의학박사 학위논문

# Emergence and spread of neutralization-escaping MERS-CoV during 2015 Korea outbreak and antibody responses in the recovered patients

# 2015년 한국 MERS 유행 시 중화항체 회피 변이의 발생과 회복 환자들의 항체반응 분석 연구

2023년 2월

서울대학교 대학원

의과학과 의과학 전공

Aigerim Abdimadiyeva

A thesis of the Degree of Doctor of Philosophy

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The Department of Biomedical Sciences
Seoul National University College of Medicine
Aigerim Abdimadiyeva

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지도 교수 조 남 혁

이 논문을 의학박사 학위논문으로 제출함
2022년 10월

서울대학교 대학원
의과학과 의과학 전공
Aigerim Abdimadiyeva

Aigerim Abdimadiyeva의
의학박사 학위논문을 인준함
2023년 1월

위 원 장 _____ (인)

부위원장 _____ (인)

위 원 _____ (인)

위 원 _____ (인)

위 원 _____ (인)

# Emergence and spread of neutralization-escaping MERS-CoV during 2015 Korea outbreak and antibody responses in the recovered patients

**Submitting a Ph.D. Dissertation of Medicine**

**October 2022**

**Major in Biomedical Sciences**
**Department of Biomedical Sciences**
**Seoul National University Graduate School**

**Aigerim Abdimadiyeva**

**Confirming the Ph.D. Dissertation written by Aigerim Abdimadiyeva**

**January 2023**

| | |
|---|---|
| Chair | _____(Seal) |
| Vice Chair | _____(Seal) |
| Examiner | _____(Seal) |
| Examiner | _____(Seal) |
| Examiner | _____(Seal) |

# Abstract

The unexpectedly large outbreak of Middle East respiratory syndrome in South Korea in 2015 was initiated by an infected traveler and amplified by several ″superspreading″ events. It has been reported the emergence and spread of mutant Middle East respiratory syndrome coronavirus bearing spike mutations (I529T or D510G) with reduced affinity to human receptor CD26 during the outbreak. To assess the potential association of spike mutations with superspreading events, I collected virus genetic information reported during the outbreak and systemically analyzed the relationship of spike sequences and epidemiology.

Zoonotic coronaviruses have emerged as a global threat by causing fatal respiratory infections. Given the lack of specific antiviral therapies, the application of human convalescent plasma retaining neutralizing activity could be a viable therapeutic option that can bridge this gap. I have traced antibody responses and memory B cells in peripheral blood collected from 70 recovered Middle East respiratory syndrome coronavirus (MERS-CoV) patients for 3 years after the 2015 outbreak in South Korea and used a mouse infection model to examine whether the neutralizing activity of collected sera could provide therapeutic benefit in vivo upon lethal MERS-CoV challenge.

Anti-spike-specific IgG responses, including neutralizing activity and antibody-secreting memory B cells, persisted for up to 3 years, especially in MERS patients who suffered from severe pneumonia. In general, antibody titers gradually decreased annually by less than 2-fold. Levels of antibody responses were significantly correlated with fever duration, viral shedding periods, and maximum viral loads observed during infection periods. In a transgenic mice model challenged with lethal doses of MERS-CoV, a significant reduction in viral loads and enhanced survival was observed when therapeutically treated with human plasma retaining a high neutralizing titer (>1/5000). However, this failed to reduce pulmonary pathogenesis, as revealed by pathological changes in lungs and initial

weight loss. High titers of neutralizing activity are required for suppressive effect on the viral replication but may not be sufficient to reduce inflammatory lesions upon fatal infection. Therefore, immune sera with high neutralizing activity must be carefully selected for plasma therapy of zoonotic coronavirus infection.

**Keyword:** Middle East respiratory syndrome coronavirus, MERS-CoV, superspreading, neutralizing antibody, plasma therapy, respiratory infections, viruses, zoonoses

**Student Number:** 2013-31362

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# List of Abbreviations

| | |
|---|---|
| **ACE2:** | Angiotensin-converting enzyme 2 |
| **ADCC:** | Antibody-dependent cellular cytotoxicity |
| **ADCP:** | Antibody-dependent cellular phagocytosis |
| **ADE:** | Antibody-dependent enhancement |
| **CCL2:** | C–C motif chemokine ligand 2 |
| **CCL3:** | C–C motif chemokine ligand 3 |
| **COVID-19:** | Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) |
| **CXCL10:** | C-X-C Motif Chemokine Ligand 10 |
| **DC:** | Dendritic cells |
| **DPP4:** | Dipeptidyl peptidase 4 |
| **ELISA:** | Enzyme-linked immunosorbent assay |
| **ELISPOT:** | Enzyme-linked immune absorbent spot |
| **HCoV:** | Human coronavirus |
| **IgA:** | Immunoglobulin A |
| **IgG:** | Immunoglobulin G |
| **IgM:** | Immunoglobulin M |
| **IFN-γ:** | Interferon gamma |
| **IL:** | Interleukin |
| **MERS-CoV:** | Middle East Respiratory Syndrome-Coronavirus |
| **MCP-1:** | Monocyte chemoattractant protein-1 |
| **MHC:** | Major histocompatibility complex |
| **MIP-1α:** | Macrophage inflammatory protein-1 alpha |
| **OD:** | Optical density |
| **$PRNT_{50}$:** | 50% plaque reduction neutralization test |
| **ppNT:** | Pseudoparticle neutralization assay |
| **PBMC:** | Peripheral blood mononuclear cell |
| **RBD:** | Receptor-binding domain |
| **RT-PCR:** | Reverse transcription polymerase chain reaction |

**SARS-CoV:** Severe acute respiratory syndrome coronavirus

**TGF-β:** Transforming growth factor beta

**Th cell:** T helper cell

**TNF-α:** Tumor necrosis factor alpha

**WHO:** World Health Organization

# 1. Introduction

The first information about human coronaviruses is dated 1960s. There were several groups of scientists working with coronaviruses. A group headed by D. A. Tyrrell succeed in the cultivation of B814 virus obtained from patients with common colds [1]. Hamre and Procknow isolated a virus called 229E from medical students and managed to grow a virus with unusual properties in tissue culture from samples obtained with colds, which they called [2]. B814 and 229E that caused human upper respiratory disease were not described in the original manuscripts as viruses belonging to the Coronaviridae family. Later, when the comparison of the morphology of viruses was done with the use of negative staining technique by electron microscopy, their similarity in general shape and spikes as well as their morphologic resemblance with avian infectious was revealed [3]. Other human strain of coronavirus that called OC43 was recovered in 1967 and its serologic studies and studies of other five viruses bearing identical morphological features to infectious bronchitis virus (IBV) and mouse hepatitis were carried out in mice model [4]. In 1968 there was a publication in Nature claiming that an informal group of virologists recognized a new group of viruses on the base of properties shared by the representatives, such as appearance, recalling the solar corona, characteristic surface structure, apparent ribonucleic acid content, essential lipid and replication in cytoplasmic vesicles. In the publication the IBV-like novel group of viruses afterwards was officially accepted as a new genus of viruses called coronavirus as there was the crown-like morphology [5]. Later, other human coronaviruses have been identified. Severe acute respiratory syndrome coronavirus (SARS-CoV-1) caused the infection with such frequently found symptoms as fever, chills, myalgia, and cough. This newly emerged coronavirus caused an outbreak in 2003 in Asia, having been firstly identified in China, and later it spread in other countries worldwide [6-8]. During the SARS-CoV-1 outbreak in 2003, the number of people affected exceeded eight thousand people. The estimated mortality level was a bit higher that 700 victims (about 10%) and was higher than 50% among 60-year aged patients [9]. The advances in scientific methods and techniques made in the XX$^{th}$ century allowed to identify the

SARS-CoV-1 genome in the same 2003 year [10]. The SARS-CoV-1 was shown to has a zoonotic nature being spread from mammals to humans and then from human to human [11]. There are other human coronaviruses that have been identified so far, including HCoV-NL63 (2003), HCoV-HKU1 (2004), MERS-CoV (2013), and SARS-CoV-2 (2019). The Human coronavirus HCoV-NL63 was first isolated by Dutch scientists in Amsterdam in 2004 from an infant suffering from bronchiolitis and conjunctivitis [12]. It was proposed that HCoV NL63 affected people for a while, differently from the SARS-CoV-1, which has only recently been introduced to the human population [13]. Later, the HCoV NL63 virus has subsequently been spread among other countries worldwide [14-17]. The next new strain belonging to Coronaviridae family was HCoV-HKU1 found in Hong Kong in 2005 in the patient hospitalized with pneumonia in the nasopharyngeal aspirates [18]. There were reports about the presence of HCoV-HKU1 in 2004-2005 in other countries as well [19-21]. In 2012, a new zoonotic MERS-CoV was identified in Saudi Arabia from the sputum of a man with acute pneumonia. This coronavirus caused the Middle East respiratory syndrome (MERS) outbreak in Saudi Arabia and Qatar in 2012 [22]. Later, the cases of MERS-CoV have been found in 27 countries in the Middle East, Africa and South Asia, that had a total of 858 registered deaths [23]. MERS-CoV virus is reported to have more likely evolutionary originated in bats, while the camels represented to be recipients of MERS-CoV and therefore acted as sources of virus for humans [24]. In spite of the fact that except for 2003 SARS-CoV-1 and 2012 MERS outbreak, most human coronaviral infections (HCoV-HKU1, HCoV-NL63, HCoV-OC43 and HCoV-229E) were relatively mild, causing common cold. The most recent type of coronavirus emerged in 2019 in Wuhan, China: Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), or COVID19, caused an outbreak declared globally a pandemic [25, 26]. COVID19 is a highly contagious viral infection that so far has initiated over 178 million cases with 3,4 million reported cases of deaths [27, 28]. Researches dedicated to newly emerged SARS-CoV-2 are ongoing, and there are many knowledge gaps about its mechanism of action, immune response, mutated types. The phylogenetic analysis of the complete viral genome indicates the high genetic similarity to SARS-like coronaviruses that has bat origin [26, 29]. Thus, it is supposed that SARS-CoV-2 can be originated as a result of natural selection in bats as animal hosts before

transmission to a human or via selection during undetected human-to-human transmission [30]. The predominant clinical symptoms of the patients suffered from severe acute respiratory syndrome coronavirus 2 infection are associated with fever, cough, breathing difficulties, and pneumonia, which may result in progressive respiratory failure, and even death [26].

Coronaviruses are a group of viruses belonging to the Nidovirales order, Cornidovirineae suborder that contains only one viral Coronaviridae family. Among eight suborders of the Nidovirales order, Cornidovirineae group includes the most epidemic viruses. According to the International Committee of Taxonomy of Viruses (ICTV) the family Coronaviridae is organized in 2 subfamilies (Letovirinae and Orthocoronavirinae), 5 genera, 26 sub-genera, and 46 species [31, 32]. The Coronavirinae includes four genera: Alphacoronavirus (α), Betacoronavirus (β), Gammacoronavirus (γ), and Deltacoronavirus (δ) [33]. Coronaviruses infect a wide range of animals; α- and β- coronaviruses infect mammals, while γ- and δ- coronaviruses mostly infect birds [29]. Described above human coronaviruses 229E and NL63 strains belong to the alphacoronavirus genus. While HCoV-OC43, HCoV-HKU1, SARS-CoV-1, MERS-CoV and SARS-CoV-2 are included in beta-coronaviruses genus [34 - 36]. Thus, currently, there are seven types of coronavirus that can infect human. Two of them (229E and NL63) are alpha coronaviruses, while the other five represent beta coronavirus. Three of the existing human coronaviruses caused infection outbreak on large scale: SARS-CoV-1, MERS-CoV, and SARS-CoV-2. Viruses of the Coronaviridae family are enveloped, positive sense single-stranded RNA viruses. They are one of the largest known RNA viruses, with genome size ranging from approximately 25 to 32 kb. Coronavirus virions are with the size varying about 118-140 nm in diameter with surface spike glycoproteins, which gave to this viral family a crown-like appearance [33, 36].

MERS-CoV emerged in 2012 in Saudi Arabia. It was called the Middle East Respiratory Syndrome-Coronavirus, as it caused an outbreak in the Middle East region that has spread across 27 countries, including Saudi Arabia, United Arab Emirates, Kuwait, Iran, Oman, Yemen, Lebanon, France, United Kingdom, Italy,

Germany, Tunis, Malaysia, Greece, the Netherlands, Egypt, South Korea and the USA [37]. 2574 cases of MERS-CoV were confirmed in laboratories by the end of May 2021. The mortality rate is 34.4%, which means that 886 deaths have been associated with MERS-CoV viral infection to date [38]. In spite the fact that there is a plenty of published studies devoted to mechanisms of epidemiology, evolution, and pathogenesis of MERS-CoV, SARS-CoV-1 and -2, these aspects are still remained unclear, and further research is necessary. All human coronaviruses are likely originated from wild animals. HCoV-NL63 and HCoV-229E, MERS-CoV, and SARS-CoV-1 were originated in bats, while HCoV-OC43 and HCoV-HKU1 have rodent origin [39, 40]. However, evolutionary MERS-CoV is closely related to coronaviruses found in bats [41]. Bats are known to chew fruits to extract the sugars as an energy component and spit out the remains that may be contaminated by biological fluids and therefore can serve as a sourse of viruses [42]. As bats rarely have close contact with people, spillover of viruses from bats can happen through intermediate animal hosts, such as pigs, civets, and other [43]. There are some studies indicating that humans could get infected with MERS-CoV from the intermediate host *Camelus dromedarius*, the dromedary camels [44, 45]. Series of studies presented data to confirm that MERS-CoV was transmitted by camels [46]. Some of them determined protein-specific antibodies against MERS-CoV spike in blood sera of camels in multiple locations, which supports the hypothesis that camels acquired MERS-CoV at some period (some specimens are dated by 1990s), and later the virus spread and was circulating among camelids [47 - 49]. Then detection of MERS-CoV was conducted by RT-PCR in rectal and nasal swabs taken from dromedary camels from different regions [50- 52]. In addition, it was revealed that MERS-CoV strains isolated from camels were almost identical genetically to human MERS-CoV. One nucleotide difference was revealed by alignment of MERS-CoV DNA fragments from camels and people [44]. This serves as strong evidence of transmission of MERS-CoV between dromedary camels and humans. As for the transmission of MERS-CoV in humans and camels, four possible routes of its transmission are suggested, namely, camel-to-human, human-to- human, camel-to-camel, and human-to-camel [53].

