A Thesis

For the Degree of Doctor of Philosophy

Development of Monitoring System for Equine Respiratory Disease and a Equine Kidney Cell Line for Equine Herpes Virus Replication

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ABSTRACT

Respiratory diseases among horses cause economic losses in foals and productivity decreases in horses being raised and in adult horses. In South Korea, fundamental studies of horses' respiratory diseases and those related to disease occurrence are insufficient. Therefore, the aim if the current study was to use diverse hematological indicators, obtained through hematological tests and measurment of blood antibody level in horses suffering form respiratory diseases, to monitor disease occurrence and prognosis evaluation.

Viral respiratory infections are common in horses, notably equine herpesvirus infection and equine influenza, which primarily initiate secondary bacterial respiratory infections such as strangles caused by *Streptococcus equi equi.* A decline in the production of stallions has been associated with these respiratory diseases leading to adverse financial implications.

Ⅰ.Investigations of infection states by horses' bacterial respiratory diseases, susceptible drugs, and resisted drugs according to pathogens showed differences between farms. Among the β-lactam-based antibiotics, cephalosporin-based antibiotics showed some efficacy against *Streptococcus* spp. To review age-based changes in erythrocytometer hemograms in hematological tests of riding horses, red blood cell (RBC) tended to gradually decrease with increasing age this tendency was statistically significant. In addition, leukocytometer hemograms analyzed by age showed that total white blood cell counts tended to decrease with increasing age..

Ⅱ.A low level of equine herpesvirus type 1 (EHV-1) (11.36%) antibodies was detected from stallions, however a high level of EHV-4 (95.84%) antibodies was detected from horses

without vaccination against this infection suggesting that EHV-4 is ubiquitous in this horse population. In case of equine influenza, ranch stallions showed low positive rate (12.06%) whereas stallions from Subtropical Livestock Research Institute displayed higher positive rate (81.32%). Antibody responses against equine influenza and strangles revealed positive rates of 26.32% and 55.12%, respectively.

Ⅲ. Immortalized primary equine epithelial cell lines for mass-producible EHV have been established by primary culturing of the equine fetal kidney. In order to set up the conditions, we separated two different cell lines, JNUEK-1 and JNUEK-2, from immortalized primary equine epithelial cells by single-cell cloning. The virus titer of EHV-1 $TCID_{50}$/ml was calculated by inoculation of serially diluted virus into a 96-well plate of Madin–Darby bovine kidney (MDBK) cells. The optimal multiplicity of infection (MOI) was approximately 0.1 for both JNUEK-1 and JNUEK-2 to obtain the highest titers: 2×10^9 $TCID_{50}$/ml and 4×10^8 $TCID_{50}$/ml, respectively. The maximum titers of EHV-4 came from JNUEK-1, at 7×10^9 $TCID_{50}$/ml. As a result, JNUEK-1 was more successful at EHV-1 and EHV-4 production than MDBK.

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LIST OF ABBREVIATIONS

WTO	world trade organization
FTAs	free trade agreements
IT	information technology
EHV-1	equine herpes virus-1
EHV-4	equine herpes virsu-4
EAV	equine arteritis virus
EVA	equine viral arteritis
PRRSV	porcine reproductive and respiratory syndrome virus
SHFV	simian hemorrhagic fever virus
LDV	lactate dehydrogenase-elevating virus
WPDV	wobbly possum disease virus
CAVV	cavally virus
NDiV	nam dinah virus
US	united states
NAHMS	national animal health monitoring system
AQH	american quarter horse
EHVs	equine herpes viruses
UL	unique long
IR	internal repeat
TR	terminal repeat
ORFs	open reading frames
URT	upper respiratory tract
CNS	central nervous system
vCKBP	viral chemokine binding protein
VEEV	venezualen equine encephalitis virus
ERV	equine rhinitis virus
HeV	equine adenovirus, or hendra virus

ERAV	equine rhinitis A virus
ERBV	equine rhinitis B virus
ES	embryonic stem
RBC	red blood cell
EVR	equine viral rhinopneumonitis
EIV	equine influenza virus
AN	amikacin
AP	ampicillin
Cefa	cefazoline
Cefo	cefotaxime
Ceft	ceftiofur
C	chloramphenicol
EF	enrofloxacin
E	erythromycin
Flo	florefenicol
GM	gentamycin
K	kanamycin
Oxy	oxytetracycline
P	penicillin
Rif	rifampin
Tri	trimethoprim
Te	tetracycline
WBC	white blood cell
PCV	packed cell volume
Hb	hemoglobin
MCV	mean corpuscular volume
MCH	mean corpuscular hemoglobin
MCHC	mean corpuscular hemoglobin concentration
TP	total protein
ALB	albumin
AST	aspartate aminotransferase
ALT	alanine aminotransferase
ALP	alkaline phosphatase
IP	inorganic phosphorus

CHAPTER 1

INTRODUCTION

In South Korea, the agricultural sector is experiencing the greatest impact from multilateralism, specifically from the World Trade Organization (WTO), and regionalism, such as from free trade agreements (FTAs). However, the influence of the agricultural sector on South Korean exports is low.

In 2013, South Korean exports reached the levels produced in 2011, which was prior to the European financial crisis. In addition, though the business world recovered slowly following this crisis, South Korea achieved trade volumes of one trillion dollars for three consecutive years and took the seventh place in trade volumes. These impressive trade volumes resulted from numerous exports in information technology (IT) products and consumer goods. Though large enterprises traditionally accounted for rises in these volumes, small and medium enterprises have made significant contributions to increases in trade volumes.

However, for exports to continue to affect economic growth in South Korea, markets and exported items must become more diverse. In South Korea, 10 main products account for approximately half (48.5%) of all exports, compared to 33% in Japan and 28.7% in China. These competing countries show greater variety in exports. In addition to diversifying industrial products, exporting to new markets is also necessary. These markets include the service and agricultural sectors. In terms of agriculture, livestock (mainly cattle, pigs, and chickens) are exported and imported. However, diseases, such as food and mouth diseases and avian influenza, often plague these domestic

species. These issues create difficulties in exporting livestock. However, horses do not typically have these diseases.

Based on the principles outlined in the Fostering of Horse Industry Act, the government intends to add vitality to the horse industry. The government will also aims to strengthen farming and fishing societies and to influence the wellbeing and green national leisure industries. Pursuant to these policies, many local governments are in the process of completing several projects. The horse industry is a new component of the livestock industry. Increases have occurred in horse breeding farms and in the popularity of horseback riding. Compared to other domestic species, horses are more valuable. The average successful bid price for a domestic racing horse is 33.3 million won, significantly higher than the average transaction value of 5.34 million for native Korean cattle. In Japan, a racing horse raised on a Hidaka fostering farm in Hokkaido is valued between seven and eight million yen. In fact, a racing horse was once exported from Japan to Arabia for 600 million yen (with a winning price of 5 billion). Though the price varies, the average cost for a riding horse exceeds 10 million won. Therefore, horse breeding can add significantly to a farm's income.

Though the horse industry is a potential alternative to the livestock industry, more information on diseases in horses is needed. To examine the causative agents of respiratory disease in Jeju horses, their ages, serological ranges, and susceptibility to drugs were measured. In addition, the antibody titers against equine herpes virus-1(EHV-1), equine herpes virsu-4(EHV-4), influenza, and strangles disease in Jeju-do horses were studied to find measures for disease prevention. Through this investigation of antibody titers, cell lines from equine kidneys were developed to create proper vaccines.

CHAPTER 2

LITERATURE REVIEW

1. EQUINE VIRAL DISEASE

Viruses, like all obligate intracellular pathogens, have to find the means to cross cellular membranes. This is the key to initiating their infectious cycle, and involves a number of discrete steps like receptor binding and entry, capsid destabilization and genome uncoating, culminating in the release of viral nucleic acids at their site of replication. Many of these changes result from conformational alterations in metastable viral structures. Virus binding to and/or cross-linking their specific receptors can also lead to activation of downstream signaling events (Greber, 2002).

These signals often induce changes that promote entry, prepare the cell for invasion and neutralize host defences. In animal cells, enveloped viruses achieve entry in two principal ways: (1) by direct fusion with the plasma membrane, or (2) by an internalization process into endosomes. For viruses using the first strategy, fusion between the viral and cellular membranes occurs after receptor docking and before the virus core penetrates the cell. Recent developments in membrane trafficking have demonstrated the existence of multiple endocytic pathways at the plasma membrane (Marsh and Helenius, 2006). Indeed, most viruses prefer to enter cells via endocytosis since the endocytic network confers an additional advantage of specific localization within the cell for a successful infection. In the case of endocytic entry, the virus must penetrate or fuse with the endosomal membrane to be

released into the cytoplasm. Studies indicate that endocytosis serves as an entry portal for both enveloped and non-enveloped viruses. While there have been many advances in understanding how enveloped viruses achieve membrane fusion, the penetration mechanisms of most non-enveloped viruses still lack clarity. This review will give an update on the viral entry pathways and highlight the common themes and key differences between the strategies deployed by enveloped and non-enveloped viruses to achieve productive cell entry.

Abortion is pregnancy loss after placental development around 40 to 45 days (may be more correctly termed “stillbirth” in term pregnancies after 320 days). Causes include bacterial, fungal, and viral organisms.

Equine arteritis virus (EAV) is a contagious arterivirus disease of horses named for the characteristic vascular lesions that develop.

EAV was first isolated from the lung of an aborted fetus following an extensive outbreak of respiratory disease and abortion on a Standardbred breeding farm near Bucyrus, Ohio, USA, in 1953 (Doll et al., 1957a and Doll et al., 1957b). After isolation of the causative virus and description of characteristic vascular lesions, equine viral arteritis (EVA) was identified as an etiologically distinct disease of the horse (Doll et al., 1957a). EAV is a small enveloped, positive-sense, single-stranded RNA virus that is the prototype virus in the family Arteriviridae (genus: Arterivirus), order Nidovirales, a taxonomic grouping that includes porcine reproductive and respiratory syndrome virus (PRRSV), simian hemorrhagic fever virus (SHFV), and lactate dehydrogenase-elevating virus (LDV) of mice (Cavanagh, 1997). Pioneering work on the distinctive replication strategy utilized by EAV originally led to the taxonomic designation of the Order Nidovirales (Cavanagh, 1997), a grouping of morphologically distinct viruses included in the families Arterviridae, Coronaviridae, and Roniviridae that all utilize a similar replication strategy that involves the generation of a nested set of subgenomic RNAs (Gorbalenya et al., 2006). The order Nidovirales has been expanded recently to include several newly identified plus-stranded RNA viruses including wobbly possum disease virus (WPDV), a close relative to the other members of the family Arteriviridae and the cause of neurologic disease among free-ranging Australian brushtail possums in New Zealand (Dunowska et al., 2012). Similarly, two

new genetically divergent SHFV variants (SHFV-krc1 and SHFV-krc2) were recently identified in a single male colobus monkey (Lauck et al., 2011). Both SHFV-krc1 and SHFV-krc2 are highly divergent from the prototypic LVR 42-0/6941 strain of SHFV (52.0% and 58.1% nucleotide diversity, respectively) and, interestingly, the two variants are also significantly different from one another and share only 51.9% nucleotide sequence identity. Subsequently, two additional highly divergent variants of SHFV (SHFV-krtg-1a/b and SHFV-krtg-2a/b [79.4% nucleotide identity]) were isolated from African red-tailed (guenon) monkeys (Cercopithecus ascanius) from Kibale National Park, Uganda. These two variants were also genetically distinct from the prototypic LVR 42-0/6941 strain of SHFV (54.1%) and the SHFV-krc1 and SHFV-krc2 (50.1%) variants. Additional novel nidoviruses have also been isolated recently from mosquitoes, including Cavally virus (CAVV) and Nam Dinah virus (NDiV). These newly identified arthropod-borne nidoviruses are provisionally placed in a new family Mesoniviridae, which is an intermediate between the families Arteriviridae and Coronaviridae and more closely related to the family Roniviridae (Lauber et al., 2012). The recent recognition of these related but distinct viruses that share similar replication strategies indicates an increasing need for reclassification of the order Nidovirales.

Like the other arteriviruses, EAV infection is highly species-specific and exclusively limited to members of the family Equidae, which includes horses, donkeys, mules, and zebras (Timoney and McCollum, 1993). The EAV associated disease, EVA, is a respiratory and reproductive disease of horses that occurs worldwide (Timoney and McCollum, 1993). Although there is only one known EAV serotype, field strains of the virus differ in their virulence and neutralization phenotype. The clinical signs exhibited by individual EAV-infected horses depend on a variety of factors including the age and physical condition of the animal, challenge dose and route of infection, strain of virus, and environmental factors. With the sole and notable exception of the experimentally derived and highly horse-adapted, virulent Bucyrus strain, other strains and field isolates of EAV very rarely cause fatal infection in adult horses (Pronost et al., 2010). The vast majority of EAV infections are subclinical, but acutely infected animals may develop a wide range of clinical signs including pyrexia, depression, anorexia, dependent edema (scrotum, ventral trunk, and limbs), stiffness of gait, conjunctivitis,

lacrimation and swelling around the eyes (periorbital and supraorbital edema), respiratory distress, urticaria, and leukopenia (Timoney and McCollum, 1993). The incubation period of 3–14 days (typically 6–8 days following venereal exposure) is followed by pyrexia of up to 41 °C (105.8 °F) that may persist for 2–9 days. The virus can cause abortion of pregnant mares, with abortion rates during field outbreaks varying from approximately 10% to 70%, depending on the virus strain (Timoney and McCollum, 1993). EAV-induced abortions can occur at any time between 3 and 10 months of gestation. Infection of neonatal foals can cause a severe fulminating interstitial pneumonia, and in 1–3 months old foals a progressive “pneumo-enteric” syndrome. A variable proportion of acutely infected stallions (10–70%) become persistently infected and shed the virus exclusively in their semen (Timoney and McCollum, 1993). There is no evidence of EAV causing persistent infection in mares, geldings, or foals. The virus persists mainly in the ampulla of the stallion's reproductive tract, and the establishment and maintenance of the carrier state in the stallion is testosterone-dependent.

Serologic surveys have shown that EAV infection has occurred among horses in North and South America, Europe, Australia, Africa, and Asia (Timoney and McCollum, 1993). Other countries such as Iceland and Japan are apparently free of the virus. Recent studies have shown that New Zealand is also free of active EAV infection. However, the seroprevalence of EAV infection of horses varies between countries and among horse breeds within a country. For example, the seroprevalence of EAV infection varies among horses of different breeds and ages in the United States (US), with marked disparity between the prevalence of infection of standard bred and thoroughbred horses (Timoney and McCollum, 1993). EAV infection is considered endemic in standard bred but not thoroughbred horses in the US, with 77.5% to 84.3% of all standard bred but only up to 5.4% of thoroughbreds being seropositive to the virus (Timoney and McCollum, 1993). Similarly, the seroprevalence of EAV infection of standard bred horses in California was 68.5% in 1991, versus less than 2% in all other breeds tested. The 1998 National Animal Health Monitoring System (NAHMS) equine survey showed that only 0.6% of the US American Quarter Horse (AQH) population was seropositive to EAV. However, the extensive US outbreak of EVA in 2006–2007 mainly involved AQHs and this very likely significantly increased the seroprevalence of EAV within this breed. The

seroprevalence of EAV infection of warmblood stallions is also very high in a number of European countries, with some 55 to 93% of Austrian warmblood stallions being seropositive to EAV. Similarly, there is high seroprevalence among mares and stallions of Hucul horses in Poland, 53.2% and 68.2%, respectively.

Equine herpes viruses (EHVs) are ubiquitous enveloped DNA viruses that have a major economic and welfare impact on the horse industry worldwide. Nine EHVs have been characterized. Five (EHV-1 to EHV-5) infect domestic horses, and two (EHV-6 and EHV-9) are associated with infections in wild equids, including asses and zebra. In domestic horses, EHV-1 is associated with respiratory disease, abortion, and neurologic disease. EHV-2 has not been convincingly associated with pathology in horses as a primary etiologic agent, but some evidence suggests that it may be associated with keratoconjunctivitis in young horses and superficial keratopathies in adult horses. EHV-3 causes coital exanthema, a venereal disease of stallions and mares (see “Venereal Diseases in the Stallion: Viruses,” in this section). EHV-4 is associated primarily with respiratory tract disease in horses. EHV-5 is epidemiologically associated with pulmonary nodular fibrosis in adult horses (Patel et al., 2005).

EHV-1 and EHV-4 are arguably the most relevant herpes viruses affecting equids and were considered subtypes of one and the same virus species until 1981 (Studdert et al., 1981). Both EHV-1 and EHV-4 harbor linear double-stranded type “D” DNA genomes, which are separated into a unique long (UL) region and a unique short (US) segment, the latter being bracketed by two inverted repeat regions called the internal repeat (IR) and terminal repeat (TR) (Telford et al., 1998 and Telford et al., 1992). The EHV-1 genome is approximately 150-kbp in size and contains at least 76 distinct genes, four of which (gene 64, 65, 66 and 67) are duplicated resulting in a total of 80 open reading frames (ORFs). EHV-4, with a slightly smaller genome size of 146-kbp, also contains 76 genes, but only three of those (gene 64, 65 and 66) are duplicated. The nucleotide identity between EHV-1 and EHV-4 genes ranges from 55% (gene 76) to 84% (gene 42) (Telford et al., 1998 and Telford et al., 1992). Although both viruses show a high degree of genetic and, hence, antigenic similarity, they are strikingly different with respect to their pathogenesis. While EHV-4 infection usually is limited

mainly to the upper respiratory tract (URT), EHV-1 has a systemic distribution affecting multiple organ systems and causing different diseases that range from mild rhinopneumonitis to abortion and lethal myeloencephalopathy (Patel and Heldens, 2005).

Both EHV-1 and EHV-4 have considerable economic impact on the horse industry. Despite widespread vaccination, the two viruses are still causing substantial problems. In recent years, efforts have been made to more comprehensively understand the mechanisms that might explain the persistence and the different pathogenicity of both viruses, and to develop more rational vaccines. In this review, we will summarize the current knowledge, focusing particularly on the developments during the past five years, and include new findings on virus epidemiology, entry, molecular pathogenesis, immune evasion and vaccines.

