral particle amounts, as well as the incubation period, were gradually reduced to increase the selection pressure for the best binders' success.

The number of PCR cycles is another critical parameter in SELEX. Every SELEX round includes PCR cycle optimization to avoid the synthesis of longer and shorter sequences, as well as increased amplification of non-specific binders, which can reduce PCR and SELEX efficiency (Musheev & Krylov, 2006). As a result, we calculated amplification cycles for each SELEX round by performing PCR on a small fraction of the bound oligos collected and analyzing the results on agarose gels. We used PCR for the rest of the material, including the reverse transcription product, once we reached the appropriate cycle number.

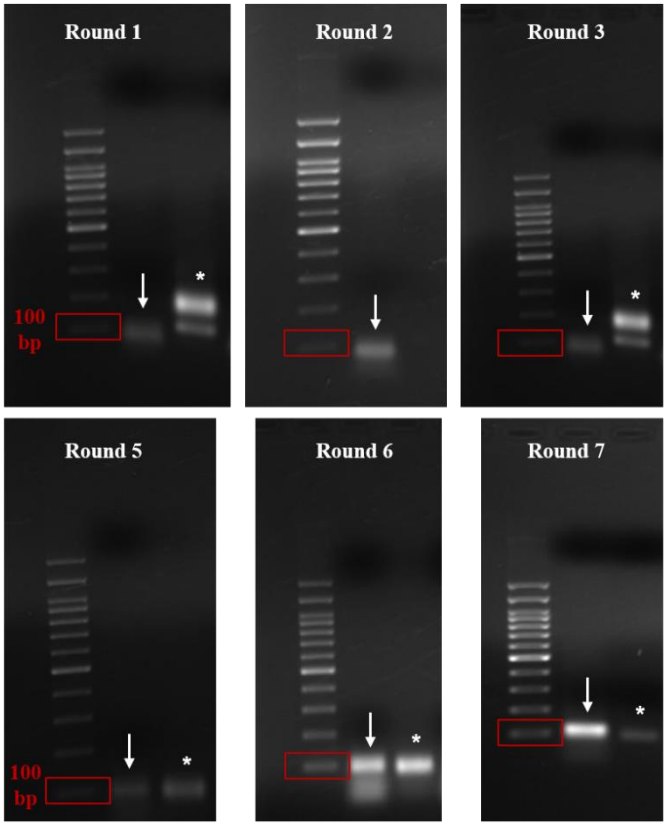

Figure 3.1. SELEX Rounds PCR Results Asterisk shows control band formed by PCR of 117 nucleotide sample, whereas arrows show band of PCR results.

3.2 Analysis of the Sequences

After SELEX was completed, the PCR products from the last round (7th round) were ligated into pJET1.2/blunt Cloning Vector and seeded into four agar plates using BL-21 cells. A total of 100 colonies were randomly selected from these plates. Plasmids were extracted from bacteria on these plates and sent to the INTERGEN to be sequenced by using the Sanger Sequencing method.

Because of insufficient plasmid isolation and ligation efficiency might result in missing or low signal, indicating incorrect or missing sequences in Sanger Sequencing results. After evaluating the signals, we collected 87 of the 100 oligo

sequences and constructed a phylogenetic tree for sequences to examine sequence similarity using the ClustalW OMEGA website (Larkin et al., 2007). Based on sequence similarity, the server generated a phylogenetic tree with 3 main branches (Figure 3.2). Then, to test the tree's efficiency, we matched some sequences from Branch-3 with CLUSTAL 0(1.2.4)(Sievers et al., 2011). Figure 3.3 shows the sequence similarities and similarity rates of oligos that are selected to test. As expected, oligos from the same sub-branch had more similarity than oligos from other branches.