MERS-CoV belongs to betacoronaviruses, lineage C found in humans and camels that is different from the other human beta coronaviruses [54], and is known as the

most lethal human coronavirus infection so far. Transmissibility rate among people is not so high as MERS-CoV has been found to circulate mostly among camel populations. As for human cases, they tended to be spread between individuals who were in close contact with the infected ones. Clinical symptoms of MERS-CoV range from mild upper respiratory to multi-system failure and pneumonia. The incubation period of MERS-CoV range is 2-14 days, median is about 5.5-6.5 days [55]. The average age of the patients suffered from MERS-CoV is around 55 years.

The functional receptor of MERS-CoV is dipeptidyl peptidase 4 (DPP4 or CD26) and its recognition is a key determinant of viral host range. DPP4 is a type-II transmembrane glycoprotein which expression is high on bronchial epithelium and macrophages [56]. It has been found that such DPP4 orthologs as mouse, hamster and ferret cell lines are not permissible to MERS-CoV, its DDP4 is not a functional receptor, while human, camel, porcine, civet, horse and bat DPP4 can be recognized by MERS-CoV [57 - 60]. DDP4 receptors were found to be expressed in various human cell lines of kidney, liver, spleen, brain, heart, lung, and intestine [61 - 63]. The size of MERS-CoV genome is 30,119 nucleotides with 10 predicted open reading frames, nine of which are possibly expressed from a nested set of seven subgenomic mRNAs [64]. The replicase gene encodes 16 non-structural proteins (nsp 1-16) that accounts approximately two-third of the genome [65]. In addition, the genome encodes the spike (S), envelope (E), membrane (M), and nucleocapsid (N) structural proteins [64]. M and E proteins are involved in viral assembly; N protein is required for RNA synthesis. The spike protein is a crucial for cross-species transmission and induces viral pathogenesis. The severity of infection has been shown to be influenced by the mutations in receptor binding domain localized on spike protein [37, 66].

It has been found that *in vitro* MERS-CoV could also infect human immune T cells, dendritic cells and macrophages bearing DDP4 receptors, which may lead to dysregulation of the immune system [67, 68], as MERS-CoV replication in immune cells shields the virus from host immunorecognition. T cells play a significant antiviral role: CD4+ T cells promote the production of virus-specific antibodies through the activation of B cells. However, cytotoxic CD8+ T cells kill virus-infected cells. MERS-CoV has been shown to be capable of infecting human T cells from spleen, peripheral blood, and tonsil. The infection of T cells by

MERS-CoV leads to T cell extrinsic and intrinsic apoptosis, which might potentially disrupt anti-viral T cell responses. In addition to T cell apoptosis, the cytokine dysregulation in severe MERS-CoV cases has been revealed as a result of an aberrant expression of inflammatory cytokines [69]. Production of pro-inflammatory cytokines occur while the virus replicates in macrophages and dendritic cells (DC). For instance, there has been identified little or delayed expression of the antiviral cytokines (interferon α and β), whereas levels of IL-6, IL-8, IL-12, IFN-γ, and tumor necrosis factor α as well as molecules of MHCI were comparatively high in MERS-CoV-infected macrophages [68]. There is also the production of chemokines such as CXCL10, CCL2, CCL3 by DC or macrophages increases during MERS-CoV-infection was observed [68]. A significant upregulation in the expression of IL-17 by Th cells has also been identified. MERS-CoV infection stimulates Th17 to secrete cytokines, which recruit neutrophils and monocytes to inflammation site resulting in activation of other downstream cytokine/chemokine cascades (IL-1, IL-6, TNF-α, TGF-β, IL-8, and MCP-1). The active replication of MERS-CoV in the immune cells leading to immune dysfunction and cytokine storm may cause a comparatively high fatality rate of MERS disease and occurrence of systemic events, such as multi organ failure [70]. Still underlying immunopathology mechanisms are not fully understood, and further research is required.

Humoral immune response on MERS-CoV as antibody production was detected in two-three weeks after the onset of the infection. The time of antibodies persistent possibly correlates with the severity of disease: antibodies could be identified in 13 months after MERS-CoV infection in patients who had pneumonia, while low levels of MERS-CoV antibodies were found in case of mild infection. In addition, 6 years after the infection, antibody responses were revealed in 100% of MERS-CoV survivors who had severe or moderate disease and in half of survivors who had mild disease [71]. The correlation between neutralizing antibody titers and levels of CD4+ has also been shown. However, virus-specific CD8+ T cells were detectable in survivors experienced mild infections with undetectable antibody levels, indicating that rapid virus clearance may precede antibody-production and

CD4+ T cell response [72]. It has been reported that activated CD8+ T cells and anti-MERS-CoV antibodies are crucial for viral clearance as response to MERS-CoV is generally implemented through antibody-mediated immunity [73]. As for the antigen presenting type I and II pathway-related genes, including MHC, diverse results have been obtained demonstrating upregulation of MHC-related genes in one case, and downregulation in another [68, 74].

The MERS-CoV outbreak in South Korea has started on May 20, 2015, when the first case has been registered officially. South Korea had the largest number of MERS-CoV cases outside the Middle East, and therefore the only outbreak outside this region. It had a resemblance in clinical symptoms with SARS-CoV-1 that emerged in 2012. A large number of MERS-CoV cases was unexpected in this region, as there is one of the most advanced medical systems in the world and therefore it attracted the attention of the government, public, and researcher [75, 55]. In total, 186 cases (111 males and 75 females) have been confirmed during the MERS-CoV outbreak in Korea, including 38 deaths. 16, 993 people have also been suspected and put at quarantine by government for 14 days to control the spread of the infection [76]. Besides, over 2,700 schools were closed [77]. The registered cases with a definitive diagnosis are people who, regardless of clinical symptoms, had positive MERS-CoV laboratory tests from sputum samples, first by real-time RT-PCR with amplification targeting the upstream E region, and then by subsequent amplification of ORF1a [78]

The index case of MERS-CoV infection was diagnosed in a 68-year-old Korean businessman who traveled about 10 days in the Middle East, namely in Bahrain, the United Arab Emirate and the Kingdom of Saudi Arabia. Seven days after returning to Seoul, the patient got hypoxia, drowsiness, fever and myalgia. 11 days after his return a new symptom (cough) appeared. A consolidation in the right lung and a diffuse ground-glass opacity was seen in his chest X-ray, and, finally, the pneumonia and an acute respiratory distress syndrome has been developed. In spite of the antibiotic treatment, the pneumonia progressed. After the laboratory tests on MERS-CoV, the infection was confirmed on May 20 [79]. A maximum temperature of 39.5°C persisted until the 27$^{th}$ day. The patient was connected to the ventilator until the 37$^{th}$ day. After a successful antiviral treatment, the patient recovered.

The genome of the first registered case (KT326819) has 29,995 nucleotides in length with 10 ORFs. Most of all, it resembled (99.59%) the virus from Riyadh, Saudi Arabia, found in February 2015 (Riyadh_KKUH_0780_20150225). The nucleotide sequence identity compared to 93 genome sequences from the NCBI ranged from 97.8-99.6%. The most variable one was a gene ORF3 with identities of 96.1-100.0%. The gene encoding the spike protein, which is crucial for the interaction of virus with host cells, was identical up to 99.8-99.9%. As a result of the phylogenetic analysis, the Korean strain was clustered in the lineage 5 of locally spreading 2015 Riyadh strains [79].

This first case became a super-spreader, and because of the improper infection control measures it caused 36 secondary cases, six of which caused deaths due to MERS-CoV. Superspreaders are the patients who caused the superspreading events. Twenty-six cases occurred in the first generation, and 10 in the second generation. The median age of the 36 cases was 51 years (ranged from 16 to 86). This first generation cases are those who were exposed to patient zero (index case) and showed symptoms within two weeks. The second generation are people who were infected after being exposed to first-generation cases or, alternatively, to patient zero with symptoms appeared more than 14 days later [80]. The most common symptoms among all patients were fever (95.2%) and chills. Hypertension, diabetes, solid organ malignancy, and chronic lung disease have been identified as the most common coexisting medical conditions with a total of 55.4% of the patients having these symptoms [76]. Mechanical ventilation was applied to 24.5% of the infected patients and extracorporeal membrane oxygenation was applied to 7%. The factors associated with high mortality risk were age over 60, smoking, pre-existing pneumonia, renal dysfunction, and comorbid conditions [81]. As for mental health, 7.6% of the quarantined (1,656) had symptoms of anxiety [82]. The median incubation period was 6.83 days, while the incubation time varied significantly between 2 and 14 days. The infectious period is reported 1-11 day from the illness onset. In total, five superspreaders were revealed to be associated with 83% of the transmission events [77]. Because of the patient movement, the MERS-CoV infection has spread within 16 clinics and hospitals. No new cases have occurred since the last case identified on June 4.

The epidemic lasted 2 months till its official end on July 6, 2015 [76]. The economic losses of Korea amounted to 8.5 billion US dollars. The clinical features of MERS-CoV outbreaks in the Middle East and the Republic of Korea were similar, but the existence of superspreaders was not reported outside South Korea [83]. Although the outbreak unveiled the weak points of medical systems, the mortality rate (20.4%) was lower than the one in the Middle East. It is thought that enhanced supervision of patients probably has been resulted in improved outcomes [76]. The MERS-CoV transmission in South Korea in 2015 showed the importance of hospital protocols for treating infectious diseases as well as a necessity of global approaches to mitigate the spread of infections and the risk of travel-related epidemics [84].

Although humoral immunity, complement system activation, and T cell immune response to SARS-CoV-1, SARS-CoV-2 and MERS-CoV have been studied and described by the global scientific community, some mechanisms are still not enough clear and some data published is controversial. To sum up, some similarities and differences in immune response during MERS-CoV, SARS-CoV-1, and SARS-CoV-2 have been found. SARS-CoV-2 (COVID-19) is genetically highly homologous to SARS-CoV, and infection may result in a similar course of the disease. In particular, SARS-CoV-2 has 79.5% sequence identity to SARS-CoV-1, while MERS-CoV has sequence identity of only 50%. In general, an adaptive immune response to coronaviral infection includes such stages as presentation of the viral antigens by dendritic cells to T cells. After that, the differentiation of T lymphocytes into subtypes occurs. CD8+ T cells, secreting interferon-gamma, eradicate infected cells. IL-4 produced by Th2 cells attracts B cells, which secrete neutralizing antibodies [85].

As by SARS-CoV-1, MERS-CoV patients initially observe mild symptoms followed by the rapid development of respiratory failure. Although SARS-CoV-1 patients may develop acute respiratory distress syndrome (ARDS) during the first week and require longer time to recover, the disease severity and mortality tended to be milder compared to MERS-CoV. On the surfaces of host cells, S glycoprotein binds to angiotensin-converting enzyme 2 (ACE2) receptors for SARS-CoV-1 and to DPP4 for MERS-CoV inducing fusion of the viral envelope and cell membranes. SARS-CoV-2 has a host cellular entry mechanism similar to SARS-CoV, which

consists in binding to the ACE2 receptor through its surface protein RBD [85]. However, the SARS-CoV-2 replication is 3 times higher compared to SARS-CoV, therefore, SARS-CoV-2 is more contagious and affects more tissues during the same period

Both MERS-CoV and SARS-CoV-1 infections cause a strong specific T cell response. Infection of human T cells by MERS-CoV induces both intrinsic and extrinsic apoptosis, resulting in the immune responses suppression. SARS-CoV oppositely infects monocyte-macrophages, DC and T cells to a lesser extent. These different mechanisms of immune response activation in MERS and SARS infections might be explained due to the high levels of DDP4 and low levels of ACE2 receptors in monocytes and dendritic cells [85]. However, rapid reduction of T lymphocytes during SARS-CoV-1 infection has been shown. There were more T lymphocytes in SARS patients compared to CD4+ T cells [86]. In MERS-CoV infected individuals, T cells undergo apoptosis, causing the suppression of immune responses. Another similarity of SARS-CoV-1 and MERS-CoV infection is that both are resulted in Th2 downregulation and high frequencies of reactive CD8+ T cells in the beginning of disease. It was revealed that in case of SARS-CoV-2, the dynamic regulation of HLA and cytokine expression was observed in SARS-CoV-2 infection, which might impair CD8+ cytotoxic T lymphocytes mediated recognition and support immune evasion.

A comparison of proinflammatory cytokines in the serum of SARS-CoV-1 and MERS-CoV patients with healthy controls has shown that, in general, chemokines and cytokines have been upregulated to a significantly greater extent and duration in MERS-CoV infection compared to SARS-CoV. Concentration of TNF-α, IL-6, IL-8, IL-10, and IL-12 was higher at the early stage of the SARS-CoV-1 infection than during the recovery from pneumonia. In MERS-CoV infected people, the increased levels of IL-1α, IL-1β, IL-6, IL-8 IL-12, and IFN-γ have been identified [87]. Patients with SARS-CoV-2 had elevated levels of inflammatory IL-6, TNF-α, IL-1, IL-2R cytokines and chemokine IL-18 that were observed in the most severe COVID-19 cases [85]. IL-8 and IL-12 cytokines levels were greater during MERS-CoV than SARS-CoV-1. Chemokine were also secreted in a greater amount

in MERS patients compared to SARS-CoV-1 infection. Increased contents of CXCL-10, MCP-1, MIP-1α, and RANTES have been observed in peripheral blood in both SARS and MERS patients [88]. The increased levels of chemokines and cytokines by MERS-CoV infection lead to intensified immunopathogenesis causing higher mortality levels [89]. Currently, attempts are being made to find any suitable therapeutic approach to overcome the cytokine storm. In a series of studie,s it has been suggested that cytokine storm is correlated with the disease severity and poor prognosis during SARS-CoVs and MERS-CoV infection.

Interferons play a crucial role to limit a viral replication. The upregulation of IFN-γ has been found in serum of SARS-CoV-1 and MERS-CoV-infected patients. However, its level was lower in serum of COVID-19 and SARS-CoV-infected patients compared to MERS patients.

