Studies on treatment and diagnostic method for infectious diseases among domestic animals

愛玩動物における感染症の治療法と診断法の研究

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1. General introduction

1.1. Viral infectious diseases in companion animals

1.1.1. Coronavirus (CoV)

1.1.1.1. General characteristics

Animal coronaviruses are classified into the order *Nidovirales*, the family *Coronaviridae.* "Corona" means a crown in Latin. The origin of its name is derived from the appearance of viral particle like a crown, because of spike proteins on viral surface. These viral members are 100-140 nm in size and enveloped and the genome is a positive-sense and single-stranded RNA. In *Coronaviridae*, there are four genera referred to as *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus* and *Deltacoronavirus*. The genome size is 25-30kb and one of the largest genomes in RNA viruses. The 5' two-thirds of the genome encode a large polyprotein including RNA dependent RNA polymerase (RdRp) and proteases. The 3' region of the genome contains open reading frames encoding structural proteins (spike (S), envelope (E), membrane (M) and nucleocapsid (N)) and accessory proteins (Sola et al.,2015). S protein includes a receptor binding domain and plays important roles in viral entry and membrane fusion. E and M proteins are an essential membrane proteins. E protein relates to viral production and maturation (DeDiego et al., 2007; Ortego et al., 2007). M protein determines the shape of viral membrane. N protein is known to bind to genomic RNA and consists of helical ribonucleoprotein (RNP) complexes termed as nucleocapsid for protection of the genome (McBride et al., 2014). The functions of some accessory proteins have been identified (for example, inhibition of immune response in host cell (Liu et al.,2014)), but the function are still unknown.

1.1.1.2. Diseases and pathogenesis

Coronavirus infection is found in many animal species including human, dog, cat, cow, pig, mouse and so on, with respiratory or digestive diseases. However, some coronaviruses cause encephalitis or granuloma. In many cases of coronavirus infection, the illness is severe in infant or juvenile animals more than in matured ones.

1.1.1.2.1. Canine enteric coronavirus infection

Canine enteric coronavirus (CECoV) belongs to the genus *Alphacoronavirus*, and it was divided into two genotypes. CECoV was first isolated from diseased dogs in Germany in 1971 (Yesilbag et al., 2004). CECoV spreads by fecal-oral route, and the dogs infected with CECoV generally show mild to severe diarrhea (Castanheira et al., 2014). Co-infection of CECoV and canine parvovirus 2 or canine adenovirus type 1 causes severe disease with high fatality rate (Decaro et al.,2006; Pratelli et al.,2001). Recently, high pathogenic CECoVs, named “pantoropic” CECoV, have been reported (Buonavoglia et al., 2006) and the characteristic disease is leukopenia (Alfano et al., 2020). CECoV infection has been detected worldwide, especially in animal shelters and kennels. In Japan, the positive rate of CECoV infection was 16.0-50.5% (Bandai et al., 1999; Soma et al., 2011; Takano et al., 2016). For infected animals, supportive treatments to dehydration and acidosis are recommended.

1.1.1.2.2. Canine respiratory coronavirus infection

Canine respiratory coronavirus (CRCoV) is a member of *Betacoronavirus* and a causative agent of canine infectious respiratory disease, which was detected in 2003 (Erles et al., 2003). CRCoV genetically has a high identity with bovine coronavirus (BCoV). Therefore, it has been considered that CRCoV is originated from BCoV (Erles

et al., 2003). CRCoV infection causes mild diseases, such as nasal drop, cough and sneezing (Mitchell et al., 2013). However, after CRCoV infection, the diseased dog sometimes becomes compromised to second viral or bacterial infections (Priestnall et al., 2009). Previous reports of RNA or antibody detection indicated that CRCoV was spread in Americas, Europe and Asia (Erles et al., 2008). Palliative treatment is usually enough for complete remission. Any vaccine for CRCoV isn't available.

1.1.1.2.3. Feline coronavirus infection

Feline coronavirus (FCoV) is classified into the genus *Alphacoronavirus*. FCoV includes two biotypes, feline enteric coronavirus (FECV) and feline infectious peritonitis virus (FIPV). Former is a low or no pathogenic virus, causing mild diarrhea or asymptomatic infection in cats (Tekes et al., 2016). The latter is a highly virulent virus associated with fatal disease, feline infectious peritonitis (FIP). Although there are two serotypes in FCoV, serotype I FCoV is dominant in natural infection (Benetka et al., 2004). Domestic cats, especially in shelters or breeder's houses, often get infected with FCoV and the seroprevalence reached to approximately 80% (Hohdatsu et al., 1992). While FCoV persistently infects to cats' enterocytes, and nucleotide mutations accumulate in the genes, especially S and 3c genes (Pedersen., 2014). It is suggested that the mutations change viral cell tropism from enterocytes to macrophages, contributing to the emergence of FIPV (Jaimes et al., 2020). FIP consists of two forms, an effusive form and a non-effusive form. The effusive form shows peritoneal or thoracic effusion with rapid diseases progression (Kipar and Meli., 2014). The non-effusive form shows parenchymal and serosal pyogranulomas in kidney, liver, omentum and intestine without effusion. Moreover, in some cases, ocular diseases or neurologic disorders, such as ataxia or

epilepsy, are observed in non-effusive form. As mentioned above, FIP is a fatal disease for cats. Although the nucleotide analog which can treat FIP was discovered, the compound is not permitted for the use in Japan (Murphy et al., 2018). Supportive treatments may improve clinical signs. Euthanasia would be one of the choices to save suffering cats. In several countries, a vaccine for FCoV is available. However, some neutralizing antibodies against FCoV enhance viral entry to macrophages and make disease severe, hence the efficacy and the benefits of vaccination remain unclear (Hartmann et al., 2005).