EHV-1 is highly contagious and usually transmitted by direct contact, mainly through infected nasal discharge. While rare, EHV-1 can also be transmitted by aerosol or contaminated feed, water, and equipment (Allen and Bryans, 1986). After inhalation, the virus replicates in epithelial cells of the URT, resulting in virus shedding and distinct herpetic lesions of mucosal membranes. In natural and experimental infections, both EHV-1 and EHV-4 can cause respiratory disease characterized by fever, anorexia, as well as nasal and ocular discharge (Patel and Heldens, 2005). After respiratory infection, EHV-1 invades the lamina propria using migrating mononuclear cells as vehicles for further distribution. As a result, EHV-1 can easily spread throughout the body via a cell-associated viremia, reaching the secondary sites of virus replication, mainly the vasculature of the pregnant uterus and/or the central nervous system (CNS). The widespread infection of endothelial cells, along with the host's repair program in response to such insult, ultimately induce abortion or neurological disease as a result of vasculitis, thrombosis, and ischemic damage. It seems likely that the leukocyte-associated viremia and the infection of endothelial cells are key prerequisites for EHV-1 pathogenicity. In contrast to EHV-1, EHV-4 infection usually remains limited to the URT and is rarely accompanied by cell-associated viremia.

Using nasal mucosal explants as an infection model, it was shown that EHV-1 could spread from epithelium to the connective tissue below the basement membrane, where mononuclear

leukocytes were infected, whereas EHV-4-infected mononuclear leukocytes were extremely rare. Interestingly, a viral chemokine binding protein (vCKBP), gG, expressed by EHV-1 and EHV-4 has different chemokine binding properties, with the former being able to modulate chemokine activity, while the latter is not. The inability of EHV-4 Ig G to modulate chemokines that are crucial for modulating leukocyte migration during both innate and adaptive immune responses was also thought to be one of the reasons why EHV-4 infection is restricted to the URT.

Considering the infection of endothelial cells, it was hypothesized that endothelial cell infection is initiated upon close contact following adhesion of PBMCs and direct cell-to-cell transfer of virus, but not by viral egress from PBMCs (Smith et al., 2002). A recent study supported the hypothesis of direct cell-to-cell spread of EHV-1 infection as the primary route of virus transfer from PBMCs to endothelial cells demonstrating successful infection in the presence of neutralizing antibodies. PBMCs circulate in the blood as non-adherent cells, but can become adherent upon stimulation, as is presumably the case after EHV-1 infection. This “on-off” adhesion behavior is controlled by the activity of adhesion molecules expressed on the cell surface. The question of how EHV-1-infected PBMCs adhere to endothelial cells and whether EHV-4-infected PBMCs have the same propensity has not been addressed. One previous study (Smith et al., 2002) has shown that the process of endothelial cell infection may be regulated by local mediators that are theoretically capable of directing tissue tropism. It has long been known that both PBMCs and endothelial cells possess a repertoire of adhesion molecules that can mediate the interactions between the two cell types. Our recent data show that EHV-4, but not EHV-1, is able to down regulate VLA-4, the receptor for the adhesion molecule VCAM-1 expressed predominantly on the surface of infected PBMCs, which may reduce the ability of EHV-4-infected PBMCs to adhere to endothelial cells. Taken together, we hypothesize that systemic spread and higher pathogenic potential of EHV-1 when compared to EHV-4 is caused by several factors including differential ability of both viruses to establish viremia and reprogram PBMCs, modulate chemokines, and regulate cellular molecules that are important for adhesion to endothelial cells.

Over the last decade, the incidence of EHM induced by EHV-1 has increased significantly in

North America and Europe. As outlined above, a single nucleotide exchange at position 2254 (A/G 2254) in the catalytic subunit of EHV-1 DNA polymerase encoded by ORF30 is strongly associated with the occurrence of EHM. Compared to the non-neurovirulent genotype of EHV-1 (A2254), the neurovirulent genotype (G2254) was found to replicate more efficiently in horses, resulting in higher levels and longer duration of cell-associated viremia. Furthermore, the production of IL-10 was significantly reduced after infection with neurovirulent Ab4, which might contribute to increased local inflammation and a higher risk of neurological manifestation. At the port of entry in epithelia of the URT, a neurovirulent EHV-1 strain was able to infect larger numbers of leukocytes under the basement membrane of nasal mucosa when compared to non-neurovirulent EHV-1 strains. It should be noted that a minority of EHM cases were shown to be associated with the A2254 genotype; on the other hand, the G2254 genotype did not necessarily lead to EHM indicating that other viral factors might also contribute to the occurrence of EHM. Lately, the UL24 (ORF37) gene product was shown to play a role in neuropathogenicity in a mouse encephalitis model. Besides viral genetics, host and environmental factors, such as age, physical condition, immune status of the host and infection route, also have a significant impact on the clinical outcome following EHV-1 infection.

Myeloencephalitis is an uncommon manifestation of infection with EHV-1 (α-herpesvirus), resulting in a diffuse and multifocal neurologic disease caused caused by widespread vasculitis, thrombosis, and ischemic necrosis of neural tissue.

Venezualen equine encephalitis virus (VEEV) has caused the most widespread and recent epidemic outbreaks of disease. Circulation in naturally occuring rodent-mosquito cycles, results in viral spread to both human and equine populations. However, equines develop a high titer viremia and can transmit the virus back to mosquito populations. As such, the early recognition and control of viral infection in equine populations is strongly associated with prevention of epidemic spread of the virus and limiting of disease incidence in human populations.

Pneumonia is lower respiratory tract infection associated with equine influenza virus, EHV, equine arteritis virus (EAV), equine rhinitis virus (ERV), equine adenovirus, or Hendra virus (HeV).

The many members that comprise the family Picornaviridae, only two species are known to

infect horses: equine rhinitis A virus (ERAV) and equine rhinitis B virus (ERBV). The equine picornaviruses were first isolated in the 1960s (Plummer, 1962) and 1970s (Hofer et al., 1972) and despite similarities that initially saw them grouped together in the genus Rhinovirus, they separate into two distinct taxonomical groups. Both ERAV and ERBV are associated with respiratory disease in horses; however, their importance as equine pathogens and the specific roles they have in respiratory disease is still unclear. The clinical manifestations of ERAV and ERBV infection remain ill-defined, with isolates recovered from clinically healthy animals, as well as those with signs of respiratory disease (Hofer et al., 1972). Their frequent detection in combination with other viral and bacterial pathogens suggests they may have a contributing role in enhancing disease length and severity.

Equine respiratory disease is of particular importance in the performance horse industry, representing a major burden due to the additional veterinary bills, lost training time and decreased performance. The high prevalence of ERAV and ERBV in horse populations indicates that further studies are required to better understand the pathogenesis and epidemiology of these widespread viruses.

The bulk of research on equine picorna viruses was performed in the 1960s–1980s, with more recent work mostly limited to studies of seroprevalence or detection by molecular methods. In more recent years, genome sequencing has resulted in a significant reclassification of the equine picorna viruses and some reassessment of the ERBV serotype groupings. This review aims to collate current knowledge of the fundamental features of the equine picorna viruses, including current approaches for their detection that may provide an insight into the pathogenesis of these viruses.

Rhinitis virus is common upper respiratory tract virus that is seldom diagnosed as a specific cause of respiratory disease in horses but may contribute to overall pathology (Studdert, 2007).

Rotaviruses were first observed in the faces of a foal with diarrhea in 1975 in Great Britain (Flewett et al., 1975). They had been detected previously in faces from a monkey in 1958, but it was not until rotaviruses were recognised as a major cause of neonatal diarrhea in calves in 1969 and children in 1973 that significant research into this pathogen in other species commenced (Malherbe

and Strickla, 1967). Initially referred to as a reovirus-like agent, the name rotavirus was later adopted from the Latin “rota” (wheel), because of the wheel-like appearance of virions by electron microscopy (EM) (Flewett et al., 1974). Rotaviruses are the most prevalent viral pathogens identified in the faces of foals with diarrhea. The frequency of detection of rotaviruses in clinical cases varies from 20 to 77% and they appear to be endemic in most, if not all, horse populations (Browning et al., 1991; Conner and Darlington, 1980). Diarrhea in young foals is a labour intensive disease that is costly to manage. An inactivated maternal vaccine has been available commercially since the mid 1990s, but despite this rotaviruses are still a major cause of diarrhea in foals.

Equine rotaviruses are ubiquitous in horse populations. The evidence for the widespread nature of rotavirus infection includes the high prevalence of rotavirus antibodies in adult horses (Conner and Darlington, 1980; Goto et al., 1981) and the detection of rotaviruses in horse populations from many countries, including the United Kingdom (Flewett et al., 1975; Strickland et al., 1982), the USA (Kanitz, 1976), Japan (Imagawa et al., 1984), Australia (Studdert et al., 1978), New Zealand (Durham et al., 1979), Germany (Elschner et al., 2005), Italy, Greece, France, the Netherlands, Venezuela (Ciarlet et al., 1994), Argentina (Barrandeguy et al., 1998) and India (Gulati et al., 2009).

Transmission is by the face-oral route via contaminated faces or fomites. Rotaviruses are highly contagious, replicate rapidly and are found in high titers in the feces of infected animals. The minimum infective dose for foals has not been published. Studies in pigs demonstrate that infection can occur with as few as 90 viral particles, while one gram of faces from an infected animal can contain up to 1010 rotavirus particles. Equine rotaviruses can be detected in the faces of dams of infected foals. Whether this is just transit of viral particles through the mare's intestinal tract or subclinical infection is not known, but seroconversion of the dam of an infected foal has been reported (Conner and Darlington, 1980).

Rotaviruses are a significant cause of diarrhea in foals. Rotaviral diarrhea has a high morbidity in foals and, although clinical disease is usually self-limiting, dehydration may lead to mortalities. Clinical disease presents as reluctance to nurse, depression, diarrhea, dehydration, pyrexia and recumbency and has been reported in foals from 3 days to 5 months of age (Conner and

Darlington, 1980; Kanitz, 1976; Strickland et al., 1982), with younger foals generally showing more severe signs of disease. The incubation period is generally short, with the onset of diarrhea usually within 1–4 days of infection (Imagawa et al., 1984; Kanitz, 1976). Virus can be shed in faces before the onset of diarrhea, during the clinical phase of disease, which may persist for 1–12 days, and after resolution of diarrhea (Conner and Darlington, 1980; Imagawa et al., 1984; Strickland et al., 1982). Subclinical infections can also occur, contributing to environmental contamination with rotavirus and the infection of other foals.

2. EQUINE INFECTIOUS DISEASE HISTORY

African horse sickness has remained endemic to sub-Saharan Africa and southern Africa with occasional incursions into the Middle East (1959-63), Spain (1966, 1987-90), Portugal (1989) and Yemen (1997) associated with the movement of infected equids, especially Old World equids such as zebra. Although African horse sickness has, thus far, remained geographically limited, the closely related Orbivirus, bluetongue, spread progressively northwards through the Mediterranean basin in the 1970s and 1980s as its main Culicoides vector was able to extend its range through the effects of climate warming. The sudden appearance of bluetongue virus in Europe in 2006 was a wake-up call to the equine industry to be vigilant and prepared for incursions of exotic diseases. In the UK, a joint Defra/equine industry working party led by the Horse Trust started work on a control plan for the UK, which culminated in the adoption of the African Horse Sickness (England) Regulations by Parliament in November 2012.

Glanders has been eradicated from many regions since the mallein test was introduced but remains a threat to the global industry because it continues to be endemic in the Middle East, Asia, Africa and in some South American countries (Khan and others 2013).

Equine infectious anaemia has been recognised since the early 1900s and was another equine infectious disease shown to be caused by a filterable agent in those early days of discovery. The

breakthrough in the control of equine infectious anaemia came in 1972 when Leroy Coggins published a method for serological diagnosis of infected horses by an immuno-diffusion test, known to this day as the Coggins test (Coggins and others 1972). The disease continues to be endemic in many countries around the world, including the USA and some European countries. Awareness of the risks posed by the virus to the European horse industry was significantly raised following an outbreak in Ireland in 2006 (More and others 2008), the accession of Romania, an endemically infected country, to the EU and then the outbreaks of disease recorded in the UK and elsewhere in Europe since 2010. The disease has spread through the movement of infected horses and equine biological products, including plasma, and is a good example of how movement and trade spread equine infectious diseases.

Equine influenza virus was first isolated in Poland in 1956 and H7N7 virus circulated widely over the next two decades until its apparent disappearance in 1979. The first H3N8 equine influenza virus was isolated in North America in 1963 and, since then, H3N8 viruses have spread throughout the world, establishing endemic disease in all horse-keeping countries except Australia, New Zealand and Iceland. In an excellent example of virus evolution and genetic and antigenic change, the H3N8 viruses rapidly diverged into European and American lineages in the late 1980s, with both virus lineages co-circulating on both continents. In the past 10 years the European lineage viruses no longer appear to be circulating and there has been continued evolution of the American lineages with Florida Clade 1 and Clade 2 viruses dominating in Europe. The continued evolution of H3N8 viruses, albeit somewhat slower than that of human influenza viruses, is a challenge to vaccine manufacturers and the horse industry. In response to the major equine influenza outbreak in the UK in 1979, mandatory vaccination was introduced by the racing authorities in 1981 and a surveillance scheme was established with support from the Horserace Betting Levy Board and carried out by the Animal Health Trust. This remains the only coordinated endemic disease surveillance scheme in operation in the UK, and is both a reflection of the importance of equine influenza to the industry and a model for other infectious disease surveillance (Elton and Bryant 2011).

A viral cause of equine abortion was first identified by Dimock and Edwards in Kentucky in

1936, who showed that a filterable agent caused the disease. The virus, equine herpesvirus type 1, was characterised in Kentucky in the 1950s and, in response to catastrophic abortion storms in Kentucky studs, rapid progress was made with the development of diagnostic tests and the first vaccines to prevent these abortions (Doll 1961), which paved the way for the later periods of intensive study into equine herpes viruses from the 1990s to the present day. Contagious equine metritis was first identified in Newmarket in 1977 and, following a combined microbiological and epidemiological approach, diagnostic tests and an industry code of practice were quickly put in place, and control of the disease achieved.

Although this is not an extensive list, it gives some sense of how the understanding of equine infectious disease has progressed. It remains a highly challenging and dynamic discipline, especially with the continuing emergence of new equine infectious diseases. Hendra virus is one such example; first identified as a disease of flying foxes in Australia in 1996, the virus is endemic in Australia and has become a significant and serious zoonotic disease that has killed at least 80 horses and four people working in contact with horses, including equine practitioners (Mahalingam and others 2012).

3.CELL CULTURE

Cell culture is the process by which cells are cultivated under controlled conditions outside the living animal (in vitro) for easier experimental manipulation and regulation of controls. This technique offers privileges of reducing the animal's need, so it avoids the legal and ethical problems of animal experimentation and related variations. In addition, it avoids interference from biological molecules that occurs in vivo, so different secretions and elements will be feasibly monitored. Furthermore, homogeneous population of cells with similar growth requirements could be obtained. Therefore, cell characteristics will be maintained over several cell generations producing consistent and reproducible results (Davis, 2008).

Albeit cell culture technology was extensively utilized as a tool in biomedical field, their

pharmaceutical applications were not so far overviewed. The rationale of the current review was to give an overview on implementations of cell culture models in pharmaceutical assessment of nanomedicines, with high emphasis on permeability assays, cytotoxicity studies and targeting efficiency as major quality attributes.

Before proceeding in cell culture applications in nanomedicine, it may be useful to summarize commonly used terminology of cell and tissue culture. The following definitions will help to explain different types of cultured cell, how to grow and expand cell population, in addition to cell distribution in monolayer and multilayer culture.

These are cells derived directly from intact or dissociated tissues or organ fragments taken directly from an animal. The cells have heterogeneous nature, and retain many of the differentiated characteristics of the original cells in vivo. However, most have a limited life span and ultimately die. Until subculture, a culture is considered a primary one, after that it is termed a cell line (Davis, 2008).

These cells are subcultured from primary cells but undergo manipulation in the laboratory so that they can be propagated and passaged indefinitely with no apoptosis. The term cell line denotes that it consists of lineages of cells that originally exist in the primary culture, it also could be referred as Established or Continuous cell line. Unlike the unmanipulated primary cells, cell line may not mimic the original cells in in vivo cells–after several passages–due to their manipulation. Tumor cell lines are often derived from actual clinical tumors, for example Coca-2 cell line which is derived from human colonic adenocarcinoma, while in transformed cell lines, the transformation may be induced using viral oncogenes or by chemical treatment. For example Hela cell line (human epithelial cervical carcinoma) is transformed by human papillomavirus 18 (HPV18) to immortalized cell line. Most normal cells undergo limited number of subcultures, or passages before they stop growing due to senescence, so that they are referred to as finite cell lines, while tumor or transformed cell lines can divide more rapidly, indefinitely, and form tumors when reintroduced into animals. Another type of cell lines includes clonal cell line which is a cell population derived from a single cell by successive mitoses forming a genetically homogeneous population (Davis, 2008).

Sub-culture is the dissociation of cells from each other and from substrate using proteolytic

enzymes. Trypsin and/or EDTA are most commonly used in this process, so it is called trypsinization. Afterward, these cells are transfered or transplanted to a new culture vessel with nutrition media so as to propagate and expand the cell population for study. Reseeding this cell suspension generates a secondary culture, which can be grown up and subcultured again to give a tertiary culture, and so on. It is essential to determine, for each cell the type, source, and suitable number of passages during subculture, as the ability of cells to re-differentiate decreases with passaging (Davis, 2008).

Cell culture is considered an important tool for nanocarrier assessment regarding three major quality attributes (permeability, cytotoxicity, and targeting efficiency). Cell line technique could circumvent drawbacks of time consuming, expensive in-vivo methods that suffer inter-subject variation. Regarding permeability assessment, cell line technique deemed superior to other in-vitro methods that lack expression of transporters and enzymes. Cell line assessment method could also exhibit superiority on ex-vivo models in view of assessment of sustained release nanocarriers. Employment of cell line in assessment of tumor targeting and cytotoxicity appraisal is a novel field currently investigated by our work group. Proper choice of cell line for tumor targeting evaluation should consider cancer etiology.

Early tissue and cell culture pioneers paid exquisite attention to the balance of nutrients, growth factors, and pH buffers used to grow cells in vitro (Carrel, 1912).

Because of the staggering complexity of the in vivo environment, systematic study of phenomena of cellular response to mechanical stimulation has relied heavily on the use of in vitro preparations. Such work frequently has involved cell culture systems with controlled delivery of a mechanical input such as hydrostatic pressure, fluid shear stress, or substrate strain. Laboratory apparatuses devised for that purpose span an appreciable range of complexity and sophistication, and they feature mechanical input signals of varied levels of precision and homogeneity (Brown, 2000).