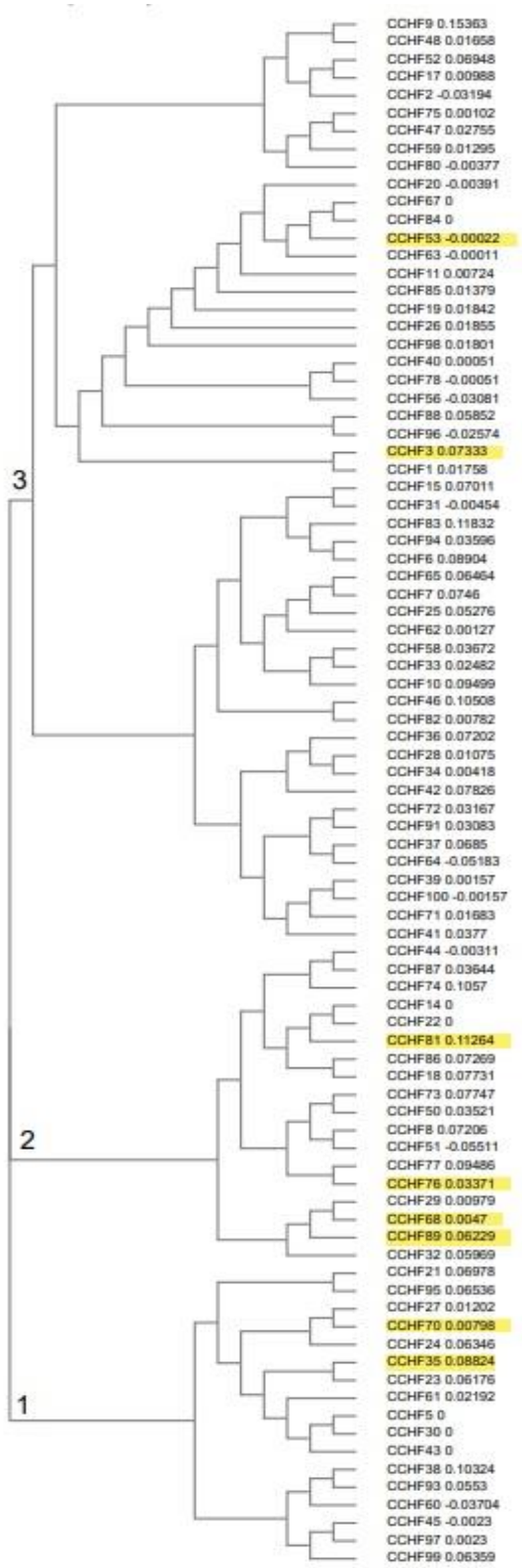

Figure 3.2. Phylogenetic Tree of Oligo Pool. Yellow oligos are selected to test.

	CCHF53	CCHF70	CCHF35	CCHF81	CCHF76	CCHF3	CCHF68	CCHF89
CCHF53	100.00	74.71	71.59	68.48	79.71	80.00	83.82	79.12
CCHF70	74.71	100.00	77.42	71.91	82.35	74.12	80.60	75.58
CCHF35	71.59	77.42	100.00	74.42	81.54	68.97	82.09	74.42
CCHF81	68.48	71.91	74.42	100.00	79.71	69.32	82.09	76.40
CCHF76	79.71	82.35	81.54	79.71	100.00	82.86	90.48	80.00
CCHF3	80.00	74.12	68.97	69.32	82.86	100.00	82.61	75.82
CCHF68	83.82	80.60	82.09	82.09	90.48	82.61	100.00	92.19
CCHF89	79.12	75.58	74.42	76.40	80.00	75.82	92.19	100.00

Figure 3.3. Similarity Rates of Selected Oligos to be tested.

3.2.1 Prediction of Secondary Structures

8 proto aptamers were chosen from 3 main branches, with the number of oligos chosen from each branch based on its size. Then, using the RNAfold online platform, we obtained the 2D structures of the oligos (Hofacker, 2003). The sequence of nucleotides and their interactions determine the 2D structure of an oligo (Figure 3.4). As expected, oligos from the same or very related branches have comparable structures, but oligos from other branches have quite distinct structures.