1.1.1.2.4. Ferret coronavirus infection

Ferret coronavirus (FRCoV) is first identified in USA in 2000 (Williams et al., 2000). The classification of FRCoV is the genus *Alphacoronavirus* like FCoV and CECoV. Ferret enteric coronavirus (FRECV) is an enteric FRCoV and related to epizootic catarrhal enteritis (ECE), which leads to vomiting, drowsiness and hypophagia. The morbidity of FRECV infection is high, but the fatality is low, and the illness is severe in old ferrets than in young ferrets (Murray et al., 2010). Ferret systemic coronavirus (FRSCV) is fetal and systemic FRCoV similar to FIPV. FRSCV infection is characterized by an abdominal mass or neurologic manifestation, accompanied by clinical signs like diarrhea, hypophagia and body weight loss (Murray et al., 2010). FRCoV infection was reported in Americas, Europe and Japan with 40-70% of the positive rates of FRCoV infection (Provacia et al., 2011; Smits et al., 2013; Terada et al., 2014). Nucleotide homologies between FRSCV and FRECV genomes are about 90%, but little is known about the emergence of FRSCV (Wise et al., 2010). There is no specific treatment for FRCoV infection and supportive treatment is carried out.

1.1.2. Calicivirus

1.1.2.1. General characteristics

Calicivirus is a non-enveloped and small virus in the order *Picornavirales* and the family *Caliciviridae* which includes 11 genera. *Sapovirus* or *Norovirus* infection cause gastroenteric diseases in human (Becker-Dreps et al., 2019; de Graaf et al., 2016). Vesivirus is a pathogen isolated from cats, pigs and seals (Love et al., 1975; Gerberg et al.,1982). Lagovirus has been found only in rabbits in the order *Lagomorpha*, causing a fatal hepatitis (Buehler et al.,2020). Calicivirus possess a positive-sense and single-stranded RNA. The genome encodes capsid proteins as structural proteins and non-structural proteins including RdRp, 3C-like protease, VPg and helicase (Smertina et al., 2021). Because of a lack of proof reading, RNA replication by RdRp generates genetic variations. The 3C-like protease cleaves viral polyprotein, NS1/2, whose functions are unclear. The VPg protein binding to the 5' end of genomic or subgenomic RNA works as a primer in genomic RNA replication (Goodfellow., 2011). The helicase has some functions as unwinding double-stranded RNA using ATP, remodeling RNA as a RNA chaperone and possessing the replication complexes (Li et al., 2018).

1.1.2.2. Diseases and pathogenesis

1.1.2.2.1. Feline calicivirus infection

Feline calicivirus (FCV), a member of the genus *Vesivirus*, is a common virus among cats. The most frequent clinical signs caused by FCV infection are oral ulceration, and nasal discharge is often observed as well (Cai et al., 2002). In kittens, FCV infection is fatal, triggering severe respiratory diseases (Radford et al., 2007). Recently, a highly virulent FCV strain has been found mainly in the USA (Pederson et al., 2000). This strain

is characterized by FCV-associated virulent systemic disease (VSD), including pyrexia, hypophagia or icterus, with high fatality (Radford et al., 2007). Due to the high density of cats, the risk of FCV infection in shelters is higher in private household. The prevalence rate in cats owned in shelters was around 30%, but that in personal owned cats was 10% (Wardley et al., 1974). FCV can keep the infectivity in the environment for a long time at room temperature (Doultree et al., 1999). Therefore, the virus can transmit indirectly from diseased cats to healthy cats. Although no specific antiviral medicine is available, broad-spectrum antibiotics are used to protect animals from secondary bacterial infection. Vaccination can be used to reduce clinical signs except for VSD, but not to prevent the infection. The antigenicity of FCV is variable depend on the viral strains, hence vaccine-induced antibodies can't neutralize all isolates. The development of broadly cross-reactive vaccine is needed.

1.1.3. Herpesvirus

1.1.3.1. General characteristics

Herpesviruses are members of the family *Herpesviridae*, consisting of *Alphaherpesvirinae*, *Betaherpesvirinae* and *Gammaherpesvirinae*. The virion has an envelope including a tegument, capsid and core, and the size of virion varies from 150 to 300 nm. In viral particle, linear ds DNA is packaged as the genome with a range of genome size from 100 to 300 kbp. The genome encodes so various genes depend on viral species. However, about 40 genes called as core genes are genetically conserved in most of herpesviruses. In addition, herpesviral genes are classified into three categories. Immediate early genes are first transcribed and the proteins translated from them regulate the transcription of other genes. Early genes, second transcribed genes, contribute to the production of DNA polymerase or other enzymes. Finally, structural proteins are translated from late genes. Since herpesviruses easily lose the infectivity in the environment, a close contact is a main route of viral transmission. Because of the highly specific host tropism, herpesviruses hardly transmit to other species. However, once they cross the species-barrier, the infections sometimes lead to fatal disease. These events can be observed in B virus or pseudorabies virus infections (Weigler et al., 1992; Ciarello et al., 2020). The latent infection in neurological tissues is one of distinctive features of herpesvirus infection, because it leads to asymptomatic carriers, which would spread the infection (Evermann et al., 2011). Until now, herpesviruses have been identified in different kinds of animals, such as fishes, amphibians, reptiles, birds and mammals. In some companion animals, diseases by herpesvirus infection are known.