Production of neutralizing antibodies restrains the infection and helps to protect from reinfection and stop the spread of virus throughout the body tissues. Coronaviruses have been shown to express surface spike (S) proteins, which are the dominant antigens stimulating a humoral immune response targeted by anbibodies. Anti- SARS-CoV-1 and anti-MERS-CoV monoclonal antibodies that had a strong affinity to S protein also facilitated ADE (antibody-dependent enhancement) viral entry into host cells [90]. Neutralizing MERS-CoV antibodies were found in all camels, which has again proved its zoonotic origin. In case of SARS-CoV-1 infection, neutralizing antibodies block the receptor-binding domain (RBD) of spike protein from interacting with the ACE2 receptor. IgG, IgM, and IgA antibody response has been detected in the patients infected with SARS-CoV- and MERS-CoV. IgG and IgM have been identified two weeks after infection with SARS-CoV, with a further significant increase in IgG antibody levels reaching the peak on the 60$^{th}$ day. The SARS-CoV-1-specific IgG antibody persists longer than IgM and IgA. The SARS-CoV-1 antibodies have been shown to persist for three years in about 95% of the examined samples, and then gradually reduced until being undetectable after 6 years [91]. However, memory T cell responses to SARS-CoV S peptides were revealed in 60% of recovered and even 17 years after the SARS outbreak [92]. Similar to SARS-CoV, the presence of MERS-CoV specific antibodies has been detected three years after the contamination. The recent studies

have found that long-lasting MERS-specific humoral immunity potentially sustains for four years after the infection and substantially declines [93]. However, long-term SARS-CoV-2 specific antibody levels and neutralizing activity are not yet clear. It was shown that the levels of anti-S IgM and anti-N IgG responses increase rapidly. Although SARS-CoV-2 specific anti-S/N IgM become undetectable in most patients 12 weeks after the disease onset, high levels of titers of IgG against S and N proteins were detected after 6 months. Humoral immune response to SARS-CoV-2 appeared in a week and neutralizing IgG specific to N protein increased in 14 days [89].

Some studies have tried to establish if there is any correlation between MERS-CoV and SARS-CoV-1 severity and antibody response. Although there are some publications claiming that SARS-CoV-1 survivors were found to have lower antibodies than patients without sequelae, lower concentration of IgG was detected in severe SARS patients compared to mild cases, which may be a result of an immune system dysfunction; others have revealed no correlation in antibody responses between patients who recovered or died [94]. On the contrary, in MERS-CoV cases, it has been confirmed that antibody responses increased during the acute phase with increasing disease severity. The correlation between the antibody level and fever duration has been observed [95].

It is difficult to provide an absolute conclusion about all mechanisms of immune responses to SARS-CoV-1, SARS-CoV-2, and MERS-CoV. However, studies examining various features of immune cells provide clues to immune responses in SARS-CoV-1, SARS-CoV-2, and MERS disease pathogenesis. Like SARS-CoVs, MERS-CoV can cause the dysfunction of infected natural immune response and induce cytokine imbalance. Though both SARS-CoV-1 and MERS-CoV infect monocyte-macrophages and dendritic cells, only MERS-CoV was found to replicate in the infected immune cells. This results in the aberrant expression of inflammatory cytokines in dendritic cells and macrophages and T cells apoptosis pathway activation. The significant increase in the expression of IL-17 by Th17 has been defined in MERS-CoVs patients, which, in turn, activates inflammatory cells to the site of infection. In addition, SARS-CoV-1, SARS-CoV-2, and MERS-CoV infection affects T cells, in particular, CD4+ and CD8+ T lymphocytes, which leads to their reduction and abnormal changes in cytokines secretion [96]. An

inadequate response to coronavirus infection is related to severe consequences for all types of coronaviruses.

**Specific aim of this study**

To assess the potential contribution of new emerging mutations to superspreading events, I have collected virus genetic information reported during the South Korean outbreak and systemically analyzed its variations, especially spike sequences, related to individual disease severity and epidemiology. I have also attempted to confirm whether the spike mutations affect virus dynamics in an in vitro infection model and virus escape from neutralizing antibody responses by using serum samples from mice immunized with wild-type spike antigen and from MERS patients in South Korea who had been infected with wild-type virus. Systemic overview of clinical and virologic data obtained during the transient but large outbreak driven by unexpected superspreading events among humans might provide new insights into understanding the evolutionary pathways of the emerging coronavirus during animal-to-human transmission.

The emergence of antibody escaping mutants under mounting immunologic pressure in a host might ensure sustained virus replication, higher virus shedding into respiratory secretions for longer periods, and delay in antigen-specific immunity, thereby increasing the probability of becoming a superspreader for a patient. Nevertheless, this evolutionary pathway of coronaviruses during human-to-human spread may result in serial decrease of host affinity and pathogenicity, as well as milder respiratory symptoms, if their transmission in the human population is not properly restricted at the initial outbreak stage.

# 2. Chapter 1

## Sequential Emergence and Wide Spread of Neutralization Escape Middle East Respiratory Syndrome Coronavirus Mutants, South Korea, 2015

## 2.1. Materials and Methods

### Middle East Respiratory Syndrome Coronavirus Culture and Plaque Assay

Wild-type Middle East respiratory syndrome coronavirus (MERS-CoV) or I529T mutant MERS-CoV isolated from patients in South Korea were cultured in a 24-well plate containing a monolayer of Vero E6 cells or 293T cells stably expressing CD26. After 1 h incubation at 37°C, viral supernatant was removed and cells were overlaid with 1 mL of 1% methylcellulose in Dulbecco modified Eagle medium, including 10% fetal bovine serum. Plates were incubated for 3 d at 37°C, and then cells were fixed with 4% paraformaldehyde and 100% methanol. The MERS-CoV plaques were detected using rabbit anti-MERS-CoV N protein antibody (Sino Biologic Inc.) and goat anti-rabbit IgG secondary antibody conjugated with horseradish peroxidase (Invitrogen; https://www.thermofisher.com/us/en/home/brands/invitrogen.html). Viral plaques were visualized by incubation with 0.05% 3′3-diaminobenzidine tetrahydrochloride and 0.01% hydrogen peroxide in 50 mmol/L Tris-HCl (pH 8.0). Cellular layers were counterstained with trypan blue dye.

### Neutralizing Antibody Assays

Pseudotyped lentiviruses with wild type or mutant spikes of MERS-CoV were generated from 293T cells (Invitrogen) by cotransfection of human immunodeficiency virus backbone plasmids expressing firefly luciferase. We used the packaging plasmids, pLP1, pLP2, and pLP/VSV-G (Invitrogen) and pLVX-Luc-IRES-ZsGreen1 (Clontech; https://www.takarabio.com). For spike protein pseudotyping, condon-optimized cDNA of spike gene (Sino Biological; https://www.sinobiological.com) was cloned into pcDNA3 after deleting an ER/Golgi retention motif and an endosomal recycling motif from the cytoplasmic tail for transfection instead of pLP/VSV-G. A plasmid carrying the gene encoding the I529T or D510G mutation in spike protein was generated by using the QuikChange kit (Stratagene; http://go.strategene.org/genetic-analysis) based on the wild-type construct, and the point mutation was confirmed by sequencing. Viral supernatants were harvested 48 h after transfection and normalized by p24 ELISA kit (Clontech) before infecting 293T cells expressing human CD26 (293T-CD26).

To assess the neutralizing activity by spike pseudoparticle neutralization assay, pseudoviruses (0.1 multiplicity of infection) were preincubated with serially diluted serum samples from mice immunized three times with wild-type spike antigen (Sino Biologic Inc.) at 4°C for 1 h. Subsequently, the infected 293T-CD26 cells were lysed 48 h after infection, and the efficiency of viral entry was measured by comparing luciferase activity. The relative luciferase activity in cell lysates was measured using a luciferase assay kit (Promega; https://www.promega.com) and Infinite 200 PRO microplate reader (Tecan; https://lifesciences.tecan.com). Neutralization titers of collected serum samples against MERSCoV were also determined by a plaque reduction neutralization titer assay. Each serum sample collected from convalescent-phase patient was serially diluted and incubated with wild-type MERS-CoV or I529T mutant MERS-CoV (0.004 multiplicity of infection) for 1 h at 4°C. The viruses were then added to a 24-well plate containing a monolayer of Vero E6 cells in duplicate. After 1 h incubation at 37°C, viral supernatant was removed and cells were overlaid with 1 mL of 1% methylcellulose in Dulbecco modified Eagle medium including 10% fetal bovine serum. Viral plaques were visualized as described above. The percentage of plaque reduction was calculated as [(no. of plaques without antibody) − (no. of plaques with antibody)] / (no. of plaques without antibody) x 100. The 50% pseudoparticle neutralization assay nd 50% plaque reduction neutralization titers were calculated by a nonlinear regression analysis (log[inhibitor] versus normalized response method) embedded in GraphPad Prism Software v5.01

**Statistical Analysis**

Data were analyzed using GraphPad Prism Software. Statistical analysis was performed using a 2-tailed Student t-test or one-way analysis of variance, followed by the Newman-Keuls Page 3 of 7 t-test for comparisons of values among different groups.

## 2.2. Results

### Emergence and spread of MERS-CoV bearing the I529T or D510G mutations in the spike protein during the outbreak in South Korea

I analyzed the genetic information of spike genes that have been reported during the outbreak in South Korea. Of these, the data regarding spike mutations in samples taken from 48 patients was studied (Table 1-1).

Two new mutants identified during the outbreak that carried D510G and I529T mutations in the RBD region were chosen for detailed research as they had reduced affinity to CD26 receptor [97]. Data about the timeline of virus exposure, the symptom onset, and the spread of MERS-CoV I529T or D510G mutants, including those shown in Figure 1-1. The first (P001) patient was found to have wild-type MERS-CoV on May 19, 2015. In 3 days, the I529T mutation was generated and detected in the same person. Early contractors (P002, P009, and P010) had wild-type alleles, while the following cases carried I529T mutations, two of them (P014 and P016) were assessed as superspreaders. D510G mutation has been revealed for the first time in P014. Therefore, a specimen isolated from P014 carried MERS-CoV with wild-type, I529T, or D510G alleles. It has been found that P014 originally had wild-type and I529T mutation. The D510G mutation with high probability has emerged in the patient later compared to MERS-CoV superspreaders (P1, P14, and P16), and only individuals infected by P014 were found to bear D510G mutation. A fluctuation of D510G and I529T and wild-type viruses was observed as I529T mutant dominated in P077 and P080 patients, but later wild-type prevailed among most patients.