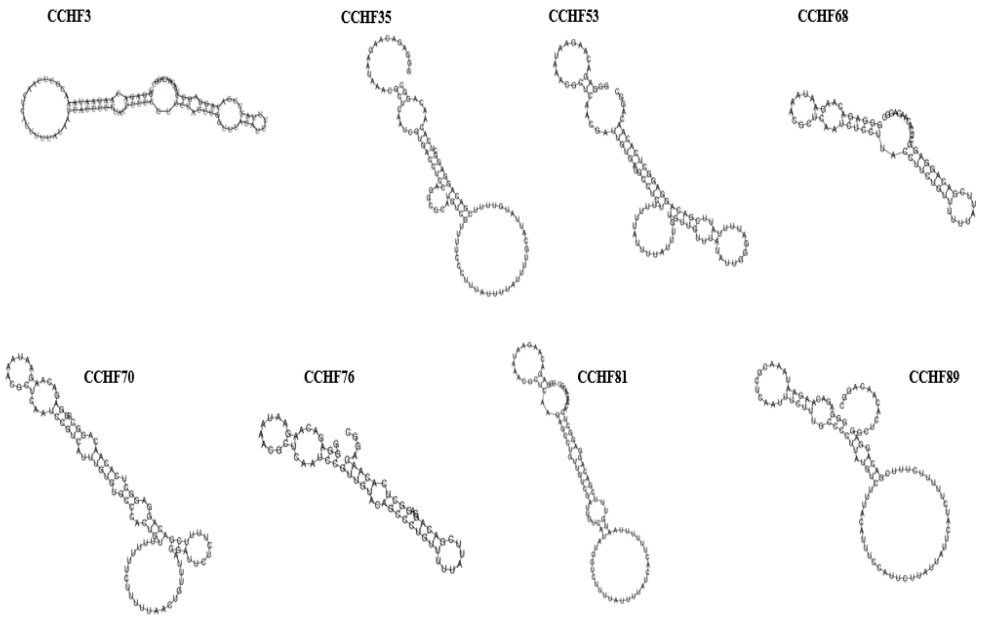

Figure 3.4. 2D Structure of Oligos Selected for Testing.

3.3 Characterization of Aptamer Candidates

3.3.1 Determination of Best Binders by CCHFV Binding Assay

We examined these eight oligos with diluted (1:10) virus particles to see if there were any changes in specificity and binding affinity between oligos from various branches and with varied nucleotide sequences. By looking at the results we can say that oligos CCHF3 and CCHF53 binds to the virüs particle more efficient.