1.1.3.2. Diseases and pathogenesis

1.1.3.2.1. Canine herpesvirus infection

Canine herpesvirus (CHV) is genetically similar with feline herpesvirus and equine herpesvirus, which belongs to *Alphaherpesvirinae*. CHV was first identified as a cause of fatal disease of puppies in the middle of 1960 (Carmichael et al., 1965). Until now, CHV has been found in not only domestic dogs, but also wild dogs all over the world, and the seroprevalence was around 90% in the case of England (Reading et al., 1999). Similar to FCV infection, the positive rate in dogs in kennels is higher than in pet dogs (Decaro et al., 2006). The clinical sign is variable depend on the age when dogs are infected. In neonates, CHV infection causes necrotizing and hemorrhagic diseases with high mortality (Decaro et al., 2006). They were generally infected with CHV from their mothers. Young or adult dogs often show asymptomatic infection, but some young animals present blindness and deafness. In the case of pregnant dogs, abortion is caused by CHV infection, or the puppies are weak and die a few days after the birth (Ronsse et al., 2004). In Japan, aged dogs in an animal medical center showed respiratory syndrome with the death (Kawakami et al., 2010). To prevent newborns from CHV infection, injection of immune sera to their mothers is more benefitable than supportive therapy. Hydration and warming are recommended to reduce the mortality in puppies. CHV is easily inactivated with disinfectants, so CHV transmits directly to uninfected dogs with oronasal discharge of infected dogs. Therefore, the restriction of viral spread to healthy puppies is necessary. A subunit vaccine is available for pregnant dogs in Europe to protect neonates from CHV infection.

1.1.3.2.2. Feline herpesvirus infection

Feline herpesvirus 1 (FHV-1) is a causative agent of severe upper respiratory

disease in cats. FHV-1 belongs to the subfamily *Alphaherpesvirinae* and the genus *Varicellovirus* with CHV and phocine herpesvirus 1. Although the host range is limited to *Felidae,* a herpesvirus similar to FHV-1 was detected in dogs (Kramer et al., 1991). There seems to be only one serotype in FHV-1 (Gaskell et al., 2007). Between the isolates, there are some small genetic changes in *Mlu*I, and *Sal*I, but it is unclear whether these differences contribute to viral pathogenicity (Hamano et al., 2005; Horimoto et al., 1992). FHV-1 transmits via oral, nasal and conjunctival routes and replicates in nasal septum, turbinate, tonsils, lymph nodes and trachea (Gaskell et al., 2007). FHV-1 infection causes multifocal necrosis in the respiratory epithelium and osteolytic lesion in the turbinate (Hoover et al., 1970; Povey et al., 1979). Diseased cats generally show oral, ocular and nasal discharges with inappetence, depression and high body temperature (Hoover et al., 1970). FHV-1 infection sometimes leads to conjunctivitis and scabbing around nostrils and eyes. In severe cases, cough and dyspnea are also observed. Unlike CHV infection, FHV-1 infection doesn't seem to be a direct cause of abortion. After acute infection, FHV-1 latently keep the infection in the trigeminal ganglia and the latently infected cats shed infectious virus as carriers (Gaskell et al., 1985; Nasisse et al., 1992). A lot of stress make latent FHV-1 reactivate and cause diseases. For the treatments of FHV-1 infection, some nucleoside analogs are considered to be effective, for examples, topical use of idoxuridine or acyclovir improved FHV-1 ocular disease (Thomasy et al., 2016; Stiles et al., 2003). Susceptibility of FHV-1 to feline and human IFN has been proven and combination of acyclovir and human IFN-alpha showed a synergetic effect (Fulton et al., 1985; Thomasy et al., 2016). Oral administration of L-lysine is safe and effective treatment to reduce viral shedding (Gaskell et al., 2007). For the prevention, two types of vaccines are available, modified live vaccine and inactivated vaccine. Although they are generally able to

improve clinical signs, FHV-1 infection and the latency are not completely prevented by these vaccines (Sussman et al., 1997; Weigler et al., 1997). Moreover, to prevent viral reactivation, whether vaccination to latently infected animals is effective is under discussion. However, the vaccine can restrict viral shedding and latency in vaccinated cats compared to control cats (Sussman et al., 1997; Weigler et al., 1997). These facts indicated that all cats should take vaccines to control the spread of FHV-1.

1.1.4. Paramyxovirus

1.1.4.1. General characteristics

Paramyxoviruses, viruses in the order *Paramyxoviridae*, possess an envelope and a single-stranded and minus-sense RNA encoding 6-10 proteins. Paramyxovirus includes mumps virus and measles virus known as highly infectious viruses for human and Hendra virus and Nipah virus associated with high mortality among human (Murray et al., 1995; Chua et al., 2000). Paramyxoviruses can be divided into four subfamilies, *Avulavirinae*, *Metaparamyxovirinae*, *Orthoparamyxovirinae* and *Rubulavirinae*. The morphology of paramyxovirus particle is various, from spherical to ellipsoidal, and the size is approximately 110-540nm (Loney et al., 2009). Paramyxoviruses have two membrane glycoproteins, fusion (F) protein and hemagglutinin (H), hemagglutinin-neuraminidase (HN) or glycoprotein (G), which are related to viral attachment and receptor binding (Chang et al., 2012). Synthesized F protein called F0 protein dose not have a fusion activity. However, after cleavage by cellular proteinases and conformational changes, F protein are activated for fusion (Paterson et al., 2000). Viral nucleoprotein (N) attaches to genomic RNA accompanied with the phosphoprotein (P) and the large (L) protein, which consists of RNP complexes (Cox et al., 2017). Matrix (M) protein contributes to the particle assembly by the interaction with N protein and glycoproteins. In addition to these proteins, several viruses also possess small hydrophobic (SH) transmembrane protein as ion channels or transmembrane (TM) protein for cell-to-cell fusion (Cox et al., 2017).