**Table 1-1.** Baseline characteristics of MERS patients and spike mutations associated with the patients.

| Patient's ID. | Sex | Age | Plausible source | Clinical severity | Fever duration | Sampling date | NCBI accession #* | MERS-CoV spike mutations** |
|---|---|---|---|---|---|---|---|---|
| P001 | M | 68 | | 3 | 54 | 19/05/2015 | KT182958.1 | WT |
| | | | | | | 22/05/2015 | KT326819.1 | I529T |
| P002 | F | 63 | 1 | 1 | 10 | 20/05/2015 | KT029139.1 | WT |
| P009 | M | 55 | 1 | 3 | 25 | 28/05/2015 | KT182953.1 | WT |
| P010 | M | 44 | 1 | 3 | 14 | 27/05/2015 | KT006149.2 | WT |
| | | | | | | 28/05/2015 | KT036372.1 | WT |
| P012 | F | 49 | 1 | 2 | 9 | 28/05/2015 | KT182954.1 | I529T |
| P013 | M | 49 | 1 | 2 | 1 | 28/05/2016 | KT182955.1 | I529T |
| P014 | M | 35 | 1 | 3 | 16 | 30/05/2015 | KX034093.1 | I529T |
| | | | | | | 31/05/2015 | KT374052.1 | I529T |
| | | | | | | 01/06/2015 | Ref. 8 | WT/I529T/D510G |
| | | | | | | 13/06/2015 | KT374053.1 | I529T |
| P015 | M | 35 | 1 | 2 | 7 | 30/05/2015 | KT182956.1 | I529T |
| P016 | M | 41 | 1 | 3 | 13 | 11/06/2015 | KT868865.1 | I529T |
| P023 | M | 73 | 16 | 4 | 5 | 11/06/2015 | KT868866.1 | I529T |
| P024 | M | 78 | 16 | 4 | 0 | 08/06/2015 | KT868867.1 | I529T |
| P030 | M | 60 | 16 | 2 | 23 | 08/06/2015 | KT868868.1 | I529T |
| P031 | M | 69 | 16 | 4 | 17 | 11/06/2015 | KT868869.1 | I529T |
| P035 | M | 38 | 14 | 3 | ? | 03/06/2015 | KT374054.1 | I529T |
| | | | | | | 08/06/2015 | KU308549.1 | |
| | | | | | | 18/06/2015 | KT374055.1 | |
| P038 | M | 49 | 16 | 4 | 19 | 10/06/2015 | KT868870.1 | WT |
| P042 | F | 54 | 1-11 | 4 | ? | 30/05/2015 | KT182957.1 | I529T |
| P048 | M | 38 | 14 | 2 | 12 | 30/06/2015 | Ref. 8 | I529T |
| P050 | F | 80 | 14 | 4 | 26 | 06/11/2015 | Ref. 8 | WT/I529T/D510G |
| | | | | | | 06/11/2015 | KX034094.1 | D510G |
| | | | | | | 26/06/2015 | Ref. 8 | I529T |
| P054 | F | 63 | 16 | 3 | 16 | 30/05/2015 | KT182957.1 | I529T |
| P061 | M | 55 | 14 | 3 | 27 | 17/06/2015 | Ref. 8 | I529T |
| P062 | M | 51 | 14 | 1 | 5 | 11/06/2015 | Ref. 8 | I529T |
| P066 | F | 42 | 14 | 2 | 16 | 04/06/2015 | Ref. 8 | D510G |
| | | | | | | 04/07/2015 | KX034095.1 | D510G |
| P068 | F | 55 | 14 | 2 | 6 | 04/06/2015 | Ref. 8 | I529T |
| P075 | M | 62 | 14 | 2 | 1 | 15/06/2015 | Ref. 8 | I529T |
| P077 | M | 63 | 14 | 4 | 10 | 05/06/2015 | Ref. 8 | I529T |
| | | | | | | 17/06/2015 | Ref. 8 | WT/I529T |
| | | | | | | 17/06/2015 | KX034096.1 | I529T |
| P078 | F | 41 | 14 | 2 | 9 | 11/06/2015 | Ref. 8 | I529T |
| P080 | M | 34 | 14 | 2 | 20 | 05/06/2015 | Ref. 8 | I529T |
| | | | | | | 11/06/2015 | Ref. 8 | WT/D510G |
| | | | | | | 17/06/2015 | Ref. 8 | WT/D510G |
| | | | | | | 17/06/2015 | KX034097.1 | D510G |
| | | | | | | 22/06/2015 | Ref. 8 | WT |
| P082 | F | 83 | 16 | 4 | 13 | 10/06/2015 | KT868872.1 | I529T |
| P085 | F | 66 | 16 | 1 | 1 | 10/06/2015 | KT868873.1 | I529T |
| P099 | M | 48 | 14 | 2 | 9 | 06/06/2015 | Ref. 8 | I529T |
| | | | | | | 11/06/2015 | Ref. 8 | I529T |
| P100 | F | 32 | 14 | 2 | 10 | 09/06/2015 | Ref. 8 | I529T |
| P101 | M | 85 | 14 | 4 | 20 | 09/06/2015 | Ref. 8 | I529T |
| P102 | F | 48 | 14 | 2 | 7 | 07/06/2015 | Ref. 8 | I529T |
| | | | | | | 12/06/2015 | Ref. 8 | I529T |
| P103 | M | 66 | 14 | 2 | 4 | 07/06/2015 | Ref. 8 | I529T |
| P110 | F | 57 | 14 | 2 | 20 | 11/06/2015 | KT868874.1 | I529T |
| P122 | F | 55 | 14 | 2 | 13 | 10/06/2015 | KT868875.1 | D510G |
| P134 | F | 68 | 14 | 1 | 1 | 12/06/2015 | Ref. 8 | I529T |
| P135 | M | 33 | 14 | 3 | 23 | 11/06/2015 | Ref. 8 | I529T |
| | | | | | | 17/06/2015 | Ref. 8 | I529T |
| P148 | F | 39 | 16-36 | 2 | 6 | 11/06/2015 | KT868876.1 | I529T |
| P155 | F | 42 | 14 | 1 | 1 | 12/06/2015 | Ref. 8 | WT/I529T/D510G |
| P157 | M | 60 | 14 | 4 | 35 | 22/06/2015 | Ref. 8 | I529T |
| P162 | M | 33 | 14-? | 3 | 18 | 22/06/2015 | Ref. 8 | I529T |
| | | | | | | 22/06/2016 | KX034098.1 | I529T |
| | | | | | | 01/07/2015 | Ref. 8 | I529T |
| P163 | F | 52 | 119 | 3 | 23 | 19/06/2015 | KT374051.1 | WT |
| | | | | | | 29/06/2015 | KT374050.1 | WT |
| P164 | F | 35 | 14-? | 2 | 11 | 21/06/2015 | Ref. 8 | I529T |
| P168 | M | 36 | 14-76 | 1 | 1 | 21/06/2015 | KT374056.1 | D510G |
| | | | | | | 24/06/2015 | KT374057.1 | D510G |
| P169 | M | 33 | 14-135 | 2 | 18 | 26/06/2015 | Ref. 8 | I529T |
| | | | | | | 26/06/2015 | KX034099.1 | I529T |
| P172 | F | 61 | 16-? | 3 | 26 | 22/06/2015 | KT868877.1 | I529T |
| P177 | F | 49 | 14 | 4 | 17 | 28/06/2015 | Ref. 8 | I529T |
| | | | | | | 01/07/2015 | Ref. 8 | I529T |
| | | | | | | 03/07/2014 | Ref. 8 | I529T |
| | | | | | | 03/07/2015 | KX034100.1 | I529T |

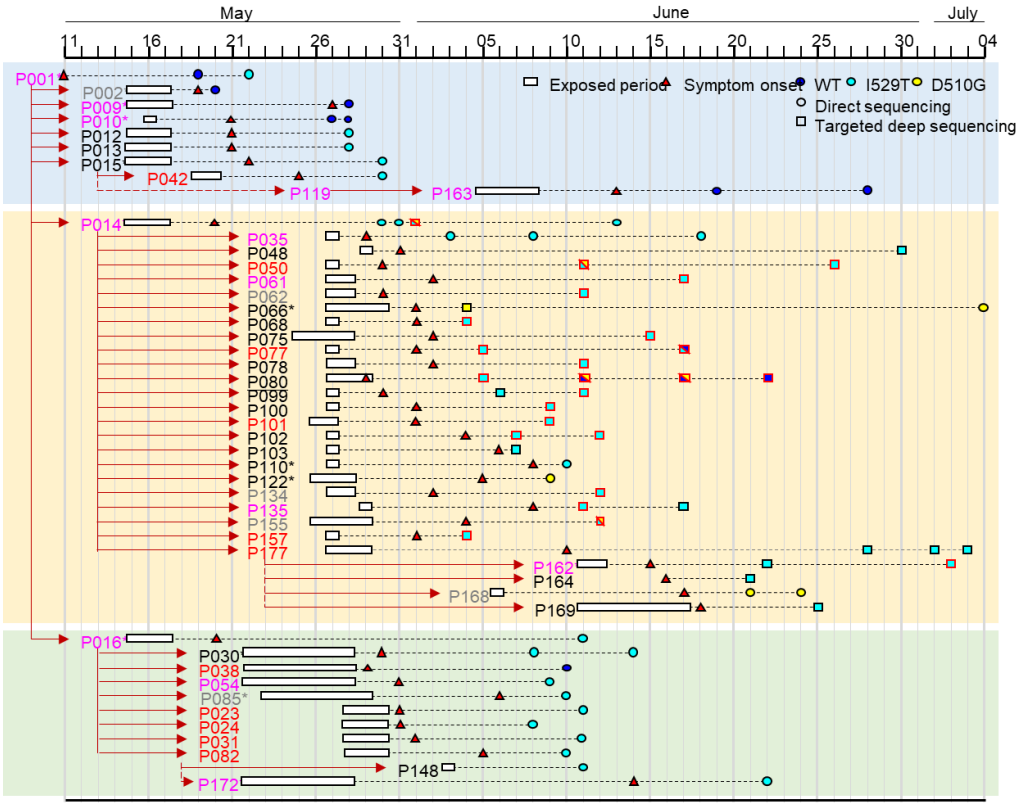


**Figure 1-1.** Emergence and spread of Middle East respiratory syndrome coronavirus (MERS-CoV) bearing the I529T or D510G mutation in the spike protein during the 2015 outbreak in South Korea. Transmission chain of infection and the timeline of potential virus exposure, symptom onset, date of specimen collection from patients, and identified mutation in the spike protein of MERS-CoV analyzed in this study. Case-patients′ IDs are colored on the basis of disease severity (gray, group I; black, group II; pink, group III; red, group IV). Spike sequences analyzed by targeted deep sequencing are denoted as a square with black (single genotype) or red (mixed genotypes with wild-type) borderline. Others are marked as circles (direct sequencing).

**Characteristics of MERS coronavirus spike genotypes reported during 2015 outbreak in South Korea**

To find if there was an association between the course of MERS infection and virus spike sequences, 48 patients under study were divided into four groups depending on the disease severity: the first one consisted of six people with fever or no symptoms and no pneumonia, the second included 19 patients who had mild pneumonia without hypoxemia, the third consisted of twelve persons who had severe, prolonged pneumonia with hypoxemia and needed oxygenation during the disease, and the fourth composed of eleven persons who died of acute respiratory distress syndrome (Table 1-2).

**Table 1-2.** Baseline characteristics of MERS coronavirus spike genotypes identified from the 2015 MERS outbreak in South Korea

| | No. (%) patients | | Patient age, y, | No. associated spike genotypes | | | | |
|---|---|---|---|---|---|---|---|---|
| Severity group | Men | Women | mean ± SD | WT | I529T | D510G | WT–I529T | WT–I529T–D510G |
| I | 3 (50.0) | 3 (50.0) | 54 ± 13 | 1 | 2 | 1 | 1 | 1 |
| II | 9 (47.4) | 10 (52.6) | 46 ± 11 | 0 | 10 | 2 | 6 | 1 |
| III | 8 (75.0) | 4 (25.0) | 48 ± 12 | 3 | 4 | 0 | 4 | 1 |
| IV | 7 (63.6) | 4 (36.4) | 68 ± 13 | 1 | 6 | 0 | 3 | 1 |

*MERS, Middle East respiratory syndrome; WT, wild-type.

**Effect of the spike mutations in MERS-CoV on fever duration and virus growth *in vitro***

I have not established any distinguishing link between D510G and I529T mutations and MERS-CoV infection severity. But the correlation of the fever duration (in days) and the spike protein mutations of MERS patients has been revealed. In particular, cases with D510G (10 ± 8, mean ± SD) and I520T (11 ± 8 days) mutations were relatively shorter than those with the wild-type allele (18 ± 6 days) or mixed genotype, including wild-type (16 ± 14 days) (Figure 1-2, panel A). Despite the fact that p = 0.0654 (possibly because of insufficient data) if comparing fever length in the individuals bearing with the wild-type virus and mixed cases (17 ± 12 days) and patients with the mutant alleles (11 ± 7 days), the increase in the fever duration in patients associated with wild-type virus allele seems to be reconcilable.

As I529T mutant MERS-CoV reduced affinity to CD26 receptor compared to wild-type, we examined whether they also reduced transmissibility and growth in plaque-assay *in vitro* in Vero E6 cells suitable for propagating viruses 293T–CD26 cells isolated from human embryonic kidneys, overexpressing human CD26. In Vero E6 cells, an average size of the plaques formed by wild-type of MERS-CoV was (mean ± SD, 0.64 ± 0.21 $mm^2$) I529T MERS-CoV mutant plaques was (mean ± SD, 0.49 ± 0.15 $mm^2$). The wild-type virus plaques were greater in size in approximately 23% than those formed by I529T MERS-CoV (Figure 1-2, panel C). To sum up, *in vitro* infection model of the spike mutation generated during the South Korean outbreak has reduced transmissibility, growth rate, or both.

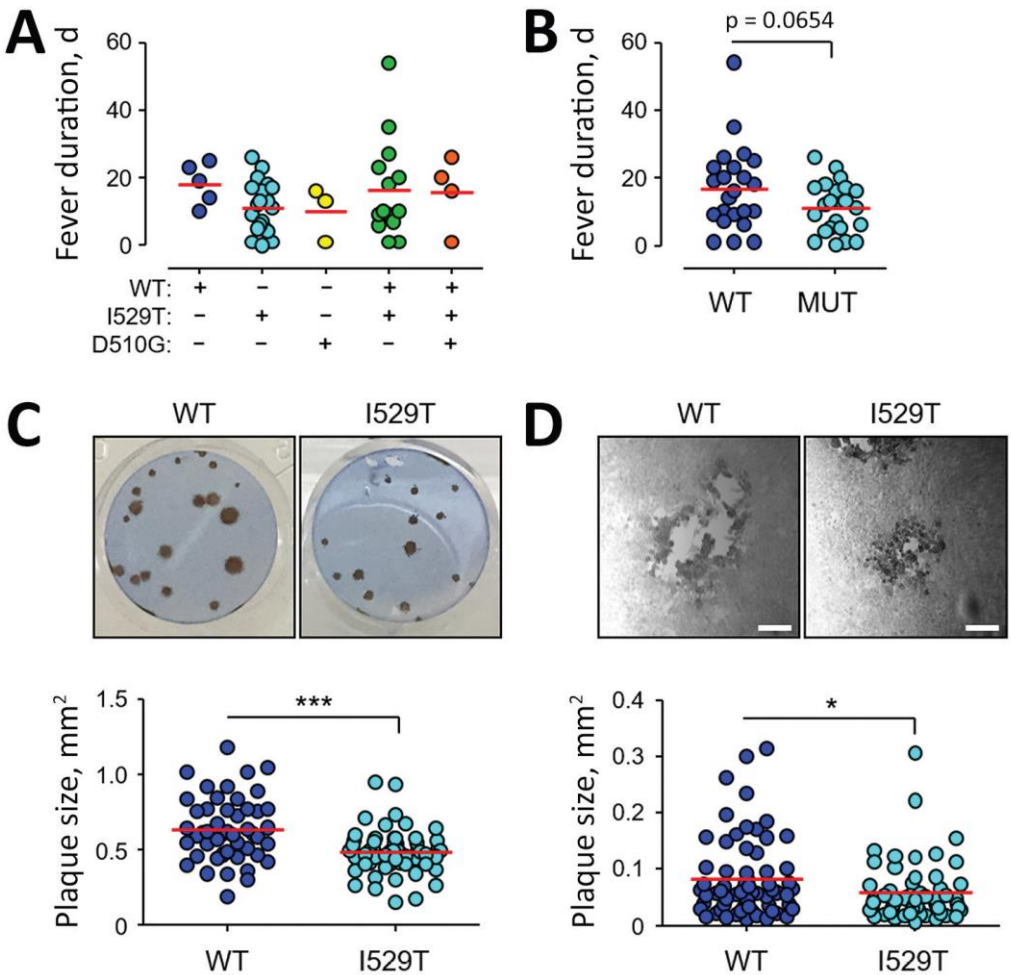


**Figure 1-2.** Effect of spike mutations in Middle East respiratory syndrome coronavirus (MERS-CoV) on fever duration and virus growth in vitro during the 2015 outbreak in South Korea. A) Fever duration of 48 patients for whom virus spike sequence information is available is presented depending on the associated spike genotypes. B) Fever duration of patient group associated with WT virus, including mixed infection (n = 23) and those infected only with either of the mutant viruses (n = 25). Mean value of each group is indicated by red lines. C, D) Distribution of viral plaque sizes in Vero cells (panel C: WT, n = 48; I529T, n = 58) or 293T–CD26 cells (panel D: WT, n = 65; I529T, n = 55) infected with MERS-CoV bearing WT or I529T mutant spike at 3 days after infection. Representative results of plaque assay are presented in the upper panels, and size

distribution of viral plaques are plotted in the lower panel. Mean values are indicated by red lines.