Embryonic stem (ES) cell lines are termed continuous cell lines and as such have the property of immortality, i.e., the cell cultures can be maintained indefinitely by continuous passages. This is in contrast to cell cultures that can be passaged for only limited periods of time and that can be referred to as finite cell lines (Freshney, 1994). The term “cell line” implies the maintenance of the

cell culture's original phenotype during continuous culture or passage, i.e., hundreds of population doublings, and also implies homogeneity of phenotype within the population of cells. For example, a muscle cell line should contain only myocytes and not myocytes and epithelial cells. For ES cell lines, this phenotypic definition is that of pluripotency, i.e., most of the cells should be capable of giving rise to somatic cells representative of the three primary embryonic germ layers (Evans and Kaufman, 1981). Furthermore, they should be able to differentiate into the germ cells, i.e., oocytes and spermatozoa (Geijsen et al., 2004). Therefore, similar to the pluripotent ICM cells of blastocysts, ES cells should be competent to contribute to all cells of the developing fetus. Also by definition, ES cell lines must be able to self-renew as stem cells; thereby maintaining their ability to differentiate into all cell types.

These definitive properties should not imply that ES cell lines must remain unchanged over time in continuous culture to be considered authentic ES cell lines. On the contrary, as with all cell lines the population of cells that comprise any ES cell line is subject to internal and external selective pressures. Stochastic events that are operating in each cell as it grows and divides will influence its comparative survival fitness within the population and within the given culture environment. So, by definition, as time in culture progresses, the various properties of the ES cell population will change; for example, the fastest growing cells will become a larger and larger proportion of the population of cells over time. Some stem cell traits are seemingly lost very early in passage, such as the ability to create live-born young that are completely ES-cell derived. Over further passages, karyotypic abnormalities become more common within the population, and cell line competence for germ line chimera contribution can also be lost. However, lost properties definitive to ES cells can probably be restored in many, if not all, ES cell lines by recognizing that the cell line is a population of individuals and that each individual cell is phenotypically and genotypically different, albeit by sometimes extremely small measures. Differences even between two daughter cells have been described. So, through single-cell cloning from the population of cells and screening the clonal populations for normal karyotypes, it has been possible to maintain the embryonic stem cell character of ES cell lines over extensive continuous culture.

CHAPTER 3

Etiologic agents of horses respiratory diseases and development of management technologies

ABSTRACT

Respiratory diseases among horses cause economic losses in foals and productivity decreases in horses being raised and in adult horses. In South Korea, fundamental studies of horses' respiratory diseases and those related to disease occurrence are insufficient. Therefore, the aim if the current study was to use diverse hematological indicators, obtained through hematological tests and measurment of blood antibody level in horses suffering from respiratory diseases, to monitor disease occurrence and prognosis evaluation.

Investigations of infection states by horses' bacterial respiratory diseases, susceptible drugs, and resisted drugs according to pathogens showed differences between farms. Among the β-lactam-based antibiotics, cephalosporin-based antibiotics showed some efficacy against *Streptococcus* spp. To review age-based changes in erythrocytometer hemograms in hematological tests of riding horses, red blood cell (RBC) tended to gradually decrease with increasing age this tendency was statistically significant. In addition, leukocytometer hemograms analyzed by age showed that total white blood cell counts tended to decrease with increasing age. These results of drug susceptibility tests and

hematological tests, which have not been presented thus far, will be useful as standardized data in future breeding management and disease tests of Jeju riding horses.

Key words : Horse, Respiratory disease, EHV, Influenza, Strangles

Introduction

As animals that have been familiar to us since ancient times, horses have carried out many tasks. In farming cultures, horses have played central roles in farm and village as transportation for cargo and people. However, the use of horses has changed significantly with economic growth. Their range of activities has been expanded to include sports and tourism, for example horse racing and horse riding. In the South Korean horse industry, the horse racing industry, the horse riding industry, and the horse breeding industry have organically engaged with each other because the scale of the overall horse industry is rapidly expanding as South Korea join the ranks of advanced nations. Because the horse industry is linked with diverse cultural projects, it differs from general livestock industries and may be considered a high grade livestock industry producing very high added values. In the case of horse racing parks, sales have been reported as six trillion, and two hundred billion has been won as of 2003. In addition, horse racing parks are important as an income source for breeding farms. As of 2004, the auction price for one horse was reported as 30 million won on average. Because of such high added value, the importance of the horse industry is growing (choi *et al*., 2011).

Despite the prosperity of the horse industry, infrastructure industries for the horse industry in South Korea are insignificant. For example, of the equine diseases, equine respiratory diseases cause reduced productivity in growing horses and adult horses, as well as economic losses due to the loss of foals. However, no study of equine respiratory diseases and their outbreak has yet been conducted. Equine respiratory bacterial diseases usually occur due to secondary infection compared to viral diseases. Infected horses generally show major symptoms, such as coughing, nasal discharge, dyspnea, bathypnea, fever, and loss of appetite (Elton and Bryant, 2011 ; MacLachlan *et al.*, 2006 ; Reed and Toribio, 2004). Studies of respiratory diseases in horses mainly evaluate lower respiratory diseases, such as pneumonia and bronchial pneumonia, and studies of upper respiratory diseases are insufficient.

Diseases that cause equine respiratory diseases include equine viral rhinopneumonitis (EVR, Rhodococcus equi or R. equi), equine influenza virus (EIV), and equine strangles. The causal agent of ERP is equine herpesvirus (EHV). When infected, adult horses show no clinical symptoms. However, fetal infection occurs at the late stage of pregnancy, resulting in miscarriage or death soon after birth. Rhodococcus equi is known as a respiratory disease occurring in foals aged one to six months. Horses infected with EIV show respiratory symptoms such as fever and nasal discharge. EIV is very strongly propagated to infect the entire stable or group of horses and causes reduced productivity leading to economic loss, although the mortality rates are reported to be low. Horses infected with equine strangles are characterized by fever, loss of appetite, and head and neck lymph node swelling. Damage increases because of the high morbidity rate, and strangles continues to occur in countries where horses are bred.

Therefore, the present study examines the conditions of infection with equine respiratory bacterial diseases using horses raised in the Jeju region. The aim is to identify effective therapeutic agents and resistant or nonsusceptible therapeutic agents, to investigate reference data to facilitate equine respiratory disease control order, and to utilize diverse serological indicators—such as serologic tests and blood antibody concentration measurements—in monitoring the occurrence of diseases and evaluating prognoses when respiratory diseases occur in Jeju horses.

Materials and methods

Animal

Twelve cotton swab samples (11 nasal discharge samples and 1 mandibular abscess sample) were used in the experiment. Blood and two pieces of lung tissue collected from four horse farms in Jeju-si and Jeju-do were also used to investigate equine respiratory bacterial disease infection conditions.

To investigate reference data for equine respiratory disease control, blood samples were collected four times from the jugular veins of 77 horses comprising 55 female horses, 9 one-year-old horses (weanlings), and 13 two-year-old horses using 10 ㎖ syringes. Part of the collected blood was treated with EDTA to analyze serological findings. The remaining blood was subjected to centrifugation to collect serum, which was kept in a freezer until analysis.

Virus separation and antibiotic sensitivity testing

The samples were cultured for 18–24 h at 37 ℃ using sheep blood agar and MacConkey agar as culture media. They were then Gram stained to identify and separate viruses using a VITEK system (BioMerieux Co., France). Antibiotic sensitivity tests of the separated viruses were conducted using the disc diffusion method (Bauer *et al*., 1966) The separated viruses were inoculated on Mueller-Hinton broth (Difco) and enriched for 2–8 h at 37 ℃. The turbidity of the viruses was adjusted to McFarland No 0.5 (1.5×108/㎖), and they were smeared on Mueller-Hinton agar (Difco) as a medium. Next, the viruses were cultured for 18~24 h at 37℃ after attaching a disc to them and the inhibition zone around the disc to judge sensitivity.

Sixteen types of antimicrobials were investigated: amikacin(AN), ampicillin(AP), cefazoline(Cefa), cefotaxime(Cefo), ceftiofur(Ceft), chloramphenicol(C), enrofloxacin(EF), erythromycin(E), florefenicol(Flo), gentamycin(GM), kanamycin(K), oxytetracycline(Oxy), penicillin(P), rifampin(Rif), trimethoprim(Tri) and tetracycline(Te)). Sensitivity to these antimicrobials was investigated.

Blood and serum tests

Red blood cell (RBC) counts and white blood cell (WBC) counts were obtained using the clinico-hematological standard method. Packed cell volume (PCV) was measured by centrifugation for 5 min at 12,000 rpm using the microhematocrit method. In addition, hemoglobin (Hb) levels were determined by measuring the optical density at 540 nm using the cyanhemoglobin method. The mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were obtained using the clinico-hematological standard method. In addition, serum chemistry—total protein (TP), albumin (ALB), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), creatinine, glucose, bilirubin, LDH, calcium, inorganic phosphorus (IP), and magnesium—was determined using an automatic chemical analyzer (Pronto Evolution; BPC Biosed, Italy) and reagents from Asan Pharmaceutical Company (Korea). Analysis of the tests was conducted at the Jeju Animal Hygiene Research Institute.

Statistical processing

The experimental results of individual groups were indicated as means and standard deviations, and the statistical significance between different classified groups was tested by conducting analyses of variance using an SAS package program (2000). The significance between different treatments was tested using Duncan's multiple range tests.

Results

Investigation of equine respiratory bacterial disease infection conditions

Cotton swab samples of purulent exudate from mandibular swellings were collected from foals that showed respiratory symptoms and laryngopharyngeal edema, mandibular edema, and high fever. Bacteria in the samples was cultured and Streptococcus equi ssp zooepidemicus (causative organism of strangles) was identified. Rhodococcus equi was identified in the blood and lung tissues of a foal that died after showing respiratory symptoms. When bacteria in 11 cotton swab samples of nasal discharge were cultured and tested, Staphylococcus spp was detected in two samples and Streptococcus spp was detected in the remaining nine samples (Table 3-1).

When tested using these samples, drugs to which bacteria are sensitive or resistant of their efficacy among farms. This is because different farms used different drugs . In the 10 cases where Streptococcus spp was detected in foals, of the β-lactam-based antibiotics, cephalosporin-based antibiotics showed some effects. When resisted drug tests were conducted with 14 cases on four farms where Streptococcus spp was detected, penicillin and tetracycline-based drugs showed resistance and thus are considered incapable of having therapeutic effects. These results are considered attributable to the fact that horse farms have frequently used penicillin and tetracycline as basic antibiotics. Of the 10 cases where Streptococcus spp was detected, 8 occurred on the same horse riding farm and showed resistance to trimethoprim-sulfamethoxazole-based antibiotics that are frequently used on most horse farms. Ampicillin and cephalosporin-based antibiotics showed sensitivity. However, although Staphylococcus spp commonly showed trimethoprim-sulfamethoxazole sensitivity, only one test sample showed sensitivity to chloramphenicol- and florefenicol-based antibiotics, and other test samples showed resistance. In the Rhodococcus equi

infected groups, quinolone-, amikacin-, and ceftiofur-based antibiotics commonly showed sensitivity. Caution is required because long-term use of quinolone-based antibiotics in foals may cause arthropathies. When foals show respiratory symptoms (except for strangles symptoms), cephalosporin-based antibiotics should be used first (Table 3-2).

Table 3- 1. Strains isolated by the rhinorrhea swab of 14 foals shown respiratory symptom

Samples	Strains	Cases(total=14)	
		n	%
Pus exudate (under jaw edma)	*Streptococcus equi* ssp. *zooepidemicus*	1	7.1
Blood & lung tissue	*Rhodococcus equi*	2	14.2
Rhinorrhea swab	*Staphylococcus* spp.	2	14.2
	Streptococcus spp.	9	64.5

Table 3-2. Antimicrobial susceptibility pattern(%) of 4 strain bacteria from foal

Antimicrobial	S equi ssp. Zooepidemicus(N=1)			Rhodococcus equi (N=2)			Staphylococcus spp (N=2)			Streptococcus sp(N=9)		
	S*	I**	R***	S	I	R	S	I	R	S	I	R
Amikacin	0	100	0	100	0	0	0	0	100	0	0	100
Ampicillin	0	0	100	0	100	0	0	0	100	33	67	0
Cefazoline	0	100	0	0	0	100	0	100	0	100	0	0
Cefotaxime	100	0	0	0	100	0	0	100	0	100	0	0
Ceftiofur	100	0	0	100	0	0	0	100	0	100	0	0
Chloramphenicol	0	0	100	100	0	0	0	0	100	0	100	0
Enrofloxacin	0	100	0	100	0	0	0	0	100	0	100	0
Erythromycin	0	100	0	100	0	0	0	100	0	0	100	0
Florefenicol	100	0	0	0	0	100	0	100	0	0	100	0
Gentamycin	0	100	0	100	0	0	0	0	100	0	0	100
Kanamycin	0	100	0	0	0	100	0	100	0	0	100	0
Oxytertracycline	0	100	0	0	0	100	0	100	0	0	100	0
Penicillin	0	0	100	0	0	100	0	100	0	33	67	0
Rifampin	0	100	0	100	0	0	0	100	0	0	100	0
Trimethorprim	0	100	0	0	0	100	50	50	0	0	0	100
Tetracycline	0	0	100	0	0	100	0	0	100	0	0	100

* : Susceptible; ** : Intermediate, ***: Resistant

Blood and serum tests

The results of tests of eight items related to red blood cells (RBCs) in riding horses' blood are shown in Tables 3, 4. RBCs (×106/㎕) showed a tendency to decrease over time, with values of 11.03 ± 1.99 in one-year-old horses, 9.58 ± 1.3 in two-year-old horses, and 8.27 ± 1.02 in female horses. These changes with age were statistically significant based on the results of t-tests of the individual classified experimental groups ($p<0.05$). MCV, MCH, and MCHC increased with age due to decreases in RBCs (Table 3-3).

White blood cells (WBCs) changes according to age are shown in Tables 6, 7, and 8. The WBC values ($\times 10^3/\mu\ell$) showed a tendency to decrease over time, with values of 13.41 ± 3.17 in one-year-old horses, 11.09 ± 1.81 in two-year-old horses, and 9.52 ± 1.67 in female horses. platelet(PLT) ($\times 10^3/\mu\ell$) also decreased over time, with values of 406.50 ± 134.57 in one-year-old horses, 202.63 ± 41.75 in two-year-old horses, and 192.73 ± 55.53 in female horses. The differences among experimental groups were statistically significant ($p<0.05$). No consistent change according to age was observed for the results of tests of other items related to WBCs (Table3-4).

Table 3. Erythrocyte profiles from Jeju crosbred horse by age during 6 months

Month	Age**	RBC ($10^6/\mu\ell$)*	Hb (g/dℓ)	HCT (%)	MCV (fL)	MCH (pg)	MCHC (g/dℓ)
	1 year <	10.57±1.05	13.38±0.81	41.62±2.48	39.37±2.62	12.64±0.98	32.16±0.56
5	1 year >	9.30±1.07	12.45±1.32	37.59±3.96	40.50±1.62	13.42±0.57	33.12±0.25
	2 year >	8.14±1.28	13.37±2.05	40.23±6.10	49.47±1.99	16.44±0.74	33.22±0.43
	1 year <	12.14±1.14	14.30±1.42	44.46±4.71	36.84±2.78	11.86±0.89	32.19±0.40
6	1 year >	8.79±2.44	11.79±3.03	35.61±9.26	40.71±1.72	13.49±0.60	33.16±0.28
	2 year >	8.47±1.05	14.06±1.61	42.40±4.72	50.19±2.05	16.65±0.76	33.18±0.38
	1 year <	11.05±3.43	13.61±1.59	40.38±10.42	37.90±5.41	14.51±8.66	32.08±0.20
7	1 year >	9.55±1.15	12.87±1.23	39.07±3.99	40.97±1.64	13.51±0.62	32.96±0.37
	2 year >	8.29±0.94	13.76±1.50	41.01±5.65	50.23±2.19	16.62±0.78	33.10±0.40
	1 year <	11.30±3.38	13.42±1.50	39.93±10.13	36.86±5.68	14.11±8.58	32.06±0.33
8	1 year >	10.22±0.93	13.98±1.12	42.98±3.54	42.11±1.70	13.70±0.56	32.52±0.29
	2 year >	8.24±0.82	13.74±1.27	41.87±3.77	50.89±2.13	16.69±0.73	32.80±0.30
	1 year <	10.95±1.95	13.59±0.95	37.33±5.20	34.47±2.83	12.76±2.11	36.88±3.87
9	1 year >	9.76±1.03	14.39±1.41	40.50±4.12	41.51±1.43	14.77±0.52	35.56±0.28
	2 year >	7.72±1.06	13.62±1.69	37.81±4.83	49.09±2.09	17.66±0.87	36.04±0.44
	1 year <	9.77±1.44	12.06±1.72	33.89±4.67	34.80±2.25	12.39±0.85	35.58±0.52
10	1 year >	10.00±1.15	15.07±1.67	42.03±4.78	42.06±1.54	15.08±0.59	35.87±0.32
	2 year >	8.33±1.06	14.94±1.88	41.65±5.27	50.02±2.00	17.95±0.77	35.89±0.46
	1 year <	11.40±1.51	14.46±1.80	40.97±5.54	35.96±2.15	12.72±0.79	35.41±0.44
11	1 year >	9.47±1.32	14.61±1.82	40.58±5.03	43.01±1.62	15.48±0.68	35.99±0.67
	2 year >	8.70±0.93	15.81±1.57	43.51±4.20	50.09±1.98	18.09±0.93	36.34±0.45
	1 year <	11.03±1.99	13.55±1.40	39.80±6.16	36.60±3.39	13.00±3.27	33.77±0.90
Average	1 year >	9.58±1.30	13.59±1.66	39.77±4.95	41.55±1.61	14.21±0.59	34.17±0.35
	2 year >	8.27±1.02	14.19±1.65	41.21±4.93	50.00±2.06	17.16±0.80	34.37±0.41

* : Mean ± S.D. ,** :1 year < ; Under 1 year ,1 year < ; over 1 year old & under 2 years old, 2 year > ; over 2 years old