1.1.4.2. Diseases and pathogenesis

1.1.4.2.1. Canine distemper virus infection

Canine distemper virus (CDV) is a member of the genus *Morbillivirus* and has been identified as a causative agent of the systematic infection termed as canine distemper in dogs since 1760 (Rendon-Marin et al., 2019). CDV uses the signaling lymphocyte activation molecular (SLAM) in the peripheral blood mononuclear cells and the nectin-4 in the epithelial cells as the receptors for viral attachment using H protein. CDV has broad cell tropism, which contributes to general clinical signs (Lempp et al., 2014). It is known that CDV has not only broad cell tropism, but also broad host range. CDV infection has been reported, for example, in many kinds of animals; racoons, ferrets, pandas and surprisingly lions and monkeys (Martinez-Gutierrez et al., 2016). During CDV infection in dogs, CDV is secreted in all body fluids and transmitted to susceptible animals (Beineke et al., 2009). After the transmission, at first, CDV replicates in macrophages and monocytes around respiratory epitheliums and tonsils and the virus spreads to other hematopoietic tissues followed by viral infection to whole-body tissues (Vandevelde et al., 2005; Okita et al., 1997). CDV infected dogs exhibit respiratory and gastrointestinal diseases, conjunctivitis, anorexia and skin rash, which are accompanied with bacterial infection or neurological diseases at acute infection (Beineke et al., 2009). Biphasic fever and demyelinating leukoencephalitis are the representative features in CDV infection (Beineke et al., 2015). Supportive treatment is recommended to control the diseases. To resolve diarrhea and vomiting, infusion is of particular importance. In the case of second bacterial infection, administration of antibiotics would be necessary. CDV vaccine is defined as a core vaccine, which should be inoculated to all dogs. The live vaccine can lead virus neutralizing antibodies which continue over for three years (Schultz., 2006). For effective immunization, vaccination schedules should be well considered by referring to the guidelines such as American Animal Hospital Association canine vaccine

guidelines.

1.1.4.2.2. Feline morbillivirus infection

Feline morbillivirus (FeMV) is a new emerging paramyxovirus, which was detected in Hong Kong in 2012 (Woo et al., 2012). This virus belongs to the genus *Morbillivirus* and, until now, FeMV infected cats have been reported in Asia, Europe and Americas (Choi et al., 2020). Virus isolation has been also succeeded in CRFK cell. In Japan, the epidemiological survey was performed using 82 urine samples and ten blood samples of cats from veterinary hospital, resulting that five urine and one blood samples were positive for RT-PCR (Furuya et al., 2014). Viral RNA can be detected not only urine samples, but also kidney samples. Although urinary and gastrointestinal diseases with fever, depression and some hametologic disorders were observed in FeMV infected cats, other viral co-infection was also observed. Therefore, the clinical signs caused by FeMV infection are still unknown. In some cases, cats were persistently infected with FeMV for more than one year (Sharp et al., 2016). Further research on viral entry mechanism and the pathogenicity are needed.

1.1.5. Orthomyxovirus

1.1.5.1. General characteristics

Orthomyxoviruses, including influenza virus, consist of the family *Orthomyxoviridae* and 7 genera *Alpha-*, *Beta-*, *Delta-*, *Ganmainfluenzavirus*, *Isavirus*, *Quaranjavirus* and *Thogoyovirus*. Influenza A viruses (IAV) have been detected from human and many animals, and cause pandemics. Influenza B and C viruses are respiratory pathogens mainly among human. Influenza D viruses have been recently reported in cows and pigs (Zhai et al., 2017). Salmon isavirus, which consists of the genus *Isavirus* alone, causes fatal anemia in atlantic salmons (Olsen et al., 2016). Viruses in the genus *Quaranjavirus* are isolated from birds and ticks (Sameroff et al., 2021). Thogotoviruses are tick-borne viruses which lead to febrile illness with neurological diseases in human and the fatal case have been reported in the USA (Kosoy et al., 2015). The form of viral particle is spherical or filamentous surrounded by a lipid envelope, and the size is at least 80-120 nm in diameter. The particle contains glycoproteins for viral entry and membrane fusion. *Alphainfluenzavirus* and *Betainfluenzavirus* possess hemagglutinin (HA) and neuraminidase (NA) and *Deltainfluenzavirus* and *Gammainfluenzavirus* have hemagglutinin-esterase-fusion (HEF) (Russell., 2016). Hemagglutinin-esterase (HE) and fusion (F) proteins exists on a surface of *Isavirus* (Kibenge et al., 2009). *Quaranjavirus* and *Thogotovirus* encodes only glycoprotein (GP) (Portela et al., 1992; Mourya et al., 2019). Orthomyxoviral genome is composed by single-stranded, linear and segmented RNA with negative polarity (Zheng et al., 2013). The number of the segments is eight (*Alphainfluenzavirus*, *Betainfluenzavirus* and *Isavirus*), seven (*Deltainfluenzavirus* and *Gammainfluenzavirus*) and six (*Quaranjavirus* and *Thogotovirus*).