**Increased resistance of MERS-CoV with the spike mutations against antibody-mediated neutralization**

The effect of spike mutations on sensitivity to antibody neutralization was measured in the 50% pseudoparticle neutralization test (PPNT50), which is a fundamental test in virology against lentiviruses bearing wild-type and mutant spikes (I529T or D510G). The ability of antibodies generated in mice immunized with wild-type MERS-CoV to neutralize the spike mutant viruses was assessed compared to the wild-type virus neutralization. The obtained results indicated that mutant viruses can escape from neutralizing antibodies as average titers of serum from the immunized mice were more efficient in the case of wild-type virus (mean ± SD, 2,629 ± 1,384) comparing to mutant viruses: neutralization of I529T (mean ± SD, 1,727 ± 897) and D510G (1,009 ±482) (Figure 1-3, panel A).

Moreover, the titers of neutralizing antibody against MERS-CoV bearing wild-type and I529T mutant spike mutation in serum from the 3 recovered patients, who had only wild-type MERS-CoV were measured. It has been found that the neutralization activity of the serum samples against the I529T mutant MERS-CoV (mean ± SD, 888 ± 723) decreased significantly compared to the activity against the wild-type (mean ± SD, 2,943 ± 2,994). Therefore, the results allow us to conclude that I529T mutant MERS-CoV can effectively escape from neutralizing antibodies against the wild-type virus (Figure 1-3, panel B). Moreover, the correlation between the infection severity and antibodies titers should be further studied as we found that titer from the serum of P002 patients who had mild symptoms was lower than in patients P009 and P010, who experienced more severe disease symptoms.

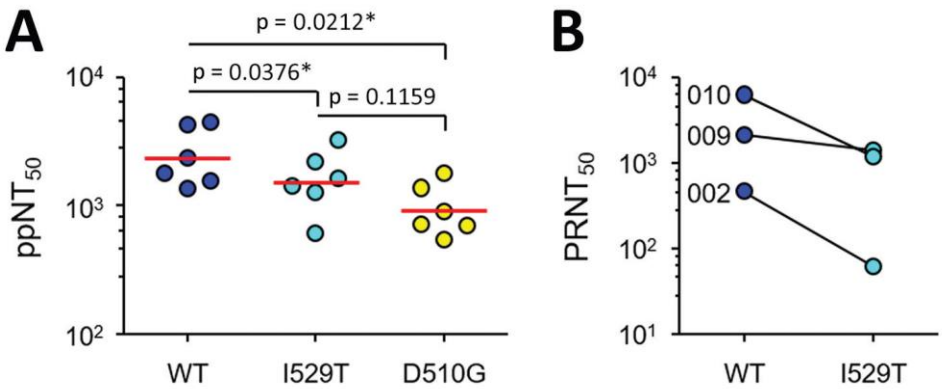


**Figure 1-3.** Increased resistance of Middle East respiratory syndrome coronavirus (MERS-CoV) against antibody-mediated neutralization by spike mutations during the 2015 outbreak in South Korea. A) Neutralizing activity of serum samples against lentiviruses bearing WT and mutant spikes. 50% pseudoparticle neutralization test titers against lentiviruses bearing WT or mutant spikes (I529T or D510G) in serum samples from mice (n = 6) immunized with WT spike antigen are plotted. Mean values are indicated by red lines. Statistical significance was calculated by using analysis of variance with Newman–Keuls post t-test correction. *p<0.05. B) Neutralization activity against MERS-CoV bearing WT or I529T mutant spike mutation in serum samples from 3 recovered patients (P002, P009, and P010) who carried only WT MERS-CoV.

## 2.3. Discussion

Overall, it is well known that the changes emerging in zoonotic coronaviruses genotype due to multiple factors might become a reason for new outbreaks in human populations. This study has investigated the information of MERS-CoV mutations emerged during the 2015 outbreak in Korea and their biological features, such as differences in spike RBD, ability to escape humoral immune response, virus growth and spread. During the study, we were focused on studying MERS-CoV genetic changes with reference to superspreading events. Superspreaders proved to be epidemiology linked to the greatest number of human-to-human transmissions for the majority of pandemic coronavirus cases [99] The consequent rise of infectious cases at the early stage of an outbreak might play a critical role in the further viral spread. The distinguishing features of superspreaders among other infected individuals are still poorly understood. In this study, we analyzed the spread of MERS-CoV bearing I529T or D5105G mutation in spike protein emerged during the outbreak and revealed the biological effects of the mutations on fever duration, virus growth, transmission etc.

Used in this study, I529T mutant isolates from Korea have shown differences in 2 amino acid (I529T and V534L) in spike receptor binding domain and 5 nonsynonymous nucleotide changes within the virus genome comparing to the wild-type variant (GenBank accession no. KT029139.1 for wild-type and KT868873.1 for I529T mutant),

As shown in Figure 1-2A the I529T or D5105G mutations of MERS-CoV resulted in decrease of fever duration in patients compared to individuals infected by the virus with wild-type or mixed (wild-type and mutant) genotypes. Such characteristics as *in vitro* virus growth and cell-to-cell transmissibility of I529T mutant MERS-CoV were analyzed in plague-forming assay and presented in Figure 1-2, panel C and D. In order to perform plague-forming assay two cell lines were chosen - human embryonic kidney cell line Vero E6 and 293T–CD26 cells overexpressing CD26 [57]. The average plaques size formed by I529T mutant MERS-CoV was notably reduced in both studied cell lines (Figure 1-2C and 1-2D). The difference in plaque sizes is primarily attributable to mutation in the spike.

Therefore, we can conclude that viral spread, growth rate, or both features simultaneously are considerably reduced in MERS-CoV bearing I529T mutation than that of wild-type viruses. The recent study has also confirmed that D510G or I529T polymorphisms led to reduced binding of CD26 and spike protein, although the viral entry into the host cells decreased only in case of low CD26 expression on target cells [100]. The D510G and I529T mutations do not modulate binding of spike protein to sialic acids, activate S protein by target cell proteases, or inhibit virus entry via transmembrane proteins induced by interferon [100].

It can be supposed that MERS-CoV benefits from the D510G and I529T polymorphisms spread during outbreak in South Korea due to increased resistance to neutralization by antibodies from serum of MERS patients. We found out that antibodies from the serum of the patients recovered from wild-type virus human had less efficiency at neutralizing the I529T or D510G mutant than the wild-type MERS-CoV. The same trend has been revealed with antibodies formed in mice infected with the wild-type spike antigen (Figure 1-3). In addition, it was established that D510G or I529T MERS-CoV variants reduced neutralization sensitivity mediated not only by antibodies from serum of MERS patients but also by monoclonal antibodies 100]. The use of MERS-CoV isolated from human patients during the Korean outbreak in this study differs from the previous research where a pseudotyped vesicular stomatitis virus system bearing mutant MERS-CoV or wild-type spike proteins was used [99]. Consequently, our results may represent more intrinsic features of spike protein in the context of the natural MERS-CoV infection. Further studies are needed for better understanding of the spike mutation effect on virus growth and spread in model systems *in vitro* and *in vivo*.

The serious need for spike mutations studying is associated with their emergence affect affinity to host cells and may be a cause of superspreading events. There was an earlier research that also supported this statement made regarding SARS epidemic during the 2002–2003 [99]. Those mutations significantly increased the affinity of S protein to human ACE2 receptor [101]. All the coronavirusal infections of the SARS epidemic in 2002–2003, MERS outbreak in 2015, SARS-CoV-2 epidemic started in 2019 proved to be associated with the mutations that change affinity to human receptors [99, 102]. However, emerged in

superspreaders mutations in SARS-CoV resulted in more efficient replication and became more pathogenic, while MERS-CoV acquired mutations, which led to the reduced affinity to human cells receptors and potentially became less pathogenic [99, 101]. As an attachment of the virus to receptors to host cells surface and their membranes fusion are mediated by the spike protein, studying of spike proteins are of particular importance. Moreover, S protein might serve as the target of neutralizing antibodies.

The two opposing ways of MERS-CoV and SARS-CoV spike protein mutants evolution might be explained studying the binding of RBD with receptors to host cells. As it has been established by cryoelectron microscopy, both SARS-CoV and MERS-CoV spike trimers have dynamic RBD structure in two conformations (in the exposed standing state position or buried lying state) [103]. It is shown that one CD26 receptor might cross-link two spike trimers of the MERS-CoV if binding to exposed RBDs (by one from each trimer), while one ACE2 molecule can interact with one spike trimer of the SARS-CoV. Therefore, SARS-CoV spike protein has lower avidity t host receptor compared to MERS-CoV [102]. These differences in avidity to the receptors on target cells may partly explain the fact that SARS-CoV mutants gained higher affinity while spreading the infection in the human population, and MERS-CoV spike mutations resulted in the affinity decrease. In particular, in case of SARS-CoV with low avidity, getting high affinity may be the only way for efficient transmission among humans. On the other hand, spike of MERS-CoV being the intrinsically more avid to the receptor may allow to gain S protein mutations resulting in its lower affinity to host cells for better escape of neutralizing antibodies. That′s why RBD should not be used as an immunogen, while conserved and surface exposed stem region can serve as a target for vaccine design [102].

This study shows three revealed superspreaders (P001, P014, and P016) in South Korea belong to III group that during the infection had severe prolonged pneumonia with hypoxemia and oxygenation. The individuals with poorer outcomes proved to have high viral loads and high viral copy numbers ($10^8$–$10^9$ copies/mL) in the respiratory secretions during the early phase of MERS infection. The superspreaders are supposed to contribute to virus transmission to susceptible

individuals within 9–11 days [77, 104]. In addition, virus continued being detected in their respiratory secretions of the superspreaders for long periods after the onset of symptoms (44, 30, and 27 days, respectively) [104, 105]. To sum up, compared to patients with milder cases of MERS-CoV infection, group III showed considerably higher levels of viral production in their respiratory secretions for longer periods [77, 105]. It is also noteworthy that superspreaders P001 and P014, who jointly infected over 80 patients for 2 weeks, were found to be the first ones bearing the newly generated I529T and D510G mutations, respectively. The P001 and P014 bearing not only mutant variants of the spike, but also wild-type spike protein sequence, spread mixed MERS-CoV variants during the early phase of the outbreak (Figure 1-1). The intrapatient MERS-CoVs heterogeneity was the highest in superspreader [106]. Therefore, it has been established that the generation of new spike genes in MERS-CoV is resulted in increased escape from neutralizing antibodies, reduced affinity of S protein to target receptors. These results prove that in some individuals spike protein microevolution might increase the number of a spreading event by prolonging period of the viral replication in the host.

The targeted deep sequencing has identified that most of the tertiary cases infected by P014 bear mixed wild-type and spike mutant viruses. Moreover, the combined frequency of the single mutations in the studied specimens was significantly higher (≈88% on average) compared to low frequency of the wild-type (≈7% on average). While studying MERS-CoV mutant polymorphisms, it was found that the frequency of D510G and I529T varied greatly among analyzed samples. These results support the hypothesis that the microevolution of spike proteins under the selective pressure of neutralizing antibodies played a key role in the generation of MERS-CoVs new genetic variants [106].

To conclude, it has been revealed that wild-type virus has higher affinity to host receptor, while mutants can effectively escape neutralizing antibodies. Thus, MERS-CoV infected with mutant and wild-type variants might contribute to stable replication of virus with higher loads. As shown in Figure 1, serum samples of P077 and P080 tertiary patients studied at the late stage of the infection prevalently had wild-type polymorphism, while in the earlier stage in the major population mutant virus dominated. Both patients suffered from other chronic

diseases such as hypotension, pancreatitis, chronic respiratory illness (P077) and lymphoma, and respiratory disease (P080) before being infected. In both cases, initially P077 and P080 patients infected by P014 showed bearing mixed variants of virus [106]. The wild-type domination that can be seen during the late viral infection suggest that P077 and P080 patients might had immunosuppression during the MERS infection either by initial high viral load (P077) or by previous cancer treatment (P080). A failure of an adaptive immunity in these patients might ensure specific conditions that allowed wild-type virus to reappear among the mixed population later during the infection. The same is confirmed by study of serial samples taken from P077 and P080, where a significant decrease in the normalized leukocyte level and an increase in the frequency of the wild-type allele was observed [106]. The obtained results also suggest that the selection pressure under the host immune system may favor mutants, but if immune pressure is decreased, the wild type variant is dominant.

These results indicate that the evolution of the spike protein MERS-CoV under immunological pressure towards neutralization antibodies escape may increase the probability of superspreading events as new mutants provide higher virus shedding into respiratory samples during the longer time, sustained replication of virus, as well as a delay in antigen-specific immunity. However, in case of MERS-CoV, spread in the human population is not restricted during the initial stage of the infection; the described way of coronavirus evolution during the transmission may lead to decrease of pathogenicity and host affinity, and milder course of respiratory infection.

# 3. Chapter 2.

## Sustained Responses of Neutralizing Antibodies Against Middle East Respiratory Syndrome Coronavirus (MERS-CoV) in Recovered Patients and Their Therapeutic Applicability

## 3.1. Materials and Methods

**Enzyme-linked immunosorbent assay (ELISA)**

An anti-MERS-CoV S1 ELISA kit (EUROIMMUN, Lubeck, Germany) for the detection of human IgG against MERS-CoV spike protein (S1 domain) was used. We assayed serum samples in duplicates and performed the assay according to the manufacturer's instructions. The assay was semi-quantitatively evaluated by calculating a ratio of the extinction value of the patient sample over the extinction value of the calibrator. Optical density (OD) ratios < 0.7 were considered negative, ratios > 1.4 were considered positive, and ratios ≥ 0.7 and ≤ 1.4 were considered as intermediate. Antibody titers against spike antigen (whole extracellular domain, Sino Biological Inc., Beijing, China) were also determined by ELISA. The cut-off titers for the ELISA was determined as the lowest titer (serial dilution from 1:100) showing an OD over the 99.0% confidence level from 4 control sera (diluted 1:100) in every assay plate.