Table 4. Leukocyte profiles from Jeju crossbred horse by age during 6 months

Month	Age**	WBC ($10^3/\mu\ell$)*	Differential Count($10^3/\mu\ell$)			
			LY(%)	MO(%)	EO(%)	GR(%)
5	1 year <	9.93±3.30	2.57±1.59	0.64±0.33	0.07±0.08	6.81±1.95
	1 year >	9.76±1.22	4.21±0.77	0.63±0.42	0.33±0.29	5.06±0.89
	2 year >	8.99±2.02	2.61±0.87	0.48±0.29	0.17±0.21	5.74±1.63
6	1 year <	13.07±4.96	3.22±1.05	1.26±0.47	0.19±0.08	8.56±3.54
	1 year >	10.81±1.86	5.19±1.22	0.33±0.23	0.85±0.61	4.61±0.72
	2 year >	10.08±1.52	3.27±0.93	0.47±0.25	0.26±0.24	6.09±1.51
7	1 year <	14.72±4.54	4.57±1.75	1.59±0.59	0.14±0.12	8.62±2.86
	1 year >	12.74±2.35	5.88±1.31	0.29±0.33	0.95±0.14	6.16±1.27
	2 year >	9.40±1.53	3.23±0.80	0.43±0.31	0.30±0.28	5.39±1.48
8	1 year <	16.31±2.18	5.29±1.57	1.53±0.41	0.16±0.25	9.33±2.21
	1 year >	12.77±1.54	6.30±1.17	0.95±0.47	0.32±0.33	5.39±1.18
	2 year >	9.80±1.45	3.45±0.70	0.37±0.19	0.32±0.28	5.69±1.32
9	1 year <	14.06±2.69	5.44±1.76	1.13±0.27	0.15±0.23	7.48±1.58
	1 year >	12.70±2.04	6.17±1.32	0.25±0.20	0.91±0.16	5.70±1.36
	2 year >	9.73±1.37	3.41±0.87	0.36±0.20	0.26±0.22	5.72±1.17
10	1 year <	15.20±2.26	6.98±1.74	0.92±0.41	0.28±0.29	7.52±2.29
	1 year >	9.95±2.24	4.35±1.70	0.39±0.17	1.10±1.19	4.79±1.80
	2 year >	9.99±2.33	3.47±1.09	0.52±0.32	0.33±0.56	5.81±2.09
11	1 year <	10.58±2.26	5.09±1.21	0.40±0.29	0.58±0.35	4.62±1.25
	1 year >	8.88±1.41	4.07±0.85	0.19±0.09	0.61±0.44	4.01±1.07
	2 year >	8.67±1.44	3.35±0.82	0.19±0.09	0.30±0.22	4.83±1.14
Average	1 year <	13.41±3.17	4.74±1.52	1.07±0.40	0.22±0.20	7.56±2.24
	1 year >	11.09±1.81	5.17±1.19	0.43±0.27	0.72±0.45	5.10±1.18
	2 year >	9.52±1.67	3.26±0.87	0.40±0.24	0.28±0.29	5.61±1.48

* : Mean ± S.D. ,** :1 year < ; Under 1 year ,1 year < ; over 1 year old & under 2 years old, 2 year > ; over 2 years old, LY : Lymphocytes, MO : Monocytes, EO : Eosinophils, GR : Granulocyte

Discussion

Equine respiratory diseases are one of the causes of economic loss in the horse industry. It is difficult to treat these diseases in horses of all ages but particularly in foals, in which these diseases cause death (Boguta *et al*., 2002). Acute or chronic infection by these diseases reduces or damages pulmonary functions (Bernard *et al*., 1991). In addition, clinical or therapeutic problems related to these diseases are known to increase globally (Boguta *et al*., 2002). Sources of infection by respiratory diseases can be largely divided into viruses and bacteria. In the case of viruses, study results on the pathogenesis and roles of EHV-1, EHV-4, EAV, EIV, etc. have been reported (Carman *et al*., 1997 ; Crabbn and Studdert, 1995). However, in the case of bacteria, studies of primary or secondary causes and roles are insufficient. In terms of clinical results when 25 farms in the UK where respiratory diseases occurred were investigated, only 8% of the diseases were found to have been caused by EHV, and 68% were not directly related to viruses (Wood JL., 1993). That is, the frequency of equine respiratory diseases was not solely related to the distribution of viruses. Most bacteria found in equine respiratory diseases are known to be secondary causative organisms that follow virus infection (Siegal and Barlough, 1996). Pathogenic streptococci, including S. equi, S. zooeppidemicus, and S. dysgalactiae, are important causative organisms of bacterial respiratory diseases that occur in most countries with a horse industry and tend to occur mainly in newborn foals, although they also are also common in breeding and racing horses of all ages. The effects range from minor respiratory symptoms to death due to septicemia (Timoney, 2003). According to previous studies, S. equi is a causative organism of equine respiratory diseases and strangles and is accompanied by mandibular lymph node abscesses. S. zooeppidemicus opportunistically infects horses to cause respiratory infection and genital infection and is recognized as the most important causative organism of bacterial respiratory infection. It has been found that approximately 40% all infections are related to S. zooeppidemicus (Newton *et al*., 2008).

Streptococcus spp comprises Str equi, Str zooeppidemicus, and Str equisimilis. Str equi causes problems such as Guttural pouch empyema in horses through secondary infection following infection by influenza, rhinovirus, or EHV-1. Rhodococcus equi causes severe pneumonia in foals and is known to be highly sensitive to penicillin G (Wintzer and Weaver, 1986). Among the antimicrobials used in horses, amikacin, ampicillin, amoxicillin, cefotaxime, cephalexin, ceftiofur, enrofloxacin, erythromycin, gentamicin, imipenem, metronidazole, ticarcillin, trimethoprim sulfa, and fluconazole are known to be effective. Penicillin, amoxicillin, streptomycin, gentamicin, erythromycin, and oxacillin are the most commonly used by most horse clinicians (Turner, 2003).

To review the results of the investigation of equine respiratory bacterial disease infection in the present study, drugs to which bacteria are sensitive or resistant differed among farms. In the case of Streptococcus spp., among β-lactam-based antibiotics, cephalosporin-based ones showed some sensitive effects. When resisted drug tests were conducted with 14 subjects on four farms where Streptococcus spp. was detected, penicillin and tetracycline-based drugs were commonly resisted and are thus considered incapable of having therapeutic effects. However, although Staphylococcus spp commonly showed trimethoprim-sulfamethoxazole sensitivity, only one test sample showed sensitivity to chloramphenicol and florefenicol-based antibiotics, and other test samples showed resistance. In the Rhodococcus equi infected groups, quinolone, amikacin, and ceftiofur-based antibiotics commonly showed sensitivity. When foals show respiratory symptoms (except for strangles symptoms), cephalosporin-based antibiotics should be used first. Future research about equine respiratory diseases should include molecular biological studies of more samples to facilitate early diagnosis and prevention.

Meanwhile, as studies involving hematological tests of riding horses at different ages for equine respiratory disease control are rare, useful standard values have not been presented. Therefore, in the present study, hematological changes in riding horses (a total of 77 horses) were divided into those of one-year-old horses (n=9), two-year-old horses (n=13), and female horses (n=55) to show changes according to age.

To review changes in red blood cell-related blood conditions found in the present study, RBC tended to decrease gradually with age from 11.03 × 106/㎕ to 8.27 × 106/㎕, and these changes were statistically significant. However, as blood cells and their components vary with seasons, specification management systems, age, genetic homogeneity, and region even in the same animal species, (Carman *et al.*, 1997 ; Curtis *et al*., 1989; Martin *et al*., 1990) blood components should be analyzed thoroughly under more long-term programs.

Based on the analysis of white blood cell-related blood conditions conducted in the present study, WBC values showed a tendency to decrease as age increased, with values of 13.41 × 10^3/㎕ in one-year-old horses, 11.09 × 10^3/㎕ in two-year-old horses, and 9.52 × 10^3/㎕ in female horses. Currently, there is no study on the hematological values and serum chemical values of riding horses being raised on Jeju. Therefore, research is necessary. In conclusion, significant changes in hematological values according to age were observed. These results of hematological tests have not been presented earlier and are considered usable as standardized data for future specification management and disease examinations of Jeju riding horses, thus facilitating appropriate treatment when respiratory diseases occur.

CHAPTER 4

A study on the formation of antibodies in horses bred in Jeju against respiratory disease through vaccination

ABSTRACT

Respiratory diseases among horses cause economic losses in foals and productivity decreases in horses being raised and in adult horses. In South Korea, fundamental studies of horses' respiratory diseases and those related to disease occurrence are insufficient. Therefore, the aim if the current study was to use diverse hematological indicators, obtained through hematological tests and measurement of blood antibody level in horses suffering from respiratory diseases, to monitor disease occurrence and prognosis evaluation.

Investigations of infection states by horses' bacterial respiratory diseases, susceptible drugs, and resisted drugs according to pathogens showed differences between farms. Among the β-lactam-based antibiotics, cephalosporin-based antibiotics showed some efficacy against *Streptococcus* spp. To review age-based changes in erythrocytometer hemograms in hematological tests of riding horses, red blood cell (RBC) tended to gradually decrease with increasing age this tendency was statistically significant. In addition, leukocytometer hemograms analyzed by age showed that total white blood cell counts tended to decrease with increasing age. These results of drug susceptibility tests and

hematological tests, which have not been presented thus far, will be useful as standardized data in future breeding management and disease tests of Jeju riding horses.

Key words : Horse, Respiratory diseases, Hematological tests, Blood antibody level

Introduction

As animals that have been familiar to us from ancient times, horses have carried out many tasks. In farming cultures, horses have worked in direct support of farming and as transportation for cargo and workers. During war, horses played a most important role in the munitions industry and were the basis of the measure of military force. The use of horses changed significantly with economic growth, and their range of activities expanded to include sports and tourism, for example, horse racing and horse riding. In addition, as the per capita income of families in South Korea has reached 30,000 dollars, people's interest and participation in horse racing and horse riding has gradually increased. To review the present situation of horse breeding in South Korea, the number of horses being bred increased slightly from 24,951 horses on 1,291 farms in 2007 to 27,881 horses on 1,528 farms in 2008. The number of horse breeding farms and the number of horses bred continued to increase, as 1,642 farms bred 28,718 horses in 2009; 1,912 farms bred 30,402 horses in 2010; and 1,912 farms bred 29,698 horses in 2012. In particular, Jeju-do accounted for at least 67% (approximately 20,000) of the horses bred in South Korea as of the end of 2012. It has 50 horse riding facilities and possesses the natural conditions necessary for horse production training; for example, Jeju has 45% (17,000 ha) of all the grasslands in South Korea. The central and local governments' attention to the horse industry has increased, as demonstrated by the enactment of the Fostering of Horse Industry Act in 2012 and the designation of special horse industrial zones. These actions are pursuant to the 2012 "Five Year Comprehensive Plan for Fostering of the Horse Industry", which was prompted by people's growing interest in horse-related activities that occurred with increased prosperity. Therefore, the scale of breeding has increased. The interest in the horse industry has mainly focused on racehorse production and the horse racing industry but has recently begun to expand to related industries, such as the horse riding industry and the horsemeat industry. Because the horse industry is linked to cultural

undertakings, it differs from general livestock industries and has been expanded from primary industries to sixth industries (Choi *et al*., 2011).

However, as the scale of the horse industry grows, issues relating to equine diseases are coming to the fore. Of the equine diseases, equine respiratory diseases result in the reduced productivity of growing and adult horses and economic loss due to the loss of foals. However, basic research into equine respiratory diseases and studies about the outbreak of those diseases are still lacking in South Korea. Equine respiratory diseases include equine pneumonia, equine influenza, and equine strangles. Equine rhinopneumonitis (ERP) is caused by equine herpes virus (EHV) that belongs to the Alpha herpes viridae subfamily of Herpes viridae. When infected by ERP, horses show no particular clinical symptoms in most cases. However, when a fetus is infected in the late stage of gestation, a miscarriage is induced or the foal dies within 24 h. When young horses are infected by ERP, loss of appetite and submandibular lymph node enlargement occur, along with fever and nasal discharge. When secondarily infected by streptococci, laryngopharyngitis accompanied by coughing and pneumonia occurs (Elton and Bryant, 2011 ; MacLachlan and Balasuriya, 2006). In addition, ERP may cause nervous diseases, such as infirmity, insanity, laryngoparalysis, and astasia. Subtypes of herpes virus that cause problems in horses are known to include EHV-1 subtype 1 (EHV-1), EHV-1 subtype 2 (EHV-4), and EHV-3 (Vail, 1993). Horses infected by equine influenza virus (EIV) show respiratory symptoms accompanied by fever, coughing, and nasal discharge. EIV spreads rapidly in highly susceptible groups of horses because its communicability is very strong; however, it is characterized by high fast relief rates and low mortality rates. However, it causes reduced productivity leading to economic losses (Yamanaka *et al*., 2009) Equine influenza is introduced and propagated when racehorses and stallions are moved from their place of origin to another location and causes problems not only in Europe and North America but also in Japan, China, and other countries close to South Korea. Equine strangles is an acute infectious disease that occurs in equidae animals due to infection by Streptococcus equi subsp. equi (Pustera *et al*., 2003) Infected horses show clinical symptoms such as fever, loss of appetite, nasal discharge, and head and neck lymph node swelling. In particular, marked swelling of mandibular and postpharyngeal lymph nodes is a distinctive clinical

symptom of this disease. Damage is significant because of the high morbidity rate, and strangles continue to occur in countries where horses are bred (Jorm, 1990). In advanced Western countries, such as Australia and the Netherlands, serological and other test methods have been used to identify EHV infection in approximately 10–30% of horse herds (Patel and Heldens, 2005). In addition, the defense efficacy and immune reactions of industrialized vaccines have been studied (Foote *et al*, 2006 ; Kydd *et al*., 2006) using fat diagnostic methods (ELISA) that can identify EHV-4 that has cross-reactivity with EHV-1. Evaluation methods have also been studied (Cravv *et al*., 1995). Strangles has also been identified in South Korea through the serologic prediction of EHV-1 in bred horses, virus separation methods, and gene detection methods. Although the occurrence of strangles in horses in the Jeju region has been reported, actual states of infection have not been investigated. In addition, although serologic investigations of EIV are conducted, vaccine titer analysis and studies on improving the administration of effective therapeutic agents are lacking.

Therefore, as respiratory diseases such as equine pneumonia, influenza, and strangles cause economic losses in the form of pneumonia and death, the present study examines whether immunity is formed after equine respiratory disease vaccinations to monitor the rates of immunity formation and the rates of antibody formation against individual diseases, to select effective therapeutic agents for bacteria that cause equine respiratory diseases, and to develop optimum programs for studies to analyze the titers of respiratory disease prevention vaccines.

Materials and Method

Animals

The blood samples used in the experiment were from 361 horses: 129 Jeju stud farm horses for which tests were requested as part of health examinations in Jeju-do between May 2012 and June 2013; 141 two-year-old thoroughbred horses belonging to the Korea Racing Authority(KRA); 91 horses obtained from the Subtropical Livestock Research Institute of the National Institute of Animal Science (NIAS) that were used to examine the rates of immunity formation by type of horse; and 55 mares, 13 two-year-old horses, 2 three-year-old horses, and 9 weanlings from the Subtropical Livestock Research Institute of the National Institute of Animal Science (NIAS) that were used to examine vaccine immunity formation and monitor changes in a one-year period. The collected blood samples were subjected to centrifugation to collect serum, and the serum was kept in a freezer until analysis.

Enzyme-Linked Immunosorbent Assay

After removing the culture medium from a culture bottle in which RK-13 cells from the rabbit kidney cell line were cultured, the culture bottle was inoculated with an appropriate amount of the virus and the virus was absorbed at 37 ℃ for 2 h. Thereafter, a culture medium was added to the bottle to culture the virus. When CPE appeared in 80–90% of the cells, the infected cells were frozen at –70 ℃ and then repeatedly thawed and re-frozen. Next, the cells were subjected to centrifugation for 30 min at 8,000×g and pellets were removed. Thereafter, only the supernatant was collected and subjected to centrifugation for 2 h at 48,300×g. Thus, the virus was obtained and then used as an ELISA antibody.

The virus antibody was diluted with a buffer solution for absorption (50 mM carbonate with a pH of 9.6 containing 0.02% sodium azide), and the diluted virus antibody solution was dispensed to individual wells of an ELISA plate (8-well strip flat-bottom microplates, Costar 2580, CA, USA) by putting 100㎕ of the diluted virus antibody solution into each well. The ELISA plate was then left at refrigeration temperatures for approximately 16 h until the virus was absorbed. Individual wells were then washed with PBS. Following this, 200㎕of PBS containing 0.5% BSA was added to each well, and the ELISA plate was left at room temperature for 30 min. Thereafter, the wells were washed with PBS and dried and were used in a later ELISA.

The serum to be tested was diluted with PBS-T, and 100㎕ of the diluted serum was added to each well. The ELISA plate was then left at room temperature for 1 h to induce reactions. The wells were washed four times, and 100㎕of goat anti-horse Ig G marked with horseradish peroxidase (HRP) was added to each well. This was left at room temperature for 1 h to induce reactions. Thereafter, the wells were washed four times, and 100㎕of color former ABTS was added to each well. When color reactions had continued for 30 min, 100㎕ of 0.005% sodium azide aqueous solution was put into each well to stop the reactions. The optical density of the reactant was immediately measured at a wavelength of 405 nm using a measuring instrument. At this point time, the optical density value of 492 nm was substituted as a control wavelength (Table 4-1).

Statistical processing

The experimental results of individual groups were indicated as means and standard deviations, and the statistical significance between different classified groups was tested by conducting analyses of variance using an SAS package program (2000). The significance between different treatments was tested using Duncan's multiple range tests.