1.1.5.2. Diseases and pathogenesis

1.1.5.2.1. Influenza A virus infection

Influenza A viruses (IAV) in the genus *Alphainfluenzavirus* is common pathogen among vertebrates. They have eight segments and HA in segment 4 and NA in segment 6 are glycoproteins. 18 HA- and 11 NA-subtypes are reported. It's considered that wild aquatic birds are the natural hosts of all IAV subtypes except for H17N10 and H18N11 in bats (Webster et al., 1992; Wu et al., 2014). Nowadays, H1N1 and H3N2 infections are seasonal diseases and other avian IAV infection is rarely observed among human (Gatherer et al., 2009; Allen et al.,2018). However, due to the segmented genome, the genomic reassortment sometimes occurred in IAVs, and this event contributes to antigenic shift and an emergence of new IAV strain (Urbaniak et al., 2014). At least five IAV pandemics were documented and particularly, Asian Flu in 1957, Hong Kong Flu in 1968 and swine Flu in 2009 emerged by the reassortants (Mostafa et al., 2020). A cause of Hong Kong Flu was H3N2, which was originated from H2N2 which leads to Asian Flu and an avian IAV (Jester et al., 2020). During the pandemic, approximately one million people were died for H3N2 infection. In 2009, swine Flu as H1N1pdm09 emerged by the unique combination of human-, avian- and swine-origin IAVs (Garten et al., 2009). H3N2 and H1N1pdm09 are globally circulating now. Therefore, IAV transmission among animals have a critical role in IAV pandemics and there are some reports of the detection of IAV infection among companion animals.

IAV-infected dogs were first reported in Florida in 2004 (Payungporn et al., 2008). These animals showed fevers and coughs, and some of them died of the hemorrhagic pneumonia caused by equine H3N8. Furthermore, another IAV infection among dogs was sporadically identified in Korea (Song et al., 2008). This virus was H3N2

and caused diseases in dogs. Besides H3N8 and H3N2, swine-origin H5N2 or H1N1pdm09 infection have been ever known among dogs (Song et al., 2013; Su et al., 2014). The seroprevalence of canine influenza virus in dogs was 8.5% in Ohio in the USA and 5.7% in Ukraine (Jang et al., 2017; Kovalenko et al., 2021).

The results of seroepidemiological surveys and animal experiments indicate that cats can be infected with some IAVs (Frymus et al., 2021). Especially highly pathogenic avian H5N1 causes severe disease in the animals in the family *Felidae*. In the case of Thailand, tigers and leopards were infected by eating chickens infected with H5N1 (Keawcharoen et al., 2004). Cats experimentally infected with the virus showed fever, dyspnea and conjunctivitis, and one of them died six days after the inoculation (Kuiken et al., 2004). H1N1pdm09 infection has been identified all over the world (Sponseller et al., 2010; Zhao et al., 2014; Fiorentini et al., 2011).

Ferrets are used for animal experiments of IAV infection, because they exhibit respiratory diseases similar to those of human (Beleser et al., 2011). Diseased ferrets develop sneezing, nasal discharge, lethargy and a fever (Reuman et al., 1989). *In vivo* experiments in ferrets can be performed for evaluation of viral pathogenicity and transmission via the droplets or the effect of vaccine and antiviral agents (Belser et al., 2018; Lee et al., 2020). For the first isolation of IAV, ferrets were inoculated with samples to replicate alive viruses (Smith et al., 1933). Ferrets are also known as pet animals like dogs and cats. Although natural infection of IAV and clinical signs have been reported in some reports, little is known about seroprevalence of IAVs in domestic ferrets (Lin et al., 2014; Swenson et al., 2010).

1.1.6. Parvovirus

1.1.6.1. General characteristics

Parvoviruses are small and non-enveloped viruses with single-stranded DNA genome. Viral particle size is around 25 nm and these viruses resist to disinfectants. The VP gene in the genome encodes capsid protein which constructs viral particle, and the NS gene encodes non-structural protein (Francois et al., 2016). Parvoviruses possess a DNA helicase called as NS1 protein, but don't have a DNA polymerase. Therefore, parvoviruses utilize host or the other viral DNA polymerase for their genome replication (Zou et al., 2018). Parvoviruses in the family *Parvoviridae* consists of three subfamilies *Parvovirinae, Densovirinae* and *Hamaparvovirinae*. Especially, the members in *Parvovirinae* infect many kinds of vertebrates, such as dogs, cats, cows, pigs, mice, minks, chickens and so on, and cause virous diseases (Cotmore et al., 2014).

1.1.6.2. Diseases and pathogenesis

1.1.6.2.1. Canine parvovirus infection

Canine minute virus, called as CPV-1, is a member of the genus *Bocaparvovirus*. Bovine parvovirus is related to mild diarrhea in cows and belongs to the same genus. CPV-1 was isolated from a healthy dog in 1967 (Binn et al., 1970). In addition to the VP gene and the NS gene, this viral genome encodes NP1 gene (Schwartz et al., 2002). CPV-1-affected dogs generally exhibit mild diarrhea, respiratory syndrome or asymptomatic infection (Carmichael et al., 1994; Pratelli et al., 1999). By contrast, CPV-1 infection is occasionally fatal in neonates (Harisson et al., 1992). The result of serological surveillance in America indicated that the seroprevalence was over 50% and the widespread of CPV-1 (Carmichael et al., 1994).