**Neutralizing antibody assays**

MERS-CoV pseudotyped lentivirus with wild type spike was generated in 293T cells (Invitrogen) by cotransfection of human immunodeficiency virus backbone plasmids expressing firefly luciferase. We used the packaging plasmids, pLP1, pLP2, and pLP/VSV-G (Invitrogen) and pLVX-Luc-IRES-ZsGreen1 (Clontech). For spike protein pseudotyping, codon-optimized cDNA of the spike gene (Sino Biological Inc.) was cloned into pcDNA3 after deleting an ER/Golgi retention motif and an endosomal recycling motif from the cytoplasmic tail for transfection instead of pLP/VSV-G. Viral supernatants were harvested 48 h after transfection and normalized by p24 ELISA kit (Clontech) before infecting 293T cells expressing human CD26 (293T-CD26). To assess neutralizing activity by spike pseudoparticle neutralization (ppNT) assay, pseudoviruses (0.1 m.o.i.) were pre-incubated with serially diluted sera from the recovered patients at 4°C for 1 h. Subsequently, the infected 293T-CD26 cells were lysed 48 h after infection, and the efficiency of viral entry was measured by comparing luciferase activity. The

relative luciferase activity in cell lysates was measured using a luciferase assay kit (Promega) and Infinite 200 PRO microplate reader (Tecan). Neutralization titers of collected sera against MERS-CoV were determined by a plaque reduction neutralization titer (PRNT) assay. Each serum sample was serially diluted and incubated with wild type MERS-CoV or I529T mutant MERS-CoV (0.004 m.o.i.) isolated from Korean patients (NCBI genome sequences: KT029139.1) for 1 h at 37°C. The viruses were then added to a 24-well plate containing a monolayer of Vero E6 cells in duplicates. After 1 h incubation at 37°C, viral supernatant was removed and cells were overlaid with 1 ml of 1% methylcellulose in DMEM including 10% FBS. Plates were incubated for 3 d at 37°C, and then cells were fixed with 4% paraformaldehyde and 100% methyl alcohol. MERS-CoV plaques were detected using rabbit anti-MERS-CoV N protein antibody (Sino Biological Inc.) and goat anti-rabbit IgG secondary antibody conjugated with horse radish peroxidase (Invitrogen). Viral plaques were visualized by incubation with Nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) (Merck). Cellular layers were counter-stained with trypan blue dye. The percentage of plaque reduction was calculated as [(no. of plaques without antibody) - (no. of plaques with antibody)] / (no. of plaques without antibody) x 100. The $ppNT_{50}$ and $PRNT_{50}$ titers were calculated by a nonlinear regression analysis (log[inhibitor] vs. normalized response method) embedded in GraphPad Prism Software v5.01 (GraphPad Software Inc., San Diego, CA, USA).

**Enzyme linked immunospot assay (ELISPOT)**

PBMCs were isolated from donated blood samples by centrifugation over Ficoll-Hypaque™ PLUS (Amersham Biosciences, Sweden) and stored in liquid nitrogen until use. The levels of memory B cells specific to spike antigen were examined by ELISPOT assay (human IgG ELISPOT BASIC kit, MABTECH Inc., Cincinnati, OH, USA) as previously described. Briefly, cryopreserved PBMCs were rapidly thawed in a 37℃ water bath and then viable cells were counted using trypan blue staining. PBMC samples retaining more than 80% of live cells (1 x $10^6$ cells/ml) were stimulated with 5 ng/ml of IL-2 and 0.5 μg/ml of imidazoquinoline

resiquimod (R848) for 5 d. Cells (5 x $10^4$ cells/well) were then transferred to spike-coated (1 μg /well) PVDF membrane plates and further incubated for 1 d. Cellular spots secreting IgG specific to spike antigen were stained according to the manufacturer's instruction and counted using a CTL ImmunoSpot reader (Cellular Technology, Cleveland, OH, USA).

### *In vivo* protection assay

Transgenic C57BL/6 mouse with mouse *DPP4* exons 10-12 replaced with human *DPP4* codons (hDPP4-Tg mouse) and a mouse-adapted MERS-CoV were kindly provided by Dr. Paul B. McCray Jr. at the University of Iowa and used for *in vivo* protection assay using sera collected from the recovered patients. Mice were maintained in the animal care facility at the Seoul National University College of Medicine. All protocols were approved by the by the Seoul National University and International Vaccine Institute Institutional Animal Care and Use Committee (Permit #: IACUC PN 2018-016). hDPP4-Tg mice were anesthetized with a mixture of 60 mg/kg alfaxalone (Careside) and 5 mg/kg xylazine (Bayer) and challenged intranasally with MERS-CoV at 2,500 PFU/mouse (5 x $LD_{50}$). Mice were then treated with pooled sera (100 ul/mouse) or therapeutic mAb (3B11, Creative Biolabs, Shirley, NY, USA; 20 ug in 100 ul of PBS/mouse) four times (1 h and 1, 2, and 3 d postinfection). Mice were monitored for weight change and survival for two weeks after infection. Infectious viral titers in lung tissues from infected mice were analyzed as described previously. viral copy numbers were also determined using total RNA extracted from the tissues as previously reported. Histopathological analysis of infected lungs was performed after fixation in 10% formalin and embossing in paraffin. Tissue sections (4 μm thickness) were stained with hematoxylin and eosin and submitted to virtual microscope scanning using Aperio ScanScope (Aperio Technologies, Vista, CA, USA). Experienced pathologist specialized in lung pathology evaluated the scanned slides under the Aperio ImageScope software (Aperio Technologies) and scored the degree of inflammation. The types of inflammatory cells were assessed based on morphology. Histopathological analysis was performed by using a 0 - 4 scoring

system, as previously described with slight modifications: scores of 0, 1, 2, 3, and 4 representing areas with 0%, less than 6%, 6%–33%, 33%–66%, and more than 66% of perivascular and interstitial inflammatory cells distribution, respectively.

**Statistical Analysis**

Data were analyzed using GraphPad Prism Software. Statistical analysis was performed using a two-tailed Student's *t*-test or one-way analysis of variance (ANOVA), followed by the Newman-Keuls *t*-test for comparisons of values among different groups. Spearman's rank test was used to analyze the correlation between variables. $p < 0.05$ was considered statistically significant.

## 3.2. Results

**Changes of antibody responses against MERS-CoV spike antigen from 12 to 36 months after infection**

The studied 73 patients who had MERS-CoV infection during Korean outbreak in 2015, were divided into three groups depending on the disease symptoms. 18 patients who had no symptoms or mild fever without any pneumonia were classified as the Group I (G-I). 37 individuals who suffered from mild pneumonia with no hypoxemia were included in the Group II (G-II). The group III (G-III) included 18 patients who had severe and prolonged pneumonia with hypoxemia and oxygenation application. The characteristics of the groups are given in Table 2-1.

**Table 2-1.** Baseline characteristics of the enrolled patients.

| | Clinical severity groups | | |
|---|---|---|---|
| | Group I (n = 18) | Group II (n = 37) | Group III (n = 18) |
| Age (years) | | | |
| Mean (± SD) | 52 (± 15.9) | 50 (± 11.5) | 48 (± 11.9) |
| Sex | | | |
| Female, n (%) | 9 (50%) | 18 (49%) | 3 (17%) |
| Male, n (%) | 9 (50%) | 19 (51%) | 15 (83%) |
| Fever duration (d) | | | |
| Mean (± SD) | 5 (± 4.7) | 11 (± 8.9) | 20 (± 11.5) |
| Person with underlying diseases[a], n (%) | 7 (39%) | 14 (38%) | 7 (39%) |
| Smokers, n (%) | - | 8 (22%) | 6 (33%) |

[a]Diabetes, chronic (heart, kidney, lung, or liver) diseases, cancer, hypertension.

In the sera of these 70 patients, the antibodies against MERS-CoV S1 spike antigen were measured by ELISA every 6 months during the period from 12 to 36 months after the onset of the first symptoms (Figure 2-1A). The average OD ratios in the studied samples maintained for three years (mean ± SD: 1.56 ± 1.22, 1.90 ± 1.69, and 1.83 ± 1.55 at 12, 24, and 36 months, respectively). It was also found that the levels of antibodies correlated with the disease severity. Thus, OD ratios in patients of G-III (mean ± SD: 2.59 ± 1.16, 3.06 ± 1.70, and 2.74 ± 1.58 at 12, 24, and 36 months, respectively) were higher compared to G-II (mean ± SD: 1.52 ± 1.06, 1.96 ± 1.58, and 1.92 ± 1.36 at 12, 24, and 36 months, respectively), and G-I (mean ± SD: 0.56 ± 0.57, 0.48 ± 0.53, and 0.30 ± 0.34 at 12, 24, and 36 months, respectively). The obtained results show that antibody levels in the sera of patients of G-II and G-III did not change, while a gradual fall in G-I was observed.

**Changes of anti-S1 OD ratio, anti-spike IgG titer and ppNT50 and PRNT50 in 3 year-period after infection and their correlation**

Sera of the 50 patients were available for the 3 years. The results of anti-S1 IgG detection were presented as positive, intermediate and negative depending on OD ratios. In particular, OD less than 0.7 were considered as negative, OD ratios higher 1.4 were indicated as positive, and intermediate levels are those with ratios from 0.7 to 1.4. It was revealed that among G-I individuals. ten out of 11 people were negative during 36 months, and the only one positive out of 11 was detected 12 months after the infection (Figure 2-1B). Among G-II consisting of 23 participants, 16 (69.6%) with persistent antibody responses were positive for 3 years. Regarding G-III, 13 out of 16 (81.3%) patients were positive throughout 36 months, one had intermediate antibody level, and two were classified as negative. To sum up, patients from G-III who suffered from MERS-CoV with severe pneumonia had considerably higher levels of anti-S1 antibody responses persisting through 36 months after the symptom onset.

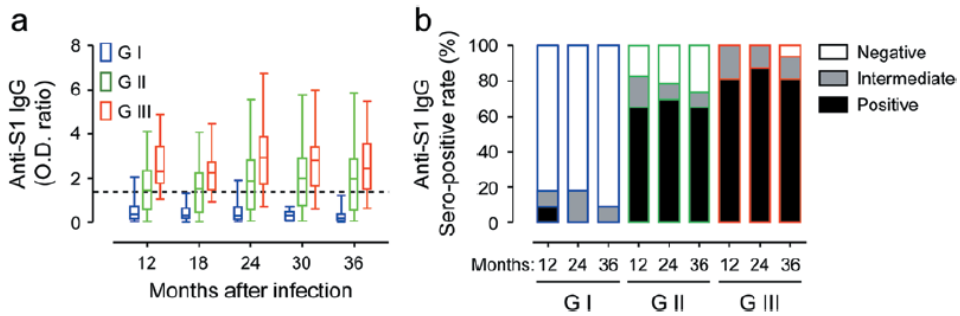


**Figure 2-1.** Kinetic changes of IgG antibody responses against S1 antigen of MERS-CoV in 70 participants from 12 to 36 months after symptom onset. A, Collected sera were tested by a commercial ELISA kit. The assay was semi-quantitatively evaluated by calculating a ratio of the extinction value of the patient sample over the extinction value of the calibrator. Optical density (OD) ratios < 0.7 were considered negative, ratios > 1.4 (dashed line) were considered positive, and ratios ≥ 0.7 and ≤ 1.4 were considered as intermediate. B, Relative proportion of sera with negative, positive, and intermediate OD ratio values is presented in clinical severity groups (GI ~ GIII) at the indicated time points (G-I: n = 9 ~ 18, G-II: n = 20 ~ 33, and G-III: n = 12 ~ 18).

Later, the antibody titers against the whole extracellular domain of spike antigens were also measured using ELISA. Moreover, assays on neutralizing antibodies against spike pseudotyped lentivirus ($ppNT_{50}$) and MERS-CoV ($PRNT_{50}$) were performed with the serum samples of 50 patients (Figure 2-2). The correlation between the values of anti-S IgG OD ratio and the neutralizing activity against the MERS-CoV and pseudotyped lentivirus was established (Figure 2, left panels). It was also found that OD ratio values of antibody titers against the spike antigen were higher in the patients who experienced more severe MERS disease than those who had milder symptoms (Figure 2, right panels). In general, the titers of anti-S IgG and the neutralizing antibodies fell gradually every year. The titers declined more considerably in G-I and G-III than in G-II. For instance, $PRNT_{50}$ titers of G-II patients in the first year were higher (2322 ± 2774) on 20.5% than in the third year (mean ± SD: 1840 ± 2350), while the percentage difference between the first and the third year in G-I and G-III accounted for 35.3% and 40.8%, respectively, in the first year (G-I: 415 ± 330, G-III: 3751 ± 3105) and in the third year (G-I: 268 ± 166, G-III: 2220 ± 1962).