Table 4-1. Examination and method by disease

Disease	Sample	Method	Result
Equine Viral Rhinopneumonitis EHV-1 & EHV-4	serum	ELISA	OD > 0.2 positive 0.1 ≤ OD ≤ 0.2 doubtful OD < 0.1 negative
Equine Influenza	serum	ELISA	S/N < 0.6 Positive S/N ≥ 0.6 negative
Strangles	serum	ELISA	Positive titer < 1:200 : negative - 1:200 : weak positive - 1:800 : moderate positive - 1:3,200 : strong positive - 1:12,800: very strong posivive

Results

EHV-1 and EHV-4

Regarding immunity formation against ERP, when EHV-1 antibody tests were conducted for the 361 horses, low antibody formation was found, as 41 horses (11.36%) were positive. When EHV-4 antibody tests were conducted, high antibody formation was found, as 346 horses (95.84%) were positive (Table 4-2). The low rate of antibodies against EHV-1 indicated the necessity of EHV-1 vaccination, while the high rates of antibody formation against EHV-4 were shown throughout the year. To review the results of EHV-1 tests, because Jeju horses and horses from the Subtropical Livestock Research Institute were not inoculated with the vaccine, 17 heads (13.18%) and 16 heads (17.58%), respectively, were identified as having the antibody. The thoroughbred horses from the Korea Racing Authority were judged as showing a low rate of antibody formation (5.76%) because they were not sufficiently inoculated with the vaccine for antibody formation as they were only two years old. In the case of EHV-4, the horses showed a high rate of antibody formation of 95.84% despite not being inoculated with any vaccine. Regarding EHV vaccination in Jeju-do, although vaccines are supplied to farms by the government, the supply is limited to farms that produce racehorses. A vaccine was therefore made of a combination of EHV-1 and EHV-4 subtypes. The low rates of antibody formation against EHV-1 are considered attributable to differences in the subtype prevailing in South Korea because the vaccine was produced in a foreign country and supplied to South Korea. In particular, in the case of the horses belonging to the Korea Racing Authority, the low rates of antibody formation seem to be attributable to the fact that the vaccinations were not continued (Fig. 4-1).

Regarding the results of antibody monitoring tests, when antibodies against EHV-1 for which no vaccination was implemented were monitored, the rates of antibodies against EHV-1 were shown

to be low, as fewer than seven heads (9.7%) on average possessed antibodies. The rates of antibodies increased greatly in April, which is assumed to be attributable to increases in virus activities due to declines in immunity at the change of seasons. On the other hand, in the case of EHV-4, high rates of antibody formation were shown throughout the year (Fig. 4-1). When compared to monthly levels of maternal antibodies after foaling, it could be seen that the levels of EHV-1 antibody formation remained a little higher at the beginning but decreased five months after foaling. In the case of monthly levels of antibody formation against EHV-4, high rates of antibody formation were still shown seven months after foaling, but these decreased 10 months after foaling (Fig. 4-2).

Equine influenza

Regarding immunity formation against equine influenza, when antibody tests were conducted for the 361 horses, 95 heads (26.32%) were antibody-positive (Table 4-2). Of the 141 horses belonging to the Korea Racing Authority, 17 heads (12.06%) were shown to be positive. Of the 91 heads from the Subtropical Livestock Research Institute, 74 (81.32%) were identified as being positive, a seven times higher level of antibody positivity. Given that high levels of antibody formation are achieved in the Subtropical Livestock Research Institute through continuous management and training of individual horses, high rates of antibody formation are assumed to occur because of booster effects (Fig. 4-3). When the results for horses from the horse farm and the Subtropical Livestock Research Institute were compared, it was found that horses were not inoculated with any influenza vaccine when they were moved to the farm of the Korea Racing Authority at six months old, indicating that foals should be inoculated with equine influenza vaccines when they are moved or sold.

According to the results of monthly influenza antibody monitoring tests, high levels of influenza immunity were formed in female horses, but the foals born to these horses showed an antibody formation of 0% because they had no maternal antibodies (Fig. 4-3). When these results are

compared with the test results of horses moved to the farm of the Korea Racing Authority, it can be seen that these horses were not inoculated with any vaccine before they were moved.

Equine strangles

Regarding immunity formation against equine strangles, when antibody tests were conducted for the 361 horses, 199 (55.12%) were antibody-positive (Table 4). Antibodies were formed in 71 (55.04%) out of 129 Jeju horses, 48 (34.04%) out of 141 horses belonging to the Korea Racing Authority, and 80 (87.91%) out of 91 horses from the Subtropical Livestock Research Institute (Fig. 6). There was an overall antibody formation rate of 55.12% for equine strangles, which is relatively higher compared to other diseases. However, at least 80% of negative horses and weak positive horses requiring additional inoculation were Jeju horses and thoroughbred horses, indicating that the horses were not sufficiently inoculated. When monthly strangles antibody monitoring tests were conducted, there was a high rate of antibody formation of 87.9%, which is relatively higher compared to other diseases. However, the number of antibody-negative horses and weak positive horses requiring additional inoculation was shown to be large around September (Fig. 4-4). Despite mother horses possessing high levels of strangles antibodies, most foals were shown to be negative and weak positive and thus in need of additional inoculation, indicating that foals are exposed to the risk of strangles. Therefore, vaccination is considered necessary for foals.

Table 4-2. Seropositive rate of EHV-1, EHV-4, influenza and strangle by Jeju stud farm, KRA and NIAS

Classification	Number of sample	Number of positive(%)			
		EHV-1	EHV-4	Influenza	Strangle
Jeju stud farm	129	17(13.18)	128(99.22)	4(3.10)	71(55.04)
KRA*	141	8(5.67)	129(91.49)	17(12.06)	48(34.04)
NIAS**	91	16(17.58)	89(97.80)	74(81.32)	80(87.91)
Total	361	41(11.36)	346(95.84)	95(26.32)	199(55.12)

*KRA : Korea Racing Atuthority, **NIAS : National Insttitute of Animal Science

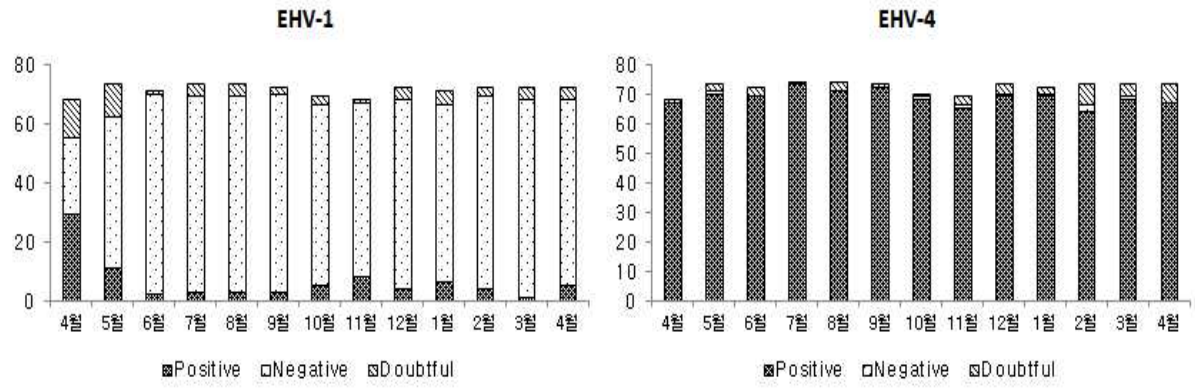

Fig. 4-1. Seropositive rate of EHV-1 & EHV-4 by Mare during one year

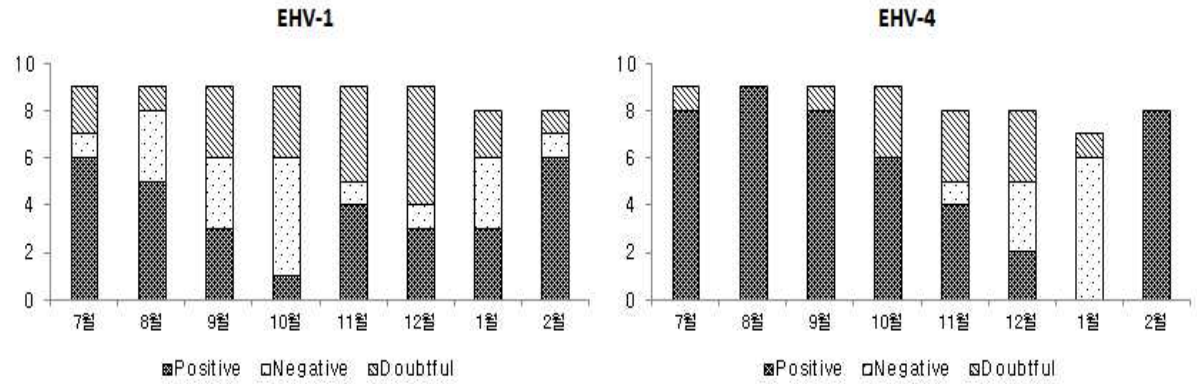

Fig.4-2. Seropositive rate of EHV-1 & EHV-4 by Foal during one year

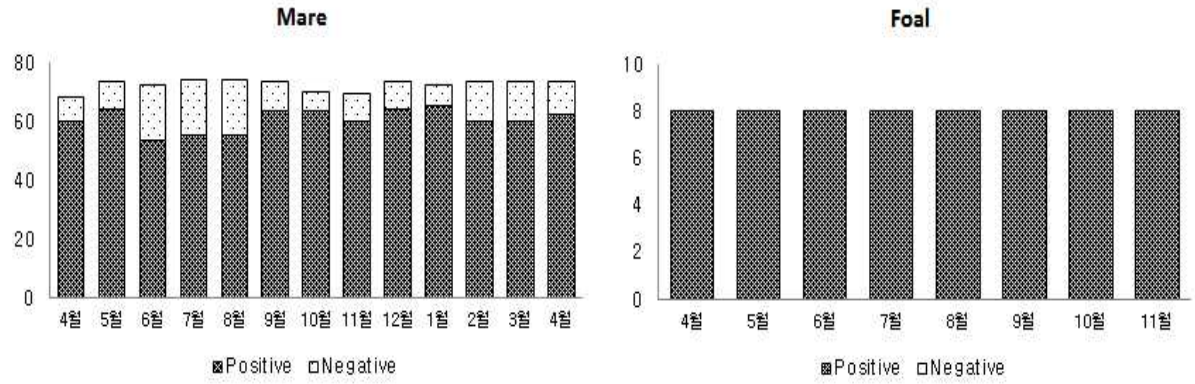

Fig. 4-3. Seropositive rate of Influenza by Mare and Foal during one year

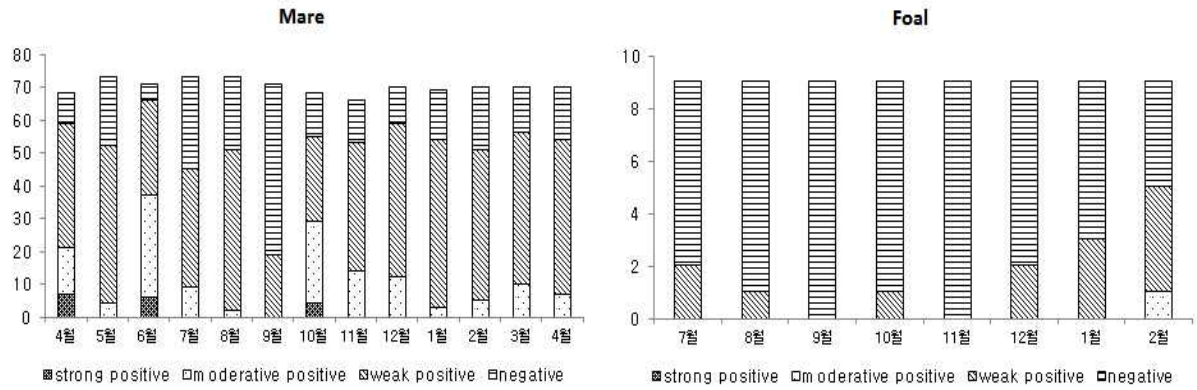

Fig. 4-4. Seropositive rate of Strangle by Mare and Foal during one year

Discussion

Equine respiratory diseases are one of the causes of economic loss in the horse industry. It is difficult to treat these diseases in horses of all ages but particularly foals, in which these diseases cause death (Boguta *et al*., 2002). Acute or chronic infection by these diseases reduces or damages pulmonary functions (Bernard *et al*., 1991). In addition, clinical or therapeutic problems related to these diseases are known to increase globally. EHV is a causal agent of equine rhinopneumonitis, which first occurred in South Korea in 1981. Recently, EHV was divided into EHV-1 and EHV-4 and methods of distinguishing these subtypes using the polymerase chain reaction(PCR)technique have been extensively studied (Matsumura *et al*., 1993). Horses infected with EIV show respiratory symptoms such as fever and nasal discharge. EIV is very strongly propagated and infects the entire stable or group, resulting in decreased productivity and economic loss, although the mortality rates are reported to be low. Horses infected with equine strangles are characterized by fever, loss of appetite, and head and neck lymph node swelling. Damage is significant because of the high morbidity rate, and strangles continues to occur in countries where horses are bred. In 2010, 647 horses were imported into South Korea and 450 horses were imported in 2011. Such imports increase every year, and horses that do not show any clinical symptoms and that have been inoculated during the waiting period of 21d are allowed to be imported. Therefore, diseases may be introduced through the import of horses with subinfection. Therefore, continuous monitoring is necessary.

Therefore, in the present study, using Jeju horses for which medical examinations were requested, thoroughbred horses belonging to the Korea Racing Authority, and horses from the Subtropical Livestock Research Institute, rates of antibody formation against equine respiratory

diseases were examined and changes throughout the year were monitored. According to the results, low rates of antibody formation against EHV-1 presented the possibility of a virus. Although horses are vaccinated against EHV-1 at some farms in South Korea, the progress of changes in antibody titers following vaccination and actual vaccination conducted at farms should be investigated to guide horse breeding farms in appropriate vaccination programs. In the case of EHV-4, a high rate of antibody formation of 95.84% was shown, despite the horses not being vaccinated against it. Given that cases of mixed infection of EHV-1 and EHV-4 were reported among miscarried horses in Jeju-do, it could be seen that the possibility of EHV-4 damage to horse breeding farms is very high. This point should be studied in more detail in future studies.

Regarding equine influenza, a low rate of antibody formation (12.06%) was found for horses bred on the horse farm of the Korea Racing Authority, and a rate of antibody formation of 81.32% was found for the 91 horses bred at the Subtropical Livestock Research Institute, which is approximately seven times higher. When the horse farm and Subtropical Livestock Research Institute horses' results were compared, it was found that horses (six months old) were not inoculated with any vaccine against influenza when they were moved to the Korea Racing Authority farm, indicating that foals should be inoculated against equine influenza when they are moved or sold. In addition, if the vaccine program being implemented by the Subtropical Livestock Research Institute is implemented on all South Korean farms, rates of antibody formation should improve so that diseases can be prevented. Based on the results of monthly influenza antibody monitoring tests, foals just born are exposed to infection as they have no antibodies against influenza viruses. Therefore, vaccination programs for foals should be implemented. As the rates of antibody formation against equine influenza and strangles were only 26.32% and 55.12%, respectively, despite the availability of vaccines, farms should be more exhaustive in this regard. In addition, studies about additional vaccination for a certain prolonged period instead of one simple vaccination and boosters should be conducted. In the case of equine strangles, to manage antibody-negative horses and weak positive horses requiring additional vaccination, rather than the autumn strangles vaccination (once per year) that is currently given, biannual preventive vaccinations should be given. In addition, no horse was

strongly positive for outdoor infection by strangles in the present tests, which is attributable to the fact that the horses tested this time were healthy with no clinical symptoms. The strangles antibody titer investigation conducted in the present study is quite meaningful because it was the first conducted in South Korea. Even the Animal and Plant Quarantine Agency has been identifying strangles only through bacterial culture and PCR tests for animals with clinical symptoms. The results of the investigations of the rates of antibody formation against individual diseases and the monthly monitoring tests are usable as basic data for studies to select effective therapeutic agents to fight bacteria that cause equine respiratory diseases and for studies to analyze respiratory disease-preventing vaccine antibody titers. They are also useful for disease prevention and vaccination programs to control respiratory disease.

CHAPTER 5

High-titer production of EHV-1 and EHV-4 using immortalized equine cell lines

Abstract

Equine herpesvirus (EHV) causes serious and often fatal disease in equines. Currently, various cells and cell lines have been used to detect or produce EHV. Immortalized primary equine epithelial cell lines for mass-producible EHV have been established by primary culturing of the equine fetal kidney. In order to set up the conditions, we separated two different cell lines, JNUEK-1 and JNUEK-2, from immortalized primary equine epithelial cells by single-cell cloning. The virus titer of EHV-1 $TCID_{50}$/ml was calculated by inoculation of serially diluted virus into a 96-well plate of Madin–Darby bovine kidney (MDBK) cells. The optimal multiplicity of infection (MOI) was approximately 0.1 for both JNUEK-1 and JNUEK-2 to obtain the highest titers: 2×10^9 $TCID_{50}$/ml and 4×10^8 $TCID_{50}$/ml, respectively. The maximum titers of EHV-4 came from JNUEK-1, at 7×10^9 $TCID_{50}$/ml. As a result, JNUEK-1 was more successful at EHV-1 and EHV-4 production than MDBK.

Key words : Equine herpes virus type 1, equine herpes virus type 2, high titer, JNUEK-1, JNUEK-2

Introduction

Among equine herpesviruses (EHV) in *Herpesviridae,* type 1, type 2, type 3, type 4, and type 5 have been identified thus far. Among them, EHV type 1 (EHV-1) and EHV type 4 (EHV-4) cause equine respiratory diseases globally (Gröne *et al*, 2002). Whereas EHV-1 sometimes causes miscarriage, stillbirth, or neurological symptoms, EHV-4 rarely does. The propagation paths of EHV-1 and EHV-4 are the respiratory organs, where these viruses are known to proliferate. To prevent EHV-1 and EHV-4 as such, immortalized mixed vaccines are used (Bannai *et al*., 2014) . However, these vaccines have inconsistent effects (efficacy is especially low in mares) and short immunity durations. Although vaccines mixed with immuno-adjuvants and subunit vaccines have been developed to solve these problems, no vaccine with reliable effects has been reported yet. To produce attenuated or immortalized vaccines, host cells that can produce viruses are necessary. Currently, EHVs are cultured using the Madin–Darby bovine kidney (MDBK) cell line and the rabbit kidney 13 (RK-13) cell lineStudies are actively in progress for methods to enhance virus production yields by making the attenuation and immortalization processes more efficient using these cell lines. The MDBK cell line consists of bovine kidney-derived cells that are known to be susceptible to EHV viruses. Cell lines that have been already created, such as MDBK, are mostly cells that have become cancerous and that have many physiological differences with general cells (Kasem *et al*., 2010).. Therefore, their productivity for viruses with strong host-cell specificity, such as EHVs, is low. Higher-level EHVs can be produced using equine fetal kidney cells. However, these primary culture cells cannot be used for virus production for long periods of time because they generally have small effective numbers of subcultures. In addition, the characteristics of these cells change as they are thawed after cryogenic freezing. One of the greatest advantages of primary culture cells is that they have the physiological characteristics of cells of individual organs, unlike cell lines produced from cancer tissues or those that become cancer cells. Although primary culture methods have been

extensively studied in various animals, studies using animal fetuses are rare. When fetuses are used, cells survive for long periods of time during the primary culture, and proliferation speeds are high because there are many cells in the mitotic phase. To increase primary culture cells' effective number of subcultures, methods such as telomerase or p53 gene regulation and hybrid cell fabrication are used. The method that overexpresses telomerase to reduce the speed of apoptosis does not change cells into cancer cells. Diverse vectors can be used to overexpress telomerase in cells. Among these, retrovirus vectors can be inserted into the chromosomes of target cells, to continuously transmit the newly inserted gene to the daughter cells after division. Due to this characteristic of retrovirus vectors, telomerase can be continuously expressed in primary culture cells, immortalizing them.