Enteric disease caused by canine parvovirus 2 (CPV-2) is a serious problem in dog populations all over the world. CPV-2 is classified into the subfamily *Parvovirinae* and the genus *Protoparvovirus*. This virus was first reported in 1978, and then antigenically divided into CPV-2a and CPV-2b (Carmichael., 2005). Furthermore, since 2000, new strain CPV-2c has been detected in many countries (Hoang et al., 2019; Battilani et al., 2019; Hao et al., 2020). Although these three strains are spreading, dominant CPV-2 is different between countries. For example, although most typical isolate is CPV-2a in Asia and Australia, CPV-2b and CPV-2c are more prevalent in North America (Meers et al., 2007; Nandi et al., 2010; Hong et al., 2007). Some epidemiological surveys showed 5.7-59.7% of positive rate in dog population and higher rate in pure breeds than that in mix breeds (Tion et al., 2018; Sayed-Afmed et al., 2020; Umar et al.,2015). Three forms, enteric, cardiac and neurologic forms, are known as the clinical signs of this viral infection. The enteric form presents pyrexia, anorexia and gastrointestinal diseases, such as vomiting and bloody diarrhea. The cardiac form indicates cardiovascular and respiratory diseases in young animals (Agungpriyono et al., 1999). CPV-2 transmits via oral route and the incubation time is approximately five days. Since this virus is able to keep the infectivity in the environments for a long time, animals are infected from the contaminated materials like clothing, shoes and feeders (Kantere et al., 2021). Administrations of fluid, antiemetics and recombinant feline omega-interferon are performed to relieve dehydration, vomiting and fever (Castro et al., 2013; Mylonakis et al., 2016). CPV-2 vaccine is included in core vaccine and the vaccination is important for all dogs to control this pathogen. Approximately 70% of vaccine coverage is necessary for prevention of outbreak (Decaro et al., 2020).

1.1.6.2.2. Feline parvovirus infection

Feline panleukopenia virus (FPV) is a cause of feline panleukopenia (FPL), known as highly virulent disease in cats (Kruse et al., 2010). FPV, a member of the genus *Protoparvovirus*, was identified from the stool sample of cats showing gastroenteritis in 1928 (Capozza et al., 2021). FPV is genetically and antigenically similar to canine parvovirus and recognized as the host range variant. It is considered that the hosts of FPV are all animals in *Felidae* and some carnivores like mink and raccoon (Steinel et al., 2001). FPV generally infects lymphoid tissues, bone marrow and intestine, where the cells grow and divide quickly, and leads lymphopenia, neutropenia, anorexia, hemorrhagic diarrhea and vomiting with 25-100% of mortality rate (Kruse et al., 2010). The sickness is severe in young animals and cerebellar hypoplasia in neonates is triggered by FPV infection (Resibois et al., 2007). However, the number of FPV outbreak is fortunately decreased by the vaccination (Stone et al., 2020). FPV vaccines are also one of the core vaccines and they are available as modified live or inactivated forms. Regardless of a place where cats are kept indoor or outdoor, all cats should be vaccinated. Treatment protocol is similar to that for CPV-2 infection. Because of no specific drug to FPL, the supportive therapy is recommended.

1.1.6.2.3. Ferret parvovirus infection

Aleutian disease is an infectious disease originally identified in minks and known as gammaglobulinemia and plasmacytosis. A cause of this illness is Aleutian mink disease virus (AMDV) classified into the genus *Ambovirus* and first reported in the USA in 1940s (Nituch et al., 2011). Virus isolation was also successful in feline kidney cells in 1977 and, until now, this virus has been detected in various animals as ferrets, skunks and

raccoons (Franzo et al., 2021). Since AMDV is as strong as other parvovirus in the environment, indirect infection sometimes occurs (Lu et al., 2021). In addition, vertical and vector-borne transmission appear to be available for AMDV (Broll et al., 1996; Shen et al., 1972). AMDV leads to different disease types depend on the age of the minks. In adult animals, AMDV persistent infection causes progressive wasting syndrome related to lymphadenopathy, splenomegaly, plasmacytosis arteritis and glomerulonephritis (Eklund et al., 1968; Hadlow et al., 1984). In pregnant animals, AMDV causes abortion, the death of embryos and low pregnancy rate (Broll et al., 1996). In contrast to the persistence in adults, AMDV infection in young animals results in acute and fatal pneumonia and the treatment using anti-AMDV antibodies is effective (Alexandersen et al., 1989). Similar to minks, AMDV-infected ferrets exhibit wasting syndrome associated with respiratory disease, arteritis and glomerulonephritis. Moreover, there are some reports of neurologic sign (Langlois et al., 2005). However, AMDV infections in ferrets are basically subclinical and not persistent.

2. CHAPTER 1

Antiviral effect of sinefungin on in vitro growth of feline herpesvirus type 1

2.1. Abstract

Feline herpesvirus type 1 (FHV-1) causes a potentially fatal disease in cats. Through the use of virus inhibition and cytotoxicity assays, sinefungin, a nucleoside antibiotic, was assessed for its potential to inhibit the growth of FHV-1. Sinefungin inhibited *in vitro* growth of FHV-1 most significantly over other animal viruses, such as feline infectious peritonitis virus, equine herpesvirus, pseudorabies virus and feline calicivirus. Our results revealed that sinefungin specifically suppressed the replication of FHV-1 after its adsorption to the host feline kidney cells in a dose-dependent manner without obvious cytotoxicity to the host cells. This antibiotic can potentially offer a highly effective treatment for animals infected with FHV-1, providing alternative medication to currently available antiviral therapies.

2.2. Introduction

Nucleoside antibiotics constitute a large family of important microbial products with antiviral, antifungal and antiprotozoal activities (Chen et al., 2016). Because nucleosides metabolites play pleiotropic roles, such as energy donors, cofactors and metabolite carriers, in most primary metabolisms and central roles in genetic inheritance, they possess high potential of targeting parasite-specific process, such as viral proliferation and protozoan parasitism (Isono et al., 1988).