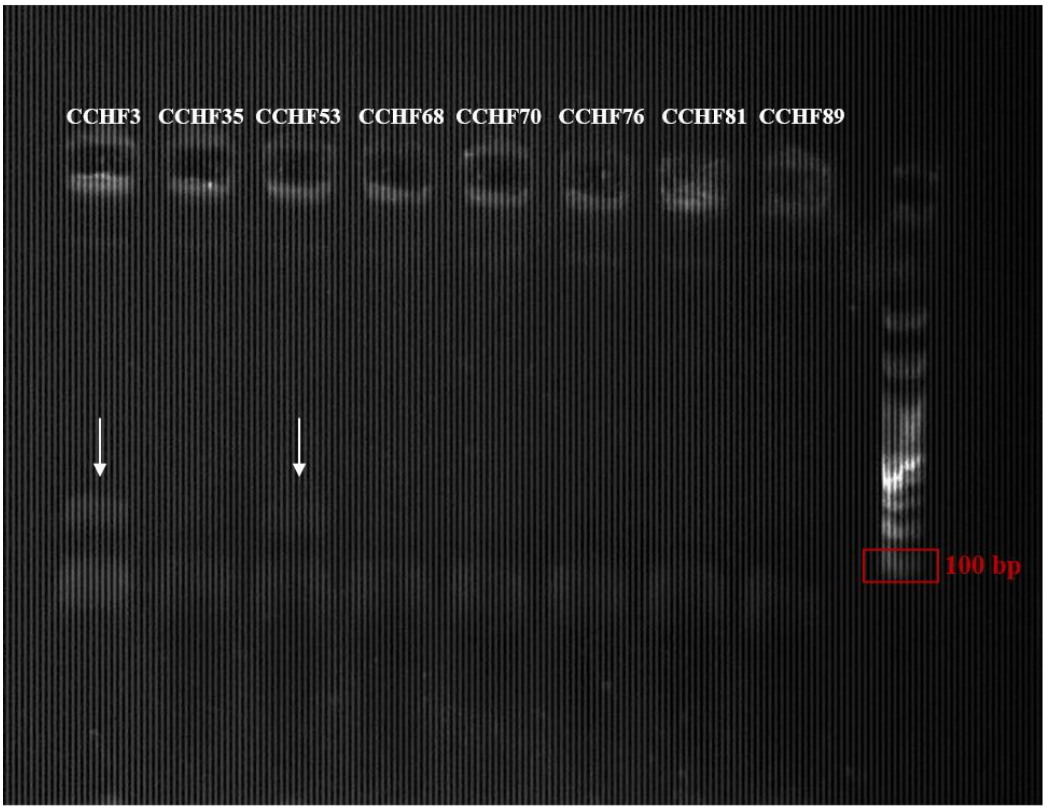

Figure 3.5. PCR Results of Selected Oligos for Testing The Hundred nucleotide region of the ladder is indicated by a red square.

Theoretically, oligos with similar structures should have similar binding patterns. The findings supported this assumption; the structures of the best binder aptamers are similar.

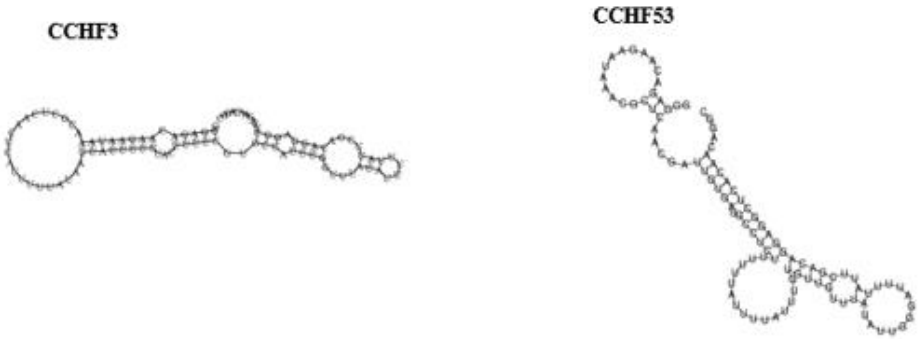

Figure 3.6. 2D Structure of Oligo CCHF3 and CCHF53.

CHAPTER 4

CONCLUSIONS AND FUTURE PRESPECTIVES

Aptamers are preferred biomolecules for therapeutic and diagnostic techniques due to their current use and high potential. Despite the fact that therapeutic uses are uncommon, their increased specificity and affinity make them effective biorecognition elements.

We were able to generate two different aptamers, CCHF3 and CCHF53, in this thesis. When compared to the aptamer CCHF3 and CCHF53 bind to CCHFV particles, CCHF3 binds more effectively. This divergence could be due to the SELEX procedure's use of the viral particle's natural conformation. As previously stated, natural conformations of the targets boost the likelihood of efficiently selecting aptamers.

Aptamers are chosen at random from large libraries, and structural data indicates that only a small portion of the sequence is required in direct interaction with the target. Because there are lots of benefits to reducing the size of the aptamer, a quick approach for determining which parts of the sequence are crucial for target binding would be quite valuable. Therefore in further studies truncation of the aptamers could be done.

Moreover, to see whether these aptamers could be used as the therapeutic agent, docking results could be checked.

In light of these findings and future researches, we believe that our aptamers have the potential to be utilized in diagnostic kits, and that one or both of them might be used as therapeutic agents.

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APPENDICES

A. In vitro Transcription Reaction

Reaction Components	Amount (µL)
2'F-Py NTP Mix (25 mM)	1.5
5X Reaction Buffer	5
DNA Template	1 µg
Apt-Get 2'F-T7 RNA Polymerase	Fill up to 25 µL
dH2O	Fill up to 25 µL
Total Volume (for each tube)	25 µL