For the development of cell lines that can produce EHV at high titers, the present study aimed to produce new equine-derived lines. The study also explored the conditions for producing high-titer EHV-1 and EHV-4 using the new lines. To this end, genes were introduced into primary culture cells to construct cell lines for EHV production. To immortalize the cells, recombinant retrovirus particles that express human telomerase reverse transcriptase (hTERT) were inserted into the primary culture in an attempt to immortalize the equine cells. In addition, EHV-1 and EHV-4 were inoculated into the immortalized cell lines to explore conditions for the production of EHV-1 and EHV-4 at high titers.

Materials and Method

Virus seed and cells

EHV-1 was separated from the blood and pleural effusions of a miscarried fetus of a mare at Daeyeong Farm in Jeju-do, South Korea, in February 2005. The blood and pleural effusions were subjected to centrifugation at 600 ×*g* for 5 min at 4°C, and the supernatant was collected. The collected supernatant was sterilized using a 0.22 μm filter and subjected to centrifugation at 3000 ×g for 15 min at 4°C, and then the supernatant was collected. EHV-4 (VR-2230) was purchased from the American Type Culture Collection (ATCC) and the MDBK cell line (KCLB-10022) was purchased from the Korea Cell Line Bank.

Equine fetal-organ harvesting

An equine fetus was secured through a slaughterhouse from a womb was aseptically harvested, and the fetus was used as a primary culture material. The womb containing the fetus was immersed in 70% ethanol for sterilization, then incised. The umbilical cord was ligated using a sterilized hemostat and the fetus was harvested. The skin of the fetus was disinfected using a potadine solution (povidone iodide, 100 mg/ml). As a secondary disinfection, the potadine solution was removed using chlorhexidine gauze. All processes for the fetal organ harvesting were implemented on a clean bench.

RBC removal and enzymatic treatment

The harvested tissues were placed in a petri dish, and 5 ml of Dulbecco's modified essential medium (DMEM; Gibco, USA) was heated in boiling water at 36°C, then added to the petri dish. The tissues were minced into small pieces using iris scissors in the medium. The criteria for red blood cell (RBC) removal from the tissues and enzymatic treatment of the tissues were determined by referring to a study conducted by Jeon and Hwang (2009). The small pieces of tissue were transferred to a 15 ml conical tube, and 5 ml of DMEM was added. The conical tube was kept static for 30–60 s to settle the pieces of tissue, and when they were determined to have settled completely, the supernatant was carefully removed. After the supernatant was completely removed, 10 ml of DMEM was added to the tube and the tissue pieces were made to float using a pipette. The conical tube was again kept static for 30–60 s to settle the tissue pieces completely, and the supernatant was again removed. This treatment was repeated 4–5 times to sufficiently remove the RBCs. Finally, 5 ml of DMEM was added to the conical tube, which was then subjected to centrifugation at 300 ×*g* for 3 min to collect the tissue pieces.All enzymatic treatments were conducted at 37°C. The tissue pieces collected after removal of RBCs were added to 5 ml of DMEM at 37°C, then treated with 0.15% collagenase for 20 min as the first enzymatic treatment, followed by a second treatment with 0.01% dispase for 15 min. When the enzymatic treatments were completed, the tissue was separated into single cells through 30–40 repetitions of pipetting. After adding 30 ml of DMEM-10 with 10% FBS (Gibco, USA), the cells were subjected to centrifugation at 300 ×*g* for 3 min and the pellets were collected. The collected pellets were made to float with 3 ml of DMEM-10 and the solution was dispensed into a 24-well cell-culture plate by placing 200 μl of the solution into each well. After adding 1 ml of DMEM-10 medium into each well, the cells were cultured for 24 h in a CO_2 incubator.

Production of recombinant retrovirus particles

pDONAI-2-hTERT, provided by yu-Kye Hwang (Department of Veterinary Medicine, JeJu National University, Jeju, Korea), was used as a retrovirus vector for cell immortalization. The retrovirus particles to be used in order to stably introduce genes into cells were made through co-transfection using the Retrovirus Packing System (TAKARA Bio, Japan). The pDONAI-2-hTERT vector, the pGP vector, and the pE-ampho vector were co-transfected into 293T cells using the calcium phosphate transfection method (Fig. 5-1). After the co-transfection, the solution was treated with 5% CO_2 for 10 h at 37°C and the medium was replaced with fresh medium. The supernatant was collected 48 h after transfection through centrifugation, filtered using 0.45 μm filters, and stored at −80°C while being used for transduction. The supernatant was collected 48 h after transfection through centrifugation, and the titer was measured with real-time PCR using a retrovirus titer set (TAKARA Bio, Japan).

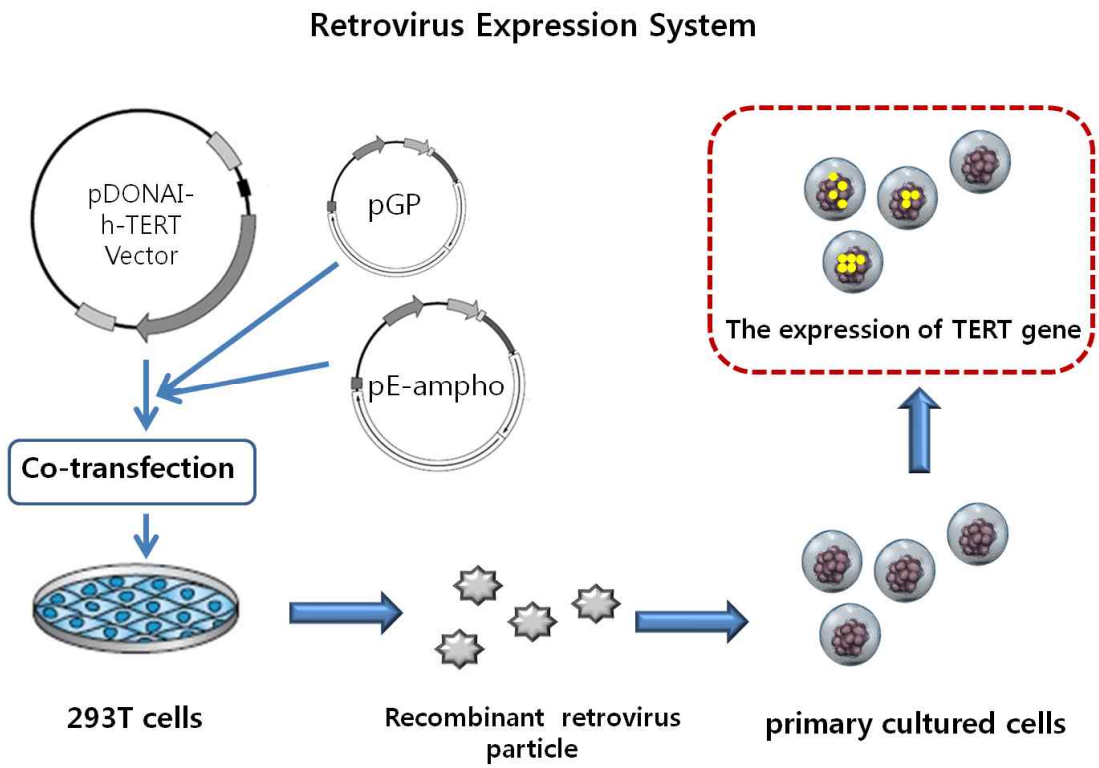

Fig. 5-1. Diagram of the gross process of the retrovirus expression system and recombinant retrovirus particle production. The recombinant retrovirus vector is co-transfected with pGP vector and pE-ampho vector. The recombinant retrovirus particles are infected with target cells and thereafter express TERT.

Gene introduction

After placing 3×10^4 primary culture cells into a T25 cell-culture flask, 5 ml of DMEM-10 was added and the cells were cultured for 24 h in a 5% CO2 incubator at 37°C. After 24 h, the medium was removed and the cells were washed three times with PBS. The washed cells were inoculated with 1 ml of recombinant retrovirus particles so that the multiplication of infection (MOI) reached 10. The cells inoculated with the recombinant virus were cultured for 4 h in a 5% CO2 incubator at 37°C. Four hours after the inoculation and culture, 4 ml of DMEM-10 was added, and the cells were cultured for 72 h in a CO_2 incubator at 37°C. Three days after the introduction of the genes, the medium was replaced with a medium made by adding 500 μg/ml of G418 to DMEM-10, which was used to subculture the cells once every four days for 16 days. From day 17, the cells were continuously cultured in the medium but with the G418 concentration reduced by half.

Cell cloning and proliferation ability evaluation

After immortalization, single cells were cloned and two cell lines were selected based on the shapes and sizes of the cells. Single-cell cloning was conducted using a 96-well cell-culture plate to select the cell lines, which were named JNUEK-1 and JNUEK-2.The proliferation ability was evaluated through radioactivity acquisition-ability experiments. The JNUEK-1 and JNUEK-2 cell lines with generation numbers of 10, 15, 20, 25, and 30 were cultured separately on 24-well cell-culture plates. After replacing the culture medium and adding 0.01 ml of epidermal growth factor (Gibco, USA) to each cell line to stimulate the cells for proliferation, the cells were cultured in a 5% CO2 incubator at 37°C. Two hours after adding the growth factor, tritium marker thymidine (0.5 μCi/ml) was added and the cells were cultured for 10 h. When the culture was complete, 0.01 ml of 1% NaN_3 was added to the medium, the cells were washed twice with PBS, 0.5 ml of 0.2 N NaOH was added, and the cells were left to react for 5 min. When the reactions ended, the cells were washed with 0.5 ml of 0.2 N NaOH solution, then neutralized with 0.2 ml of 2 N HCl. After neutralization was

complete, 0.15 ml of 5% trichloroacetic acid (TCA) at 4°C was added, and the cells were subjected to centrifugation for 3 min at 300 ×*g*. The precipitate was transferred to a glass filter, 10 ml of scintillation was added, and the radioactivity was measured with a beta counter.

Immunofluorescent staining

To identify whether the produced immortalization cell lines were epithelial cells, the expression of cytokeratin was checked. The JNUEK-1 and JNUEK-2 cell lines were separately transferred to 24-well cell-culture plates and cultured for 48 h, then the medium was removed and the cells were washed twice with PBS. The cells were then treated with 4% paraformaldehyde for 15 min and washed twice with PBS with 1% BSA added. The cells were then treated with PBS added to 0.25% triton X-100 for 10 min, and again washed twice with PBS. After blocking with 0.2% BSA for 30 min, the cells were made to react with anti-cytokeratin 18 antibody (Abcam®, USA) diluted to 1:200 as a primary antibody for 2 h. When the reactions ended, the cells were washed twice with PBS and treated for 50 min with anti-mouse IgG FITC (Abcam®, USA) diluted to 1:400 as the secondary antibody. After being washed, the cells were treated with DAPI (Sigma, USA) diluted to 1:3000 for 5 min. When all of the treatments were completed, the cells were mounted on ProLong® Gold Antifade Reagent (Sigma, USA) and observed through a fluorescence microscope.

Virus seed production and susceptibility test

Viruses were received from a detailed study conductor in the same research-project group as that of this author, and seed viruses were made for use in the study. JNUEK-1 cells were subcultured in a T75 cell-culture flask to form a 70%–80% monolayer by 24 h later. The medium was removed and the cells were washed three times with 15 ml of PBS, then inoculated with 2 ml of PCV-2 virus added to 2 ml of DMEM. After being cultured for 5 h in a CO_2 incubator at 36°C, 11 ml of DMEM medium was added with 2% FBS and the cells were continuously cultured. Five days after the virus

inoculation, the medium was collected, placed into a 50 ml conical tube, and subjected to centrifugation for 5 min at 130 ×*g*. Then, only the supernatant was collected. The collected supernatant was subjected to centrifugation for 10 min at 3000 ×*g*, and again, only the supernatant was collected. The viruses in the collected supernatant were purified using 45 um filters, and the supernatant was divided into 0.5 ml units in 1.5 ml microtubes and kept in a −80°C freezer until being used.

Virus seed production and susceptibility test

The MDBK cells were subcultured in a T75 cell-culture flask to form a 70%–80% monolayer by 24 h later. The medium was removed and the cells were washed three times with 15 ml of PBS. The washed cells were inoculated with 2 ml of EHV-1 and EHV-4, 2 ml of DMEM was added, and the cells were cultured for 5 h in a 5% CO2 incubator at 37°C. Five days after virus inoculation, the medium was collected, placed into a 50 ml conical tube, and subjected to centrifugation for 5 min at 130 ×*g*. Then, only the supernatant was collected. The collected supernatant was subjected to centrifugation for 10 min at 3000 ×*g*, and again, only the supernatant was collected. The collected supernatant was sterilized using a 45 um filter, dispensed into 0.5 ml units, and kept in a −80°C freezer until being used.

The JNUEK-1 and JNUEK-2 cell lines were cultured in a T25 culture flask to form an 80% monolayer by 24 h later. After identifying the formation of the monolayer, the medium was removed and washed three times with PBS. After inoculating 50 μl of EHV-1 and EHV-4 into each well, 1 ml of medium was added. Four hours after inoculation, 4 ml of medium was added and the cells were cultured for 7 days. CPE was checked during the culture period.

MOI selection and titer test

The JNUEK-1, JNUEK-2, and MDBK cell lines were cultured for 20 h so that approximately 50%, 70%, 90%, and 100% monolayers were formed. EHV-1 and EHV-4 seed viruses were diluted with DMEM-2 added to 2% FBS, and inoculated into the cell lines at MOI of 0.01, 0.1, 1, and 10. Four hours after the virus seed inoculation, the inoculated solution was removed and the cells were washed twice with PBS. DMEM-10 was added and the cells were cultured for 72 h before the virus titer was measured.

To test the titer, the MDBK cell line was cultured in a 96-well culture plate so that an 80% monolayer was formed by the next day. After the monolayer was identified, the medium was removed and the cells were washed three times with 15 ml of PBS. After washing, 50 μl of the collected medium was diluted in 1.95 ml of DMEM, and 200 μl of the solution was inoculated into each well. The medium was inoculated into each well step-by-step while diluting by 1/10 at each step, so that the final amount of the medium was 200 μl. The medium was cultured for 3 days in a 5% CO_2 incubator at 36°C, and then the CPE and virus titers were checked.

Mycoplasma infection test

Whether the immortalized cells were infected by mycoplasma was verified using PCR (Mycoplasma PCR Detection Kit; iNtRON, Seoul, Korea).

Results

Cell cloning

After cell immortalization, single-cell cloning was conducted to select the cell lines, which were named JNUEK-1 and JNUEK-2 (Fig. 5-2). Morphologically, the JNUEK-1 cells were longer than those of JNUEK-2.

To select immortalized cells, G418 (geneticin) was added to the medium at a concentration of 500 μg/μl and the culture was continued for approximately 2 weeks, replacing the medium with fresh medium and G418. The surviving cells were then cultured using the same medium but with the concentration of G418 reduced to half, while checking whether the cells were immortalized. During the subculturing, we observed whether the shapes of epithelial cells were maintained and whether there were phenomena such as contact inhibition or proliferation into multilayers, in order to determine whether the cells had become cancerous. Fig. 2B shows the shape of cell line JNUEK-1, with its longish cytoplasm. Fig. 2C shows cell line JNUEK-2, consisting only of cells with round cytoplasms.

The proliferation abilities of cell lines JNUEK-1 and JNUEK-2 at the 10^{th}, 15^{th}, 20^{th}, 25^{th}, and 30^{th} generations after immortalization were evaluated, and the results indicated that there were no differences in proliferation ability between different generation numbers. However, the primary culture cells showed proliferation abilities similar to those of cell lines JNUEK-1 and JNUEK-2 until the 15^{th} generation, which then rapidly decreased starting with the 20^{th} generation.

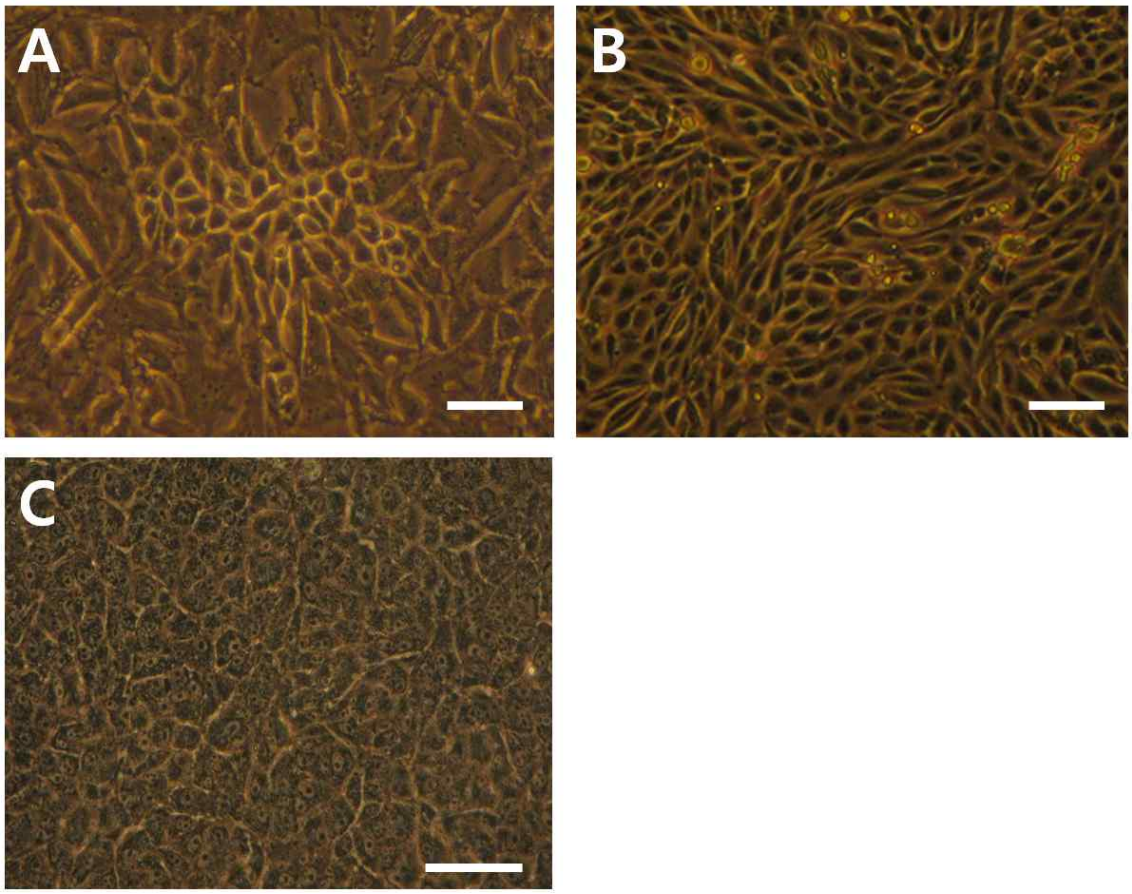

Fig. 5-2. Primary equine cell lines and immortalized equine cell lines (JNUEK-1 and JNUEK-2). A: Primary culture cells in a mixture. B: JNUEK-1 cell line, separated by single-cell cloning. C: JNUEK-2 cell line, separated by a single-cell cloning. Scale bar: 40 μm.