Sinefungin is a nucleoside antibiotic and structural analogue of S-adenosyl-L-methionine, and it inhibits the methylation of DNA, RNA, proteins and other molecules (Fuller et al., 1978; Paolantonacci et al., 1985; Phelouzat et al., 1992; Zhen et al., 2006). It has been suggested that the potent antiviral activity of sinefungin might be due to selective inhibition of cap-methylation in maturing mRNA molecules, particularly by RNA (guanine-N7) methyltransferase, which adds a methyl group to Gppp-RNA for the formation of the m7GpppRNA cap. This process is essential for the initiation of translation and protection of RNA molecules from degradation by 5′-exonucleases (Bouvet et al., 2010; Li et al., 2007; Selisko et al., 2010; Zheng et al., 2008).

In this study, we assessed the antiviral activity of sinefungin on some major animal viruses. Feline herpesvirus type 1 (FHV-1) is an enveloped DNA virus, classified into the family *Herpesviridae*, subfamily *Alphaherpesvirinae*, genus *Varicellovirus*. This virus causes viral rhinotracheitis in cats. This commonly diagnosed clinical disease is characterized by upper respiratory diseases and conjunctivitis (Kruger et al., 1996; Povey et al., 1979; Spradbrow et al., 1971; Stiles et al., 1997). Although most kittens can recover from primary infection with FHV-1, the virus can latently infect the trigeminal ganglion, often becoming reactivated in old and/or immunocompromised

cats. When FHV-1 infects newborn, debilitated and immunodeficient cats, secondary infections with other pathogens occur and sometimes the disease becomes fatal.

In addition to FHV-1, we evaluated some other viruses which can infect animals. Feline infectious peritonitis virus (FIPV) is a member of the family *Coronaviridae*, subfamily *Coronavirinae*, genus *Alphacoronavirus*; it has a single-stranded positive-sense RNA genome. FIPV is a causative agent of feline infectious peritonitis, which is a systemic and fatal immune-mediated disease (Montali et al., 1972). Equine herpesvirus type 1 (EHV-1) and Pseudorabies virus (PRV) belong to the family *Herpesviridae*, genus *Varicellovirus*; they possess double-stranded DNA genome similar to FHV-1. EHV-1 causes severe respiratory disease, neurological disease, abortion or death in horses (Lunn et al., 2009; Mochizuki et al., 2009). PRV causes Aujeszky's disease, a neurological and respiratory disease in pigs and a lethal disease in other animals (Thiry et al., 2013). Feline calicivirus (FCV), which contains a single-stranded positive-sense RNA genome, belongs to the family *Caliciviridae*, genus *Vesivirus*; it causes acute oral and upper respiratory disease in cats (Reubel et al., 1992).

Here we report the in vitro antiviral effect of sinefungin on the growth of FHV-1, FIPV, EHV-1, PRV and FCV. In addition to its antiviral activity, the cytotoxicity of sinefungin toward host cells was assessed to determine its potential as an antiviral treatment for infected animals.

2.3. Materials and methods

2.3.1. Cells and viruses

A feline kidney cell line, CRFK (Crandell et al., 1973), Fcwf-4 cell line (Norimine et al., 1992) and our established fetal horse kidney (FHK)-Tcl3.1 cell line (Andoh et al., 2009) were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen Carlsbad, CA, USA) containing 10% heat-inactivated fetal calf serum (FCS) (GE Healthcare Life Science, UK), 100 U ml^{-1} of penicillin and 100 μg ml^{-1} of streptomycin (Thermo Fisher Science, USA) in a humidified atmosphere with 5% CO_2 at 37 °C. FHV-1 C7301 strain (Mochizuki et al., 1977), FCV F9 strain (Bittle et al., 1976), FIPV M91-267 strain (Mochizuki et al., 1997), PRV Indiana strain (Fukusho et al., 1981) and EHV-1 89c25p strain (Matsumura et al., 1998) were used in this study. FHV-1, FCV and PRV were propagated in CRFK cells, FIPV was propagated in Fcwf-4 cells (Pederson et al., 1981) and EHV-1 in FHK-Tcl3.1 cells cultured in DMEM with 2% FCS at 37 °C in 5% CO_2 until cytopathic effect was observed and then were stored at −80 °C. Sinefungin was purchased from Sigma-Aldrich (MO, USA), and the purity of >98% was confirmed by our analytical HPLC system implemented with Hitachi photodiode-array detector and a CAPCELL PAK SCX column (Shiseido, Japan) as previously described (Fukuda et al., 2010).

2.3.2. Virus inhibition assay

Host cell lines, CRFK, Fcwf-4, and FHK-Tcl3.1, were cultured at 37 °C on 12-well culture plates (Sumitomo Bakelite, Tokyo, Japan) in DMEM containing 10% FCS. Next, the medium was removed and cells in each well were inoculated with FHV-1,

FCV, FIPV, PRV or EHV-1 at a multiplicity of infection (M.O.I.) of 0.01 in 200 μl of DMEM containing 2% FCS with/without sinefungin (100 μg ml^{-1}; 262 μM). After incubation for 1 h at 37 °C, cells were washed twice with DMEM and overlaid with media containing 2% FCS with/without sinefungin (100 μg ml^{-1}). After inoculation at 37 °C, FCV was collected at 48 h post-infection (h.p.i.), FIPV at 36 h.p.i. and FHV-1, PRV and EHV-1 at 72 h.p.i. The supernatant was collected and stored at −80 °C, and virus titres were determined by plaque assay. The percentage of plaque number was calculated in comparison with that of the untreated control. Each experiment was repeated at least twice.