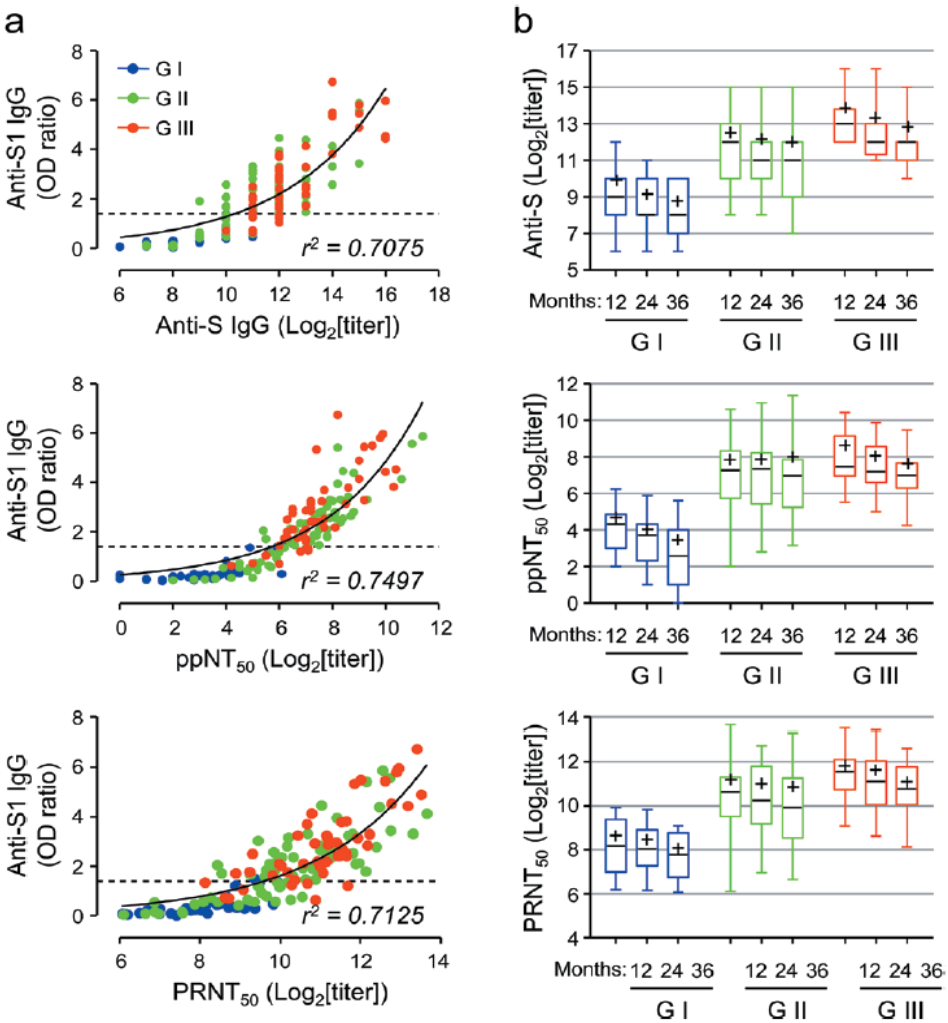


**Figure 2-2.** Correlation of anti-S1 OD ratio with anti-spike IgG titer and neutralizing activity ($ppNT_{50}$ and $PRNT_{50}$) in sera from 50 patients. A, Correlation of OD ratio values against S1 antigen with the antibody titers against spike antigen and the neutralizing titers against the pseudotyped lentivirus ($ppNT_{50}$) or MERS-CoV ($PRNT_{50}$) were assessed (left panels). Nonlinear regression curves (exponential growth) and goodness of curve fit (r 2 value) are presented. B, Kinetic changes of anti-S IgG titers and neutralizing activity ($ppNT_{50}$ and $PRNT_{50}$) in sera samples are presented in clinical severity groups (GI ~ GIII). Box and whiskers (min to max) plots including median (black line) and mean (+) values of each plot are presented at the indicated time points (G-I: n = 11, G-II: n = 23, and G-III: n = 16).

**Quantification of spike-specific memory B cell responses on a cellular level from 12 to 36 months after symptom onset by the ELISPOT**

After that, the levels of memory B cells against the spike antigen of MERS-CoV were assessed by ELISPOT to confirm the spike-specific antibody responses. In this experiment PBMC from 36 recovered patients were evaluated in the 1st and 3rd year after the initial MERS infection (G-I included 7, G-II- 16, and G-III-13 individuals) (Figure 2-3A). Based on the test, a direct correlation was established between the number of cellular spots secreting IgG specific to spike antigen in the samples and OD ratio against antigen Spike 1 (P = p = 0.0001). Besides, the same trend was found when studying G-II samples (P = 0.0150), while specimens from G-I and G-III showed no significant correlations between OD ratio and spots number (Figure 2-3B). Counts anti-S IgG-secreting B cells have been found to have no significant decrease in all samples in 36 months (mean ± SD: 127.6 ± 91.0 cells/$10^5$ PBMCs) compared to those in 12 months (mean ± SD: 154.6 ± 112.5 cells/$10^5$ PBMCs). To sum up, the numbers of B-cell spots secreting IgG specific to spike antigen were considerably lower in G-I (mean ± SD: 36.6 ± 34.4 cells/$10^5$ PBMCs) than those in G-II (mean ± SD: 203.2 ± 104.7 cells/105 PBMCs) and G-III (mean ± SD: 158.4 ± 106.3 cells/105 PBMCs) in 12 months after the infection and in 36 months (Figure 2-3C). The results obtained clearly demonstrate that the levels of memory B cells specific to spike antigen and OD ratio values against S1 antigen are higher in samples from people who had MERS disease associated with pneumonia than from those who had no symptoms or experienced mild symptoms. Moreover, the antibody responses decreased inconsiderably and persisted for at least 36 months after the infection onset.

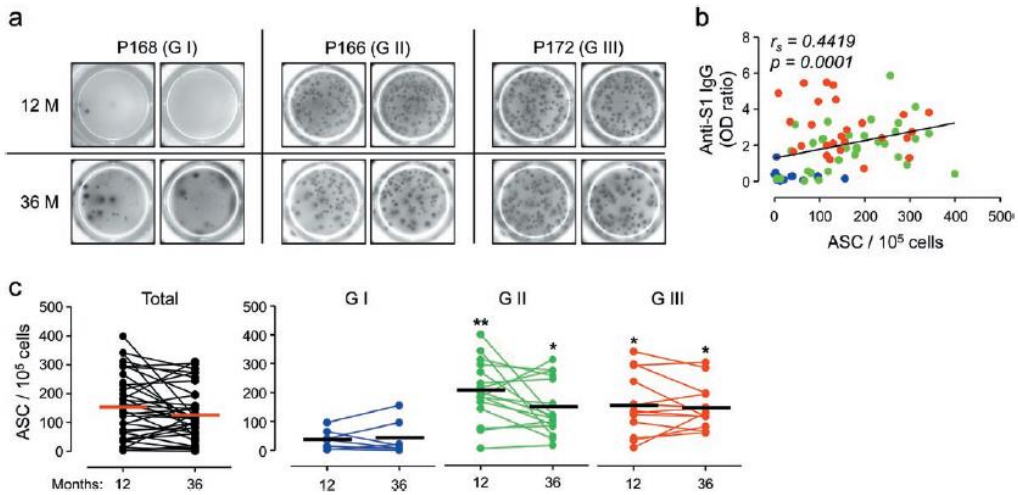


**Figure 2-3.** Quantification of spike-specific memory B cells in peripheral blood mononuclear cells (PBMCs) taken at 12 and 36 months after infection in 36 subjects. A, Representative images of B cell ELISPOT results. B, Correlation of OD ratio values against S1 antigen with anti-S IgG-secreting B cell counts were assessed by linear regression (black line) and Spearman′s rank test (r s and P value). PBMCs were taken at 12 and 36 months after infection from 36 subjects (G-I: n = 7, G-II: n = 16, and G-III: n = 13) and applied for analysis of spike antigen-specific IgG secreting memory B cells. C, Kinetic changes of anti-S IgG-secreting B cells in PBMCs are presented. Statistical analysis was performed using one-way ANOVA, followed by the Newman–Keuls t-test for comparisons of values among the severity groups at the indicated time points.

**Correlation of antibody levels with fever duration, viremia period, and maximum viral loads during infection period**

An effect of antibody levels measured in one year after the duration of MERS disease, viremia, or maximum viral loads in respiratory secretions during infection was assessed (Figure 2-4). Moreover, in the samples of the 50 subjects, a positive correlation of neutralizing antibodies $PRNT_{50}$, $ppNT_{50}$ and antibody titers against spike antigen with viral clearance, fever duration, and maximum viral loads was established. In particular, the levels of $PRNT_{50}$, $ppNT_{50}$ and anti-S antibodies correlated positively with the maximum viral loads. However, no positive correlation in individuals from G-III was revealed while comparing $PRNT_{50}$, $ppNT_{50}$ with the duration of fever and viremia measured in days. Average duration of fever and viremia of 2 patients from G-II and 4 patients from G-III with the highest levels of neutralization antibodies ($PRNT_{50} > 1/5000$ or $ppNT_{50} > 1/1000$) was 22.3 days (SD: ± 4.4) and 19.3 days (SD: ± 8.1), respectively.

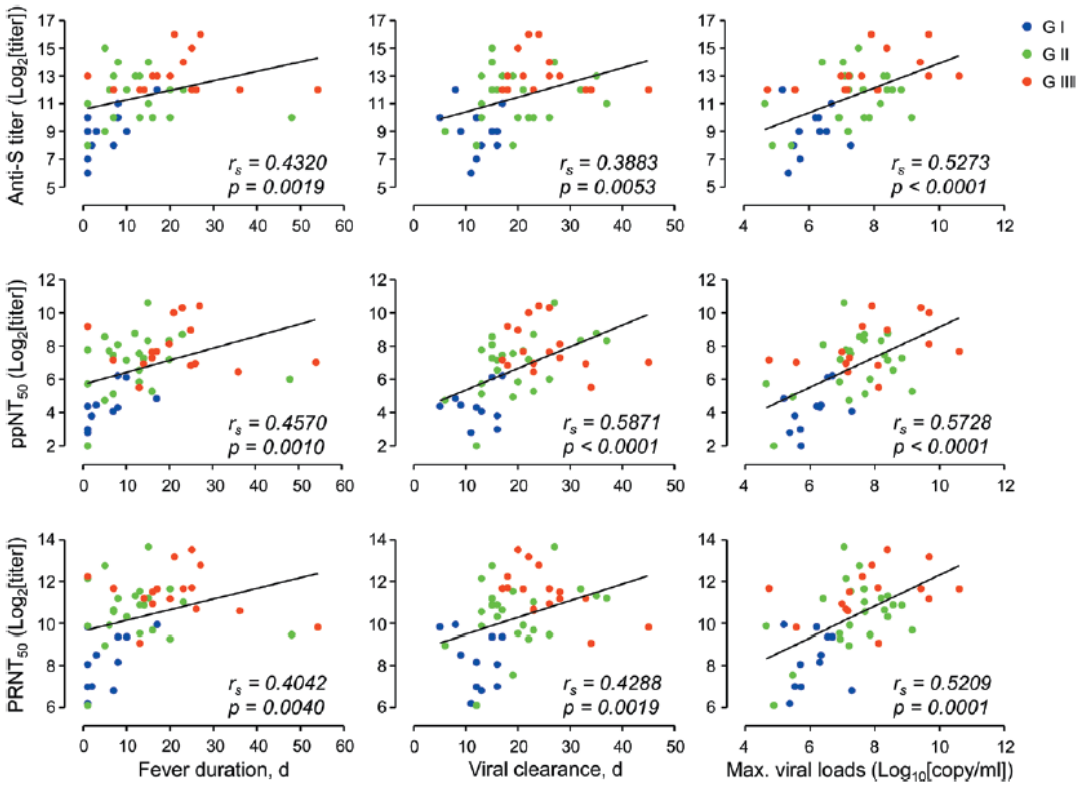


**Figure 2-4.** Correlation of antibody levels with fever duration, viremic period, and maximum viral loads during infection period. Correlations of antibody levels (anti-S IgG titer, ppNT50, and PRNT50 in sera collected at 1 year after infection) with the indicated parameters observed during infection periods in 50 subjects (G-I: n = 11, G-II: n = 23, and G-III: n = 16) were assessed by linear regression (black line) and Spearman′s rank test (r s and P value).

**Evaluation of use of pooled sera for therapy (on infected hDPP4-Tg mice)**

After that, the therapeutic efficiency of the sera of recovered individuals with neutralizing antibodies was studied by evaluating the change in hDPP4-Tg mice weight and their survival during 2 weeks after the MERS-CoV infection. First, hDPP4-Tg mice were intranasally infected with MERS-CoV at 2500 plaque-forming units (PFU)/mouse (5 × $LD_{50}$). Then, four variants of neutralizing antibodies were used to treat the mice: serum with negative, moderate, and high titers and monoclonal antibodies. For the experiment, 3 serum samples containing high $PRNT_{50}$ titers (> 1/5000) and the same number of serum-containing moderate $PRNT_{50}$ titers (~ 1/1000) were taken and mixed for obtaining pooled sera. As a negative control, a pooled serum from the healthy donors who have not been infected with MERS-CoV was taken. As a positive control, we used a 3B11 human therapeutic monoclonal antibody that targets the receptor-binding domain of the MERS-CoV spike protein [98]. The levels of antibodies in the collected pooled sera were estimated by measuring the anti-S1 OD ratio, anti-spike IgG titer, and $PRNT_{50}$ titer (Table 2-2).

**Table 2-2**. Summary of anti-spike Antibody titers and neutralizing titers in pooled sera for therapy

| Groups | OD ratio | Anti-S titer | $PRNT_{50}$ |
|---|---|---|---|
| Negative control | 0.029 | - | - |
| Moderate titer sera | 1.946 | 4096 | 1081 |
| High titer sera | 4.997 | 32 786 | 7046 |

Abbreviation: OD, optical density.

After the infection, therapeutic mAb (20 µg in 100 µL of PBS/mouse) or pooled sera (100 µL/mouse) were administered in the mice 4 times (in one hour, one, two, and three days after the infection). Then, survival, body weight changes and viral loads of virus-challenged mice were observed for 14 days (Figure 2-5).

The obtained results indicated that the mice that were administered with non-immune sera intermediate titer sera had high death rates during 8 days after infection – 100% and 87.5% (7/8), respectively. In contrast, mice treated by sera with high PRNT50 titer and therapeutic mAb had considerably increased survival rate (75.0% [6 out of 8] and 87.5% [7 out of 8], respectively). The mice that died also experienced weight loss; among the survived ones, some (1/8 in the therapeutic mAb group and 3/8 in the high titer group) had decrease (25 - 30%) in the initial body weight during 8 days after the infection before being recovered. Mice that received intermediate PRNT50 titer sera lost weight more rapidly at the beginning of infection compared to those treated with non-immune sera.

MERS-CoV viral loads and copy numbers of RNAs were measured in lung tissues in 4 days after infection during the acute phase to evaluate the effect of the sera on MERS replication (Figure 2-5B). It was revealed that the value of productive viral infection and copies of RNA in hDD4-Tg mice treated with negative serum were $2.7 \times 10^4 \pm 1.4 \times 10^4$ PFU/g of lung tissue and $5.0 \times 10^7 \pm 4.6 \times 10^7$ copies/μg of RNA, while administration of sera with high titer of neutralizing antibodies resulted in the suppression of viral loads and replication (mean ± SD: $4.1 \times 10^3 \pm 1.4 \times 10^3$ PFU/g of lung tissue and $1.0 \times 10^7 \pm 2.0 \times 10^7$ copies/μg of RNA). Transfer of serum with moderate neutralizing activity did not decrease replication of viral RNA ($2.0 \times 10^4 \pm 1.5 \times 10^4$ PFU/g of lung tissue and $4.3 \times 10^7 \pm 4.1 \times 10^7$ copies/μg of RNA).