Proliferation ability evaluation

The proliferation abilities of cell lines JNUEK-1 and JNUEK-2 at the 10^{th}, 15^{th}, 20^{th}, 25^{th}, and 30^{th} generations after immortalization were evaluated, and the results indicated that there were no differences in proliferation ability between the different generations (Fig. 5-3). However, the primary culture cells showed a proliferation ability similar to those of cell lines JNUEK-1 and JNUEK-2 until the 15^{th} generation, which rapidly decreased after the 20^{th} generation. The proliferation ability of the primary culture cell line was highest in the 10^{th} and 15^{th} generations. In the 20^{th} and later generations, the JNUEK-1 cell line showed the highest proliferation ability while the primary culture cell line showed the lowest. In the 25^{th} and 30^{th} generations, the primary culture cell line was barely proliferating. However, cell lines JNUEK-1 and JNUEK-2 continued to show high proliferation abilities.

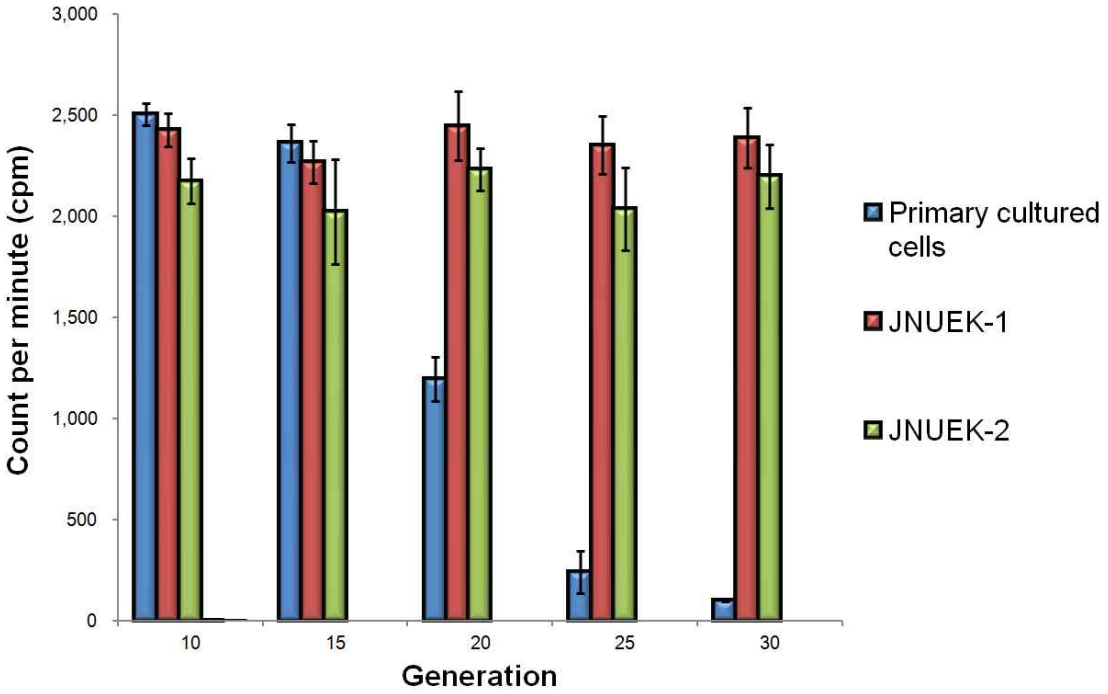

Fig. 5-3. The cell division abilities of immortalized equine cell lines and primary cultured cells. Among the equine cell lines, those with active proliferation until the 30th generation were JNUEK-1 and JNUEK-2. The division speed of the primary equine cell lines rapidly decreased, and they did not proliferate after the 25th generation.

Immunofluorescence staining

To verify that the produced immortalization cell lines were epithelial cells, the expression of cytokeratin was checked(Fig. 5-4). The JNUEK-1 and JNUEK-2 cell lines were separately transferred to 24-well cell-culture plates and cultured for 48 h to conduct immunofluorescence staining. When the 5^{th} generation of JNUEK-1 and the 10^{th} generation of JNUEK-2 were stained with cytokeratin, at least 80% of the cells were identified as secreting cytokeratin. The JNUEK-1 and JNUEK-2 cell lines were separated from single cells through single-cell cloning. Since cytokeratin was secreted by most cells and the cell lines were separated from single cells, it could be verified that JNUEK-1 and JNUEK-2 were epithelial cells. Although no photo of the results is attached, in the present study, it was determined that both of these cell lines continued to secrete cytokeratin, even after the 30^{th} generation.

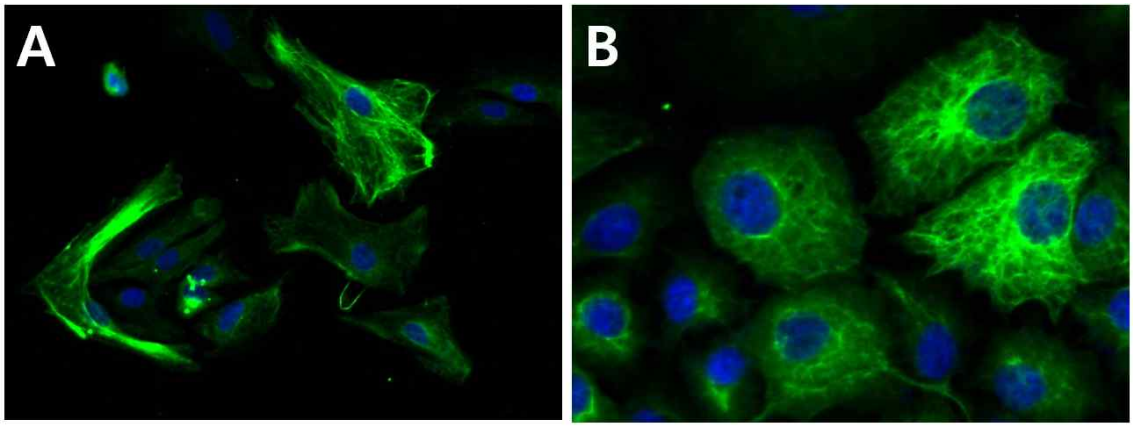

Fig. 5-4. Immunofluorescence analysis of cytokeratin in the JNUEK-1 and JNUEK-2 cell lines. A: The expression of cytokeratin (green color) in the JNUEK-1 cell line (5^{th} generation). B: The expression of cytokeratin (green color) in the JNUEK-2 cell line (10^{th} generation). The nuclei are dyed blue.

MOI selection and titer test

The JNUEK-1, JNUEK-2, and MDBK cell lines were cultured for 20 h so that approximately 80% cell monolayers could form. EHV-1 and EHV-4 seed viruses were diluted with DMEM-2 and 2% FBS, and inoculated into the cell lines at MOIs of 0.01, 0.1, 1, and 10. Four hours after the virus seed inoculation, the inoculated solution was removed, the cells were washed twice with PBS, DMEM-10 was added, and the cells were cultured for 72 h before the virus titer was measured. CPE was observed 4 days after the inoculation for both EHV-1 and EHV-4.

The results of EHV-1 inoculation experiments showed that the titer of cell line JNUEK-1 was the highest, with a value of 2×10^9 $TCID_{50}$/ml. The EHV-1 titers were different among the different MOIs of inoculation. When inoculated at MOI 0.01, whereas JNUEK-1 showed a titer of 1.5×10^9 $TCID_{50}$/ml, the control group (cell line MDBK) showed a titer of 5×10^6 $TCID_{50}$/ml, a difference of approximately 500 times. All cell lines showed the highest titer when inoculated with EHV-1 at MOI 0.1.

In the case of EHV-4, all cell lines showed the highest titer when inoculated at MOI 0.1. When inoculated at MOI 0.1, cell line MDBK showed a titer of 8×10^6 $TCID_{50}$/ml. On the other hand, JNUEK-1 and JNUEK-2 showed titers of 5×10^9 $TCID_{50}$/ml and 7×10^8 $TCID_{50}$/ml, respectively.

Mycoplasma infection test

The produced cell lines maintained the shapes of epithelial cells, even when generation numbers increased and no growth into multiple layers was observed. PCR (Mycoplasma PCR Detection Kit; iNtRON, Seoul, Korea) was used to determine whether the immortalized cells were infected by mycoplasma, and all cells were shown to be negative (Fig. 5-7).

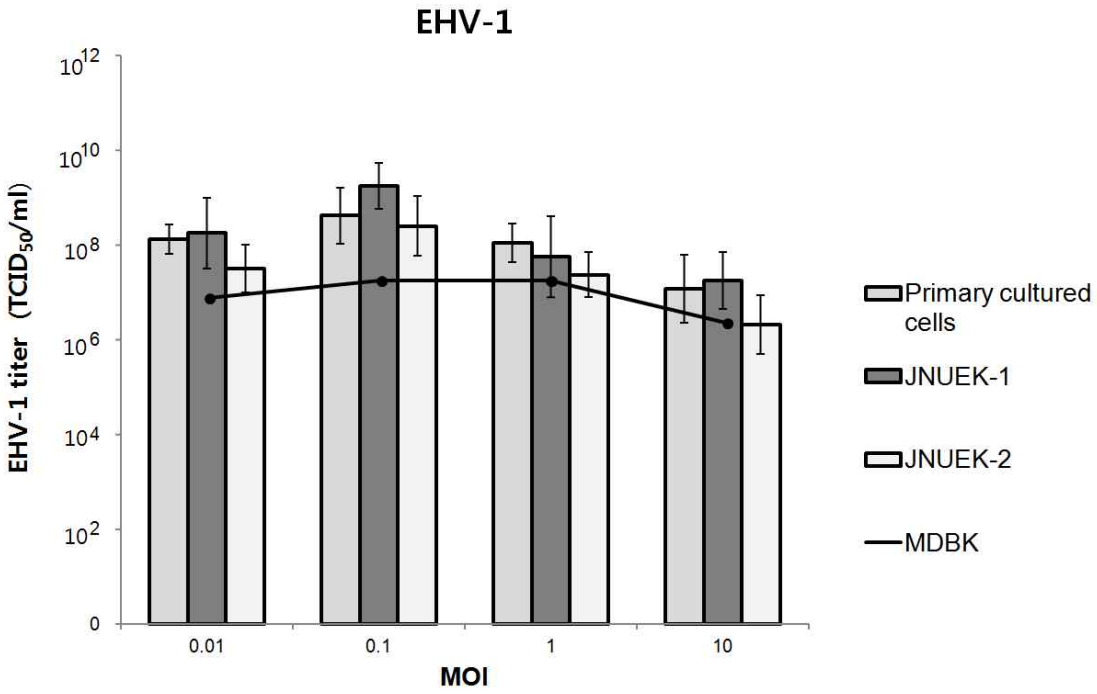

Fig. 5-5. Virus titer of EHV-1 inoculated with various MOIs. The JNUEK-1 cell lines showed the highest titer.

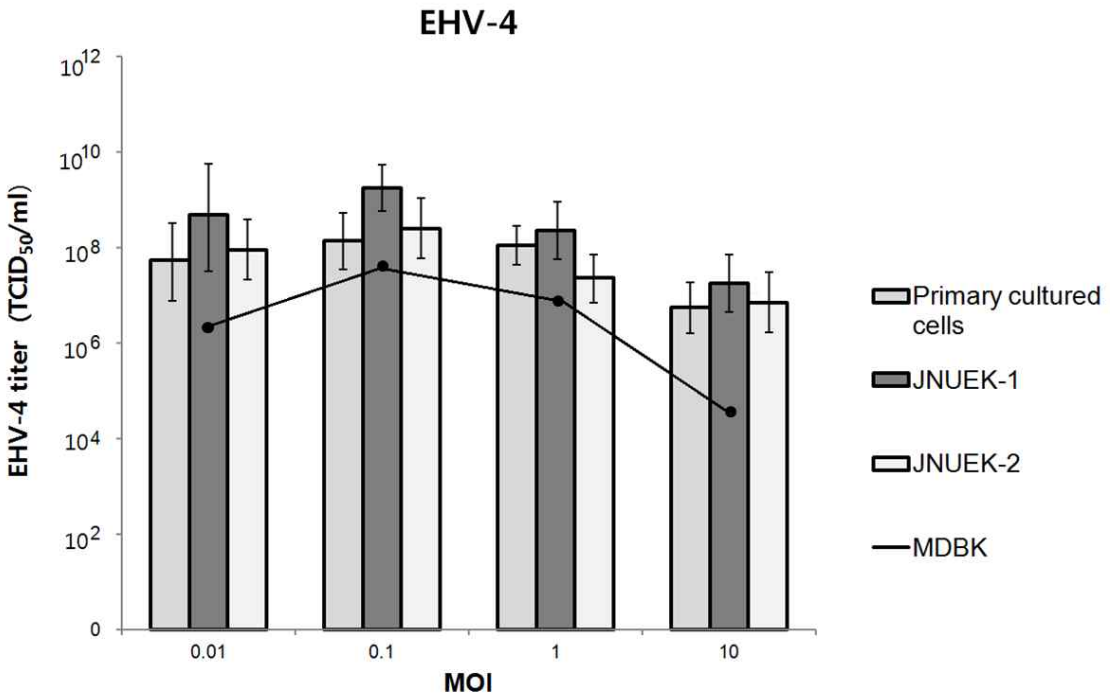

Fig. 5-6. Virus titer of EHV-4 inoculated with various MOIs. The JNUEK-1 cell lines showed the highest titer.

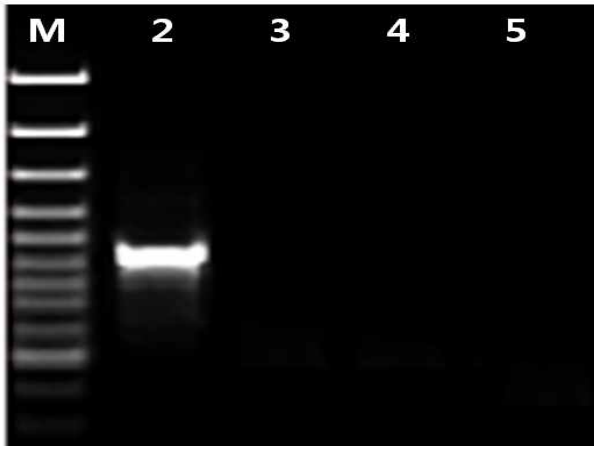

Fig. 5-7. PCR results for mycoplasma detection. Line M: 100 bp DNA marker; Line 2: mycoplasma positive control; Line 3: mycoplasma negative control; Line 4: JNUEK-1 cell line; Line 5: JNUEK-2 cell line. All cell lines were confirmed as negative.

Discussion

EHV-1 and EHV-4 are viruses that belong to the Alphaherpesvirinae subfamily and the Varicellovirus genus. They cause respiratory diseases in horses around the world. Typically, only symptomatic treatment is applied for diseases caused by EHV. There is no specific treatment for EHV, and it has been shown that once the disease occurs, it is very difficult to be completely cure due to the continuous latent infection (21). Therefore, the best method at present is to prevent infection of EHV-1 and EHV-4 through vaccines.

For EHV, it is primarily inactivated vaccines and modified live vaccines that are being produced and used (22, 23). To produce inactivated vaccines or modified live vaccines, host cells that produce viruses with high titers are required. The characteristics of the host cells and the titers of the produced viruses have a major influence on the quality and cost of production for the final vaccines. Therefore, this study was conducted to develop new cell strains derived from horses that can produce viruses with high titers, as these have high susceptibility to EHV-1 and EHV-4.

The kidney cells of fetal horses were primarily cultured, and the cells were immortalized by inoculating recombinant retrovirus particles that can over express telomerase. Then, their susceptibility to EHV-1 and EHV-4 was checked. Furthermore, after the susceptibility was confirmed, the characteristics of the developed cell strains were determined, and in order to mass-produce EHV-1 and EHV-4, experiments were conducted to determine the optimal inoculation for the multiplicity of infection (MOI).

The proliferation capacity of the JNUEK-1 and JNUEK-2 cell strains, which were selected through single-cell cloning after the immortalization of the cells, was evaluated, and both cell strains were found to have a high proliferation capacity of more than 2,000 cpm continuously up to 30 generations (Fig. 3). In order to use these cell strains for the virus culture, they must have a constant and steady proliferation capacity even when the generations increase. Furthermore, if the proliferation capacity of the cell strains drops or is inconstant, the yield of viruses will also be inconstant, and it will become difficult to manage the cell strains. Therefore, the constantly high proliferation capacity of the JNUEK-1 and JNUEK-2 cell strains is a highly advantageous characteristic.

EHV-1 and EHV-4 are generally known to be susceptible to epithelial cells (24). In order to check if the immortalized cell strains that have been produced are epithelial cells, the expression of cytokeratin, which is specific to epithelial cells, was checked. Following the immunofluorescence staining of the JNUEK-1 and JNUEK-2 cell strains with anti-cytokeratin, both cell strains secreted cytokeratin (Fig. 5-4). It seems that the JNUEK-1 and JNUEK-2 cell strains showed a high susceptibility to EHV 1 and EHV-4, as only the epithelial cells with a high division rate among the many colonies were cloned through the single-cell cloning.

For the mass production of EHV-1 and EHV-4, the optimal inoculation MOI was examined. The JNUEK-1 and JNUEK-2 cell strains and the conventional MDBK cell strain, which is typically used as the EHV host cell, were inoculated with EHV 1 and EHV 4 at 0.01, 0.1, 1, and 10 MOI. In the case of EHV 1, when it was inoculated at 0.1 MOI, the JNUEK-1 cell strain showed a titer that was about 500 times as high as that of the MDBK cell strain (Fig. 5-5). Furthermore, in the case of EHV-4, the JNUEK-1 cell strain showed a titer that was about 600 times as high as that of the MDBK cell strain (Fig. 5-6). Thus, the JNUEK-1 cell strain showed a very high titer compared to the conventional MDBK cell strain that is used as the host cell. This is a highly encouraging result that suggests the potential of the JNUEK-1 cell strain as a host cell for the mass-production of EHV-1 and EHV-4. To

further increase the yield, additional experiments are needed on subdividing the inoculation MOIs and on comparing the titers in the cells and culture medium.

In conclusion, in considering the JNUEK-1 and JNUEK-2 cell strains that were developed, the JNUEK-1 cell strain was found to be an excellent host cell for culturing EHV-1 and EHV-4. Although lower than that of the JNUEK-1 cell strain, the JNUEK-2 cell strain also showed a higher titer than that of the MDBK cell strain. Therefore, both the JNUEK-1 and JNUEK-2 cell strains can be used as host cells for culturing EHV-1 and EHV-4 in place of the MDBK cell strain. Based on the experimental results, the JNUEK cell strains can be used as the cell strain for EHV-1 and EHV-4 vaccine production or for virus detection or separation. In general, the susceptibility and titer depend on whether the virus is a wild type or an attenuated virus type as well as on the differences in the host cells for the culture. Therefore, based on the findings of this study, more research should be conducted to determine the susceptibility and culture conditions for various virus strains.

SUMMARY

Part 1. Etiologic Agents of Horses Respiratory Diseases and Development of Management Technologies

말의 호흡기 질환은 말 산업에 있어서 경제적 손실을 초래하는 원인 중에 하나로서 특히 망아지의 폐사를 초래하는 등 모든 연령의 말에서 치료에 어려움을 겪고 있는 것으로 알려져 있다. 급성이나 만성적인 감염은 폐 기능을 손상하거나 저하시키는 역할을 한다. 또한 이 질병은 전 세계적으로 임상적 혹은 치료적인 문제가 증가되고 있는 것으로 알려져 있다. 호흡기 질환의 전염원은 바이러스와 세균으로 크게 구분할 수 있다. 바이러스의 경우 EHV-1, EHV-4, EAV(equine viral arteritis), 및 EIV(equine influenza) 등의 발병기전과 역할에 관해서 많이 보고 되어 있으나 세균은 일차적 혹은 이차적 원인과 역할에 대해서 미진한 상태이다. 임상적인 면에서 영국에서 호흡기 질환이 발생된 25개의 목장을 조사한 결과 EHV는 8%에 불과하였고 68%는 바이러스와는 직접적인 관계가 없는 것으로 보고한 바 있다.

말에서 중요시 되는 Streptococcus spp.는 S. equi, S zooeppidemicus, S. equisimilis로서 S. equi는 influenza, rhinovirus, EHV-1의 감염에 따른 이차적인 감염으로 인해 Gyttural pouch empyema 등 말에서 문제시되며 R. equi는 망아지에서 심한 폐렴을 유발하나 penicillin G에 감수성이 높은 것으로 알려져 있다. 말에서 주로 사용하는 항균제는 amikacin, ampicillin, amoxicillin, cefotaxime, cephalexin, ceftiofur, enrofloxacin, erythromycin, gentamicin, imipenem, metronidazole, ticarcillin, trimethoprim sulfa, fluconazole 등이 유효한 것으로 알려져 있고, 대부분의 말 임상가들이 가장 공통적으로 사용하는 항균제는 penicillin, amoxicillin, streptomycin, gentamicin, erythromycin,

oxacillin 등으로 알려져 있다.

말 호흡기 세균성 질병 감염양상 조사에 대한 본 연구결과를 보면 병원체에 따른 감수성 약제와 내성약제는 농장간의 차이를 보였다. Streptococcus spp.는 β-lactam계의 항생제 중 cephalosporin계열의 항생제가 다소 감수성 효과를 나타내었으며, 검출된 4농가에서의 14건의 내성약제 검사결과 공통적으로 penicillin계와 tetracycline계열은 내성을 나타내어 치료효과를 나타내지 못할 것으로 사료된다. 반면, Staphylococcus spp.에서는 공통적으로 trimethoprim-sulfamethoxazole 감수성을 나타내었으나 chloramphenicol과 florefenicol의 항생제는 하나의 검사시료에서만 감수성을 나타낸 반면 다른 검사시료에서는 내성을 나타내었다. R. equi감염군에서는 quinolone계 항균제와 amikacin계 항생제, ceftiofur계 항생제가 공통적으로 감수성을 나타내었다. 선역 증상을 보이는 호흡기 증상을 제외한 자마 호흡기증상 발생에서는 우선적으로 cephalosporin계 항생제를 우선적으로 사용되어져야 할 것으로 나타내었다. 말 호흡기 질환에 관한 연구는 향후 더 많은 시료에 대해서 분자생물학적 연구도 병행하여 조기진단 및 예방에 노력을 기울려야 할 것으로 사료된다.

한편 말 호흡기 질병관리를 위한 승용마의 연령별 혈액학적 검사에 관한 연구보고는 극히 미비한 실정이었기 때문에 이에 관련된 유용한 표준 수치를 제시해 주지 못하고 있다. 따라서 본 연구에서는 승용마의 혈액학적 변화상을 1세마(n=9), 2세마(n=13), 암말(n=55)으로 구분(총 77두)하여 연령에 따른 변화상을 보여주고자 실험하였다.

본 연구의 결과에서 적혈구계 혈액상의 변화상을 보면, RBC는 연령이 증가함에 따라 $11.03 \times 10^6/\mu\ell$에서 $8.27 \times 10^6/\mu\ell$로 점차적으로 감소하는 경향이었고, 이의 통계적 유의성이 인정되었다($p < 0.05$). 다만, 혈액 성분은 동일 축종의 같은 품종일지라도 계절과 사양 관리 시스템 그리고 나이와 유전적 동종성, 지역에 따라 혈액의 구성 세포와 그 성분에 변화가 달라지므로, 좀 더 장기적인 계획을 가지고 다양한 분석을 하여야 할 것으로 생각된다.

혈액 내 백혈구계의 연령에 따른 본 연구의 결과 역시 1세마에서 13.41 × 103/㎕로, 2세마에서 11.09 × 10^3/㎕로, 기초축 암말에서는 9.52 × 10^3/㎕로 연령이 증가함에 따라 총 백혈구 수치가 감소하는 경향을 보였다. 현재 제주에서 사육되고 있는 제주산마에 대한 혈액학치 관한 연구는 전무한 실정이다. 따라서 제주에 사육 중인 제주산마와 관련하여서는 보다 추가적인 연구가 요구된다고 본다. 결론적으로 혈액학적 검사 결과는 연령에 따라 각각 그 수치가 유의성 있게 변화됨을 관찰할 수 있었다. 이러한 혈액학적 검사 결과는 이전에 제시되지 않았던 것으로 앞으로의 제주산마의 사양 관리 및 질병 검사 시에 표준화된 유용한 자료로 사용될 수 있을 것으로 사료된다.

Part 2. A study on the immune response in horses bred in Jeju against respiratory diseases vaccination

말의 호흡기 질환은 말 산업에 있어서 경제적 손실을 초래하는 원인 중에 하나로서 특히 망아지의 폐사를 초래하는 등 모든 연령의 말에서 치료에 어려움을 겪고 있는 것으로 알려져 있다. 급성이나 만성적인 감염은 폐 기능을 손상하거나 저하시키는 역할을 한다. 또한 이 질병은 전 세계적으로 임상적 혹은 치료적인 문제가 증가되고 있는 것으로 알려져 있다. EHV는 말의 비강폐렴을 일으키는 원인체로서, 1981년 국내에서 말의 비강폐렴의 발생을 처음으로 보고 하였다. 제주지역의 경우 이 등이 혈청검사를 실시하여, 제주마의 양성율이 24.3%라고 보고한 적 있으며, 제주도내 EHV의 양성율이 80.5%임을 발표한 바 있다. 최근 EHV가 EHV-1과 EHV-4로 분류되었고, PCR기법을 이용한 이들의 구분법이 광범위하게 연구되고 있다. 말 인플루엔자(EIV)에 감염된 말은 발열, 콧물 등과 같은 호흡기증상을 나타내며 매우 강하게 전파되어 마방 또는 마군 전체에 감염을 일으키며 폐사율은 낮은 것으로 보고되나 생산성 저하를 나타내어 경제적 손실 또한 야기한다. 말 선역에 감염된 말은 발열, 식욕부진 및 두경부 림프절 종창을 특징으로 높은 이환율로 인한 피해가 증가하며 말을 사육하는 국가에서는 선역이 지속적으로 발생하고

있다.

따라서 본 연구에서는 제주특별자치도에서 건강검진으로 의뢰된 제주산마와 한국마사회의 더러브렛, 난지축산연구소의 공시마를 이용하여 말 호흡기 질병의 항체 형성율을 조사하였다. 그 결과 EHV-1의 낮은 항체 형성율은 바이러스 감염을 유발할 가능성을 제시해 주며, EHV-1은 국내 농가에서는 백신을 접종하고 있으나, 차후 백신 접종에 따른 항체가 변화 추이 조사 및 농가에서 실제로 이루어지는 백신접종 실태 조사를 통해 말 사육농가에 적절한 백신 접종 프로그램에 대한 지도를 해나가야 할 것으로 판단된다. EHV-4는 95.84%의 높은 항체 형성율을 보이고 있으며, 제주도내 유산마에서 EHV-1과 EHV-4의 혼합 감염사례가 보고됨을 볼 때 말 사육농가에 EHV-4에 의한 피해가 발생하고 있을 가능성이 높은 것을 알 수 있었다.

말 인플루엔자의 경우는. KRA과 NIAS의 결과를 비교해보면 마사회입사(6개월령)시 인플루엔자에 대한 백신접종이 이루어 지지 못한 것으로 분석됨에 따라 자마의 이동 및 판매시 말 인플루엔자에 접종이 실시되어져야 할 것으로 사료된다. 또한 NIAS에서는 일년에 두번 상반기와 하반기에 인플루엔자 백신 접종을 매년 실시하고 있으며 이와 같은 백신프로그램을 국내 농가에서도 유사하게 진행하면 높은 항체형성율을 나타냄으로써 질병예방이 가능할 것으로 보여진다. 말 인플루엔자와 선역 백신은 KRA을 통해 공급되고 있음에도 불구하고 양성율은 각각 26.32%와 55.12%에 불과한 항체 형성율을 나타냄에 따라 질병발생을 일으킬 수 있음에 따라 농가에서는 백신접종에 보다 철저히 실시해야 한다고 생각된다. 또한 단순 백신 접종만이 아닌 일정한 기간에 대한 추가 접종 및 부스터효과에 관련된 연구가 진행되어야 한다. 말 선역에 대해서는 추가접종이 요구되는 항체음성 개체와 낮은 양성(weak positive : SeM 역가 1:200~400)개체관리를 위해 현행 이루어지는 가을철 선역 백신접종(1회/년)보다는 6개월 단위의 예방접종 프로그램이 실시되어져야할 것으로 판단된다. 또한 이번 검사에서는 선역 야외감염이 의심되는 매우 강한 양성반응 (very strong positive: SeM 역가 > 1:12,800)을 보이는 개체는 확인 되

지 않았으며, 이는 이번 검사개체 들이 임상증상이 없는 건강한 개체를 대상으로 실시하였기 때문으로 판단된다. 본 연구에서 선역의 경우는 해외에서 분석하고 있지만, 국내 농림수산검역검사 본부에서도 임상증상축에 대한 세균배양 및 PCR 검사를 통해 양성을 확인하는 방법으로만 사용되고 있었으며, 선역항체가 조사를 실시한 경우는 국내에서는 처음으로 실시하였기에 그 의의가 크다고 할 수 있다. 각 질병의 항체 형성율 조사와 월별 모니터링 검사는 말 호흡 질환 유발균에 대한 유효 치료제 선발 연구 및 호흡기 질환 예방 백신 항체가 분석연구 등의 최적 프로그램 개발에 대한 기초자료가 될 수 있을 것으로 사료되며 호흡기 질병관리를 통하여 질병예방 및 백산 면역 역가이용 접종프로그램 설정에도 크게 도움이 될 수 있을 것으로 생각된다.

Part 3. Development of a Equine kidney cell line for equine herpes virus replication

EHV에 대한 백신은 주로 inactivated vaccines과 modified live vaccine이 생산 및 사용이 되고 있다. inactivated vaccines 혹은 modified live vaccine을 제작하기 위해서는 바이러스를 고역가로 생산하는 숙주세포가 필수이다. 숙주세포의 특성 및 생산되는 바이러스 역가는 최종적으로 제작되는 백신의 품질과 생산 단가에 큰 영향을 미친다. 따라서 본 연구는 EHV 1과 EHV 4에 높은 감수성이 있어 고역가로 바이러스를 생산할 수 있는 말 유래의 새로운 세포주를 개발하고자 하였다.

말(horse) 태아(fetal)의 신장(kidney)세포를 초대배양하고 telomerase를 과발현 시킬 수 있는 recombinant retrovirus particle을 접종하여 세포를 불멸화 시킨 후 EHV 1과 EHV 4에 대한 감수성을 확인하였다. 또한 감수성을 확인한 후 개발된 세포주의 특성을 확인하고 EHV 1과 EHV 4를 대량생산하기 위하여 최적의 접종 MOI를 실험하였다.

세포의 불멸화 이후 single cell cloning 통하여 선발된 JNUEK-1 세포주와 JNUEK-2 세포주의 증식능력을 평가 결과 두 세포주 모두 30 세대(generation) 까지 지속적으로 2,000 cpm 이상의 높은 증식능력을 보유하고 있음을 확인하였다 바이러스 배양용 세포

주로 사용하기 위해서는 세대가 증가하여도 일정하며 꾸준한 증식능력을 보유하여야 한다. 또한, 숙주세포의 증식 능력이 떨어지거나 일정하지 않으면 바이러스의 수율도 일정하지 않게 되며, 숙주세포를 관리하기도 힘들게 된다. 따라서 JNUEK-1 세포주와 JNUEK-2의 일정하게 높은 증식능력은 숙주세포의 특성으로 매우 유리한 점이라고 사료가 된다.

EHV 1과 EHV 4는 일반적으로 상피세포에 감수성이 있다고 알려져 있다(24). 제작된 불멸화 세포주가 상피세포임을 확인하기 위하여 상피세포가 특이적으로 가지고 있는 cytokeratin의 발현을 확인하였다. JNUEK-1과 JNUEK-2 세포주를 anti-cytokeratin으로 면역형광염색을 한 결과 두 세포주 모두에서 cytokeratin의 분비를 확인하였다. JNUEK-1과 JNUEK-2 세포주가 EHV 1과 EHV 4에 높은 감수성을 보이는 것은 single cell cloning을 통하여 여러 colony 중에서 분열속도가 빠른 상피세포만을 cloning한 것이 주요했다고 사료가 된다.

EHV 1과 EHV 4의 대량생산을 위하여 최적의 접종 MOI를 알아보았다. JNUEK-1 세포주와 JNUEK-2 세포주 그리고 기존에 EHV 숙주세포로 일반적으로 사용되고 있는 MDBK 세포주에 EHV 1과 EHV 4를 MOI 0.01, 0.1, 1 그리고 10으로 접종을 하였다. EHV-1의 경우 MOI 0.1로 접종하였을 때 JNUEK-1 세포주는 MDBK 세포주 보다 약 500배 높은 역가(titer)를 나타냈다(Fig. 5). 또한, EHV-4의 경우에는 JNUEK-1 세포주가 MDBK 세포주 보다 약 600배 이상 높은 역가를 나타냈다(Fig. 6). 이처럼 기존에 숙주세포로 사용되고 있는 MDBK 세포주에 비하여 JNUEK-1 세포주는 매우 높은 역가를 나타내었다. 이는 매우 고무적인 결과로 JNUEK-1 세포주가 EHV 1과 EHV 4의 대량생산을 위한 숙주세포로 높은 가능성을 제시하는 결과로 사료가 된다. 보다 높은 수율 향상을 위한 추가 연구로 접종 MOI를 보다 세분화 하거나, 세포내부와 배지속(culture medium)의 바이러스 역가를 비교하는 추가 실험이 필요할 것이라 사료가 된다.

결과적으로 개발된 JNUEK-1 세포주와 JNUEK-2 세포주 중에서 특히 JNUEK-1 세

포주는 EHV 1과 EHV 4를 배양하기 위한 숙주세포로 매우 뛰어난 것을 확인할 수 있었다. JNUEK-1 세포주와 비교하면 낮았지만 JNUEK-2 세포주 또한 MDBK 세포주와 비교하면 높은 역가를 나타냈다. JNUEK-1 세포주와 JNUEK-2 세포주 모두 MDBK 세포주를 대체하여 EHV 1과 EHV 4를 배양하기 위한 숙주세포로 사용이 가능한 것을 확인하였다. 이러한 실험 결과를 바탕으로 JNUEK 세포주들은 EHV 1과 EHV 4의 백신 생산용 혹은 바이러스 검출 및 분리용 세포주로 이용이 가능하리라 판단이 된다. 일반적으로 바이러스는 배양하는 숙주세포의 차이뿐만 아니라 바이러스 자체가 wild type 혹은 attenuated virus type 이냐에 따라서 감수성 및 titer가 달라진다. 따라서 본 연구를 바탕으로 보다 다양한 바이러스주에 대하여 감수성 및 배양 조건을 찾기 위한 연구가 계속되어야 할 것이라 사료가 된다.

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