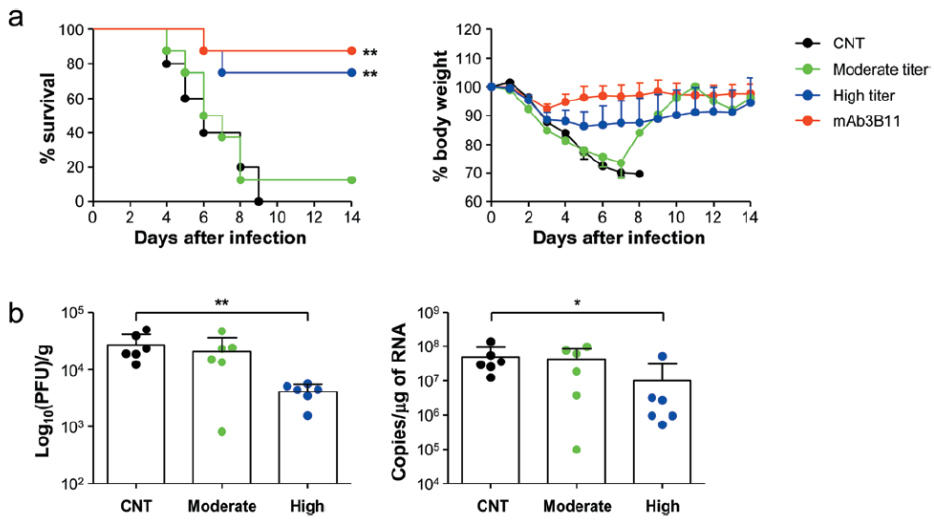


**Figure 2-5.** Evaluation of therapeutic efficacy of pooled sera from recovered patients in hDPP4-Tg mice. A, hDPP4-Tg mice were challenged intranasally with MERS-CoV at 2500 PFU/mouse (5 × LD50) and then treated with pooled sera (100 µL/mouse) or therapeutic mAb/3B11 (20 µg in 100 µL of PBS/mouse) four times (1 hour and 1, 2, and 3 days post infection). Virus-challenged mice were monitored for 14 days to evaluate survival rate (left) and body weight changes (right). The body weight data are presented as means + SD of mice in each group (CNT: n = 5, moderate, high titer, and mAb/3B11: n = 8). Significant differences between the experimental group and control group (CNT) treated with non-immune sera are indicated (**, P < .01). B, MERS-CoV viral loads were assessed by measuring PFU (left) and copy numbers of viral RNA (right) in lung tissues collected at 4 days after infection. Statistical significance between the experiment group and control group was tested by using a two-tailed Student′s t-test.

**Lung pathology of hDD4-Tg mice treated with pooled serum after MERS-CoV infection**

Besides, histopathological changes in lungs were studied in four days after the mice infection. Lung inflammation was found in all experimental groups treated with serum at varying degrees. Inflammation was characterized by the presence of edema fluid in the alveolar or interstitial lung and infiltration of monocytes/macrophages, lymphocytes, plasma cells, and a few neutrophils in the pulmonary and perivascular parenchyma. No notable difference among the researched groups was identified in pulmonary pathology; however, mice treated with the serum enriched by neutralizing antibodies with intermediate titers had more variations in pathological grades of lung inflammation and damage (Figure 2-6A and B). Therefore, it means that sera with moderate levels of neutralizing antibodies cannot control MERS replication as efficiently as immune-sera with high activity, and also resulted in a more variable degree of lung inflammation and damage compared to the negative control. The comparison of lung pathological changes and viral replication revealed no correlation between them (Figure 2-6C). The obtained results show that the administration of high neutralizing activity immune-sera had a positive impact via suppression of MERS replication, but was no efficient for alleviation of lungs inflammation during acute phase.

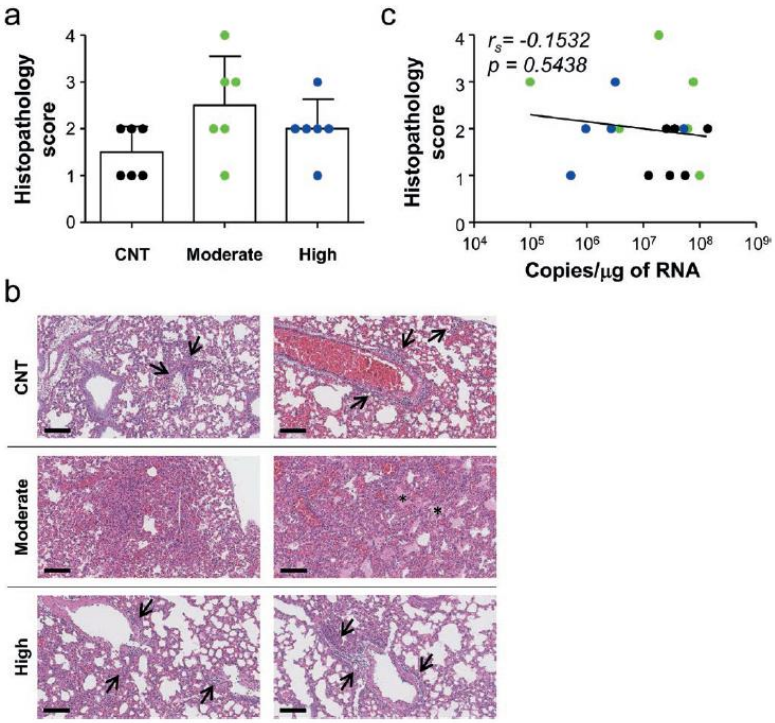


**Figure 2-6.** Pathological changes in lungs of hDD4-Tg mice infected with lethal dose of MERS-CoV. A and B, Lung tissue sections collected from mice at 4 days after infection were stained with hematoxylin and eosin. Pathological scores of infected lungs (n = 6/group) (bar graphs: mean + SD, A) and representative scanned images are presented (B). Bar, 100 μm. C, Correlation of histopathological scores with viral loads (copy numbers of viral RNAs) was assessed by linear regression (black line) and Spearman′s rank test (r s and P value).

## 3.3. Discussion

This study has investigated the quality and longevity of anti-spike-specific IgG responses in a large-scale follow-up study in 70 recovered MERS-CoV-infected patients. The complete clinical and virological datasets during the 2015 Korean outbreak were also collected and analyzed.

Responses specific to spike antigen antibody persisted for up to 3 years after the MERS infection, especially in patients who suffered from the pneumonia, hypoxemia, and treated with oxygen during hospitalization. However, from Figures 2-1 and 2-2 it can be seen that antibody titers gradually fell every year by less than twofold. The similar results have also been obtained in other long-term studies (less than 3 years) of MERS-CoV antibody kinetics in smaller studied groups [107, 108]. Levels of MERS specific IgGs have shown significant dependence on the duration and severity of the infection [107- 109].

As shown in Figure 2-4, there is a clear correlation between the levels of antibodies and fever duration, viremic periods, and maximum viral loads. It is noteworthy that the best correlation of antibody levels with maximum viral loads in respiratory secretions taken in the acute phase of MERS-CoV disease was revealed regardless of the severity of patient group. In addition, the presence and persistence of memory B cells in PBMC secreting spike-specific antibodies was also confirmed by the ELISPOT immunoassay (Figure 2-3). Persistence of neutralizing antibodies, and memory B cells in the recovered individuals may protect against further reinfection [110]. It was shown in experiment that infected animals were protected from reinfection of MERS-CoV [111]. Although the evidence of natural reinfection of camels was identified earlier, prior infection does not protect immunity from reinfection [112]. Prior infection and generation of neutralizing antibodies or passive transfer of neutralizing antibodies proved to protect animals from subsequent reinfection [113, 114]. Since there were limited studies on reinfection in humans, careful monitoring of potential reinfection cases needs to be continued.

Earlier it was established that during the SARS-CoV infection, specific antibodies in 21 of the 23 studied samples of recovered patients and antigen-

specific memory B cell response in all patients became undetectable in six year after the disease. Besides, patients with more severe clinical symptoms had a higher level of Ag-specific memory T cell response [115]. Therefore, to fully understand the persistence and longevity of the MERS-specific antibodies, the further studies are required.

Passive therapy with the use of antibodies enriched with convalescent plasma can be used in urgent cases and applied during epidemics if there is lack of time and resources to generate immunoglobulin therapy [116]. Clinical benefits of convalescent plasma therapy expressed in improving clinical symptoms and reducing viral loads in patients infected with all known coronaviruses SARS-CoV-1, MERS-CoV and COVID-19 were reflected in several studies [117-120]. It was found that despite the fact that most of the studies had limitations, such as insufficient samples size, simultaneous use of various medicals as anti-inflammatory and/or antiviral drug, transfer of immune plasma to patients during the coronaviral infection is safe and leads to a mild course of the disease and might be recommended for wider use in case of emergency as current and newly appearing pandemics [116, 120].

The use of immune plasma for patients infected with coronaviruses should be properly evaluated in a set of clinical trials to define titers of neutralizing antibody appropriate for passive therapy as in our study the use of plasma with moderate titers of neutralizing antibodies did not provide any clinical benefit. The antibodies in immune sera not only bind to a specific pathogen, directly neutralizing it, but there are also other antibody-mediated effector functions that might also contribute to its therapeutic effect such as antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity, and antibody-dependent cellular phagocytosis (ADCP) [116]. The results obtained in this study showed that mice treated with lethal dose of MERS-CoV after administration of the pooled sera with high antibody titers and neutralizing activity had considerable decrease in viral loads and increased survival (Figure 2-5). However, the use of moderate plasma not only caused no noticeable clinical improvements but also caused more variation in lung lesions. Therefore, only pooled sera with high titers of neutralizing activity can suppress replication of following spread of MERS-

CoV, but it cannot efficiently decrease inflammatory pulmonary pathogenesis during fatal infection. Figures 5 and 6 demonstrate overall picture of pathological changes in lungs and weight loss in different experimental groups. Similar trends were observed in *in vivo* studies of common marmosets that received monoclonal antibodies against MERS-CoV or hyperimmune plasma [121], and mice treated with immune sera from camels [122].

However, such neutralizing effects of antibodies as ADCP, activation of complement, and ADCC, may have not only protective, but detrimental consequences as well [123]. For example, it was reported that elevated ADCC or enhanced complement activation may also contribute to the lung pathogenesis during acute respiratory viral infection [124]. Antibody-dependent enhancement (ADE) of coronavirus entry into host cells was also reported [125], proposing that ADE might occur *in vivo* under specific conditions that depends on levels of antibodies used, their binding affinity, viral expression, and Fcγ receptors. Moreover, vaccine-induced antibodies may also directly contribute to the disease enhancement via macrophage-induced inflammatory cytokines and chemokines, resulting in lung leisure in acute SARS-CoV infection [126]. Consequently, antibody response to emerging coronaviruses should be further studied to propose exact required properties such as antibody titer, dosing range for effective application of immune sera in therapeutics and for vaccine generation safely with positive clinical effect [123].

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# 국문 초록

치명적인 호흡기 감염을 일으키는 인수공통 바이러스인 코로나바이러스는 세계적인 위협이 되고 있다. 효과적인 항바이러스제가 없는 현재의 상황에서 바이러스 중화능이 있는 회복기 사람 혈장은 효과적인 치료수단이 될 수 있을 것이다. 본 연구에서는2015년 대한민국에서의 Middle East respiratory syndrome coronavirus (MERS-CoV) outbreak 발생 당시 유행하던 바이러스의 유전체를 분석하여 SPIKE 유전자에서 두 가지의 중화항체 회피 변이들(D510G, I529T)이 발생한 것을 확인하였으며, 이후 3년간 70명의 환자에서 PBMC를 분리하여 항체반응과 기억 B 세포를 추적하였고 마우스 감염 모델을 이용하여 수집된 plasma의 중화능을 MERS-CoV 감염 치료에 이용할 수 있을지 평가하였다. MERS-CoV spike에 특이적인 IgG 반응과 중화항체 반응, 항체분비 기억 B 세포는 3년동안 지속되었고 특히 중증도가 심해질수록 더 높은 반응성을 보였다. 평균 항체가는 해마다 2배 이하로 줄어들었다. 항체반응의 정도는 발열기간과 유의한 경향성을 보였다. 유전자 변형 마우스를 MERS-CoV로 감염 시키고 높은 중화능(> 1/5000)이 있는 사람 혈장으로 치료한 결과 생존률이 향상되고 감염량이 감소하였으나 폐 병증과 초기 체중감소를 고려하면 폐의 병리기전을 막지는 못하였다. 높은 중화 항체가가 바이러스의 증식을

제어하기 위하여 필요하나 치명적인 감염상황에서 염증 병변을 줄이기에는 불충분 하였다. 따라서 인수공통 코로나바이러스 감염의 혈장 치료를 위한 중화능을 가진 면역 혈청은 신중하게 선택되어야 한다.

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- 본논문의 Chapter1의 내용은 (Kim, Yeon-Sook, et al. "Sequential emergence and wide spread of neutralization escape Middle East respiratory syndrome coronavirus mutants, South Korea, 2015." Emerging infectious diseases 25.6 (2019): 1161.), Chapter 2의 내용은 (Kim, Yeon-Sook, et al. "Sustained Responses of Neutralizing Antibodies Against Middle East Respiratory Syndrome Coronavirus (MERS-CoV) in Recovered Patients and Their Therapeutic Applicability." Clinical Infectious Diseases 73.3 (2021): e550-e558.) 에 출판되었습니다.

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**주요어:** 중동 호흡기 증후군 바이러스, MERS-CoV, 슈퍼 전파, 중화 항체, 혈장치료, 호흡기 감염, 바이러스, 인수공통

**학번:** 2013-31362