2.3.3. EC_{50} determination

CRFK cells were cultured on 12-well culture plates in DMEM containing 10% FCS in a humidified atmosphere with 5% CO_2 at 37 °C. Next, the media was removed and cells in each well were inoculated for 1 h with FHV-1 at an M.O.I. of 0.01 in 200 μl of DMEM containing 2% FCS with/without twofold diluted sinefungin or acyclovir (3–100 μg ml^{-1}) at 37 °C or without sinefungin at 4 °C. The cells were then washed twice with DMEM. After washing, the cells were overlaid with media containing 2% FCS with/without the antiviral agent (3–100 μg ml^{-1}) and incubated at 37 °C. After incubation, the supernatant was collected and stored at −80 °C until virus titration assay. Each experiment was repeated at least twice.

2.3.4. Virus titration

Cell lines, CRFK or FHK-Tcl3.1, cultured on six-well plates (Sumitomo Bakelite, Tokyo, Japan), were inoculated with 200 μl of tenfold diluted viral solutions

and incubated at 37 °C for 1 h. After incubation, the media were removed, the cells were washed twice with DMEM and then overlaid with 0.8% agarose (SeaPlaque GTG Agarose; Lonza, Basel, Switzerland) in DMEM containing 6.7% FCS. The plates were incubated at 37 °C until plaques were observed. Next, the cells were fixed with phosphate-buffered formalin and stained with crystal violet to count the number of plaques. The percentage of plaque numbers was calculated by comparison with that of the untreated control

2.3.5. Cytotoxicity assay

The cytotoxicity of sinefungin was assessed using MTT assay as described in a previous report (Elshabrawy et al., 2014). CRFK cells were inoculated at a density of 10^4 cells/well in 96-well plates. After 24 h, the cells were incubated with different concentrations (3–100 μg ml^{-1}) of sinefungin. Following incubation for 72 h, the cells were washed once with phosphate-buffered saline and 100 μl of fresh DMEM was added to the wells. Subsequently, 10 μl of MTT reagent was added to each well and the plates were incubated for 4 h at 37 °C. Following incubation, 100 μl of 10% sodium dodecyl sulphate in 1 mM HCl was added to each well and the plates were incubated for 4 h at 37 °C. The optical density was measured at 570 nm using a spectrophotometer (Bio-Rad, Tokyo) and the percentage cell viability was calculated in comparison with that of the untreated control after subtracting the background. Each experiment was repeated at least twice.

2.4. Results

2.4.1. Antiviral effects of sinefungin

A variety of viruses were used to assess the antiviral effects of sinefungin. CRFK cells were inoculated with FCV, FIPV, FHV-1, or PRV, and FHK-Tcl3.1 cells were inoculated with EHV-1. After incubation, the supernatants were collected, and the virus titre was determined by plaque assay. Figure 2-1 presents the percentage of plaque number standardized with plaque number of the untreated control. The plaque numbers for FCV and FIPV decreased to 67.7% and 7.8%, respectively. Thus, sinefungin was most effective against FHV-1 infection (0.04%). Alternatively, PRV and EHV-1, which belong to the genus *Varicellovirus* similar to FHV-1, were less sensitive to sinefungin, and the plaque yields were 52.4% and 21.9%, respectively. The antiviral activity of sinefungin was reproduced at the similar levels on another FHV-1 isolate, Tokyo/181128, and when the host was Fcwf-4 cell (data not shown).

2.4.2. EC_{50} of sinefungin against FHV-1 infection

To investigate whether sinefungin inhibits viral adsorption to host cells, CRFK cells were incubated with FHV-1 and with/without different concentrations of sinefungin (3–100 μg ml^{-1}) at 37 or 4 °C for 1 h and washed with DMEM. After incubation for 72 h with/without sinefungin, the viruses were collected, and the titres were determined. The result showed that sinefungin specifically suppressed FHV-1 propagation in CRFK cells in a dose-dependent manner. The EC_{50} values at each incubation temperature were nearly identical (EC_{50} at 37 °C: 9.5 μg ml^{-1}, at 4 °C: 11.2 μg ml^{-1}) (Fig. 2-2). Sinefungin may not affect the attachment of FHV-1 to the host cells,

but it may affect the biochemical processes after adsorption. The EC_{50} value of acyclovir (18 μg ml^{-1}) was determined by our in vitro assay (Fig. 2-3), suggesting that sinefungin is as effective as the anti-herpes drug, acyclovir, which is used for the treatment of shingles.

2.4.3. Cytotoxicity of sinefungin toward CRFK cells

The cytotoxicity of sinefungin toward CRFK cells was assessed using an MTT assay (Fig. 2-2). There was no significant difference in cell viability at concentrations of 3–100 μg ml^{-1}, which suggested that sinefungin has almost no cytotoxicity toward CRFK cells at effective antiviral concentrations.

2.5. Discussion

The antiviral activity of sinefungin has been reported to be because of the potent inhibition of mRNA-methyltransferase and multiplication of the vaccinia virus (Pugh et al., 1978) or inhibition of cell transformation by the Epstein–Barr virus (Long et al., 1987). In this study, we assessed the antiviral effects of sinefungin on some major animal viruses. The results indicate that FHV-1 is more sensitive to sinefungin than other cat and herpes viruses. The mechanism underlying inhibition remains unclear; however, this antiviral effect appears not specific to the genus *Varicellovirus* because FIPV and EHV-1 were also inhibited by sinefungin, while PRV and FCV were less sensitive to sinefungin in CRFK (Fig. 2-1). It is unknown whether the mechanism of action of sinefungin against FHV-1 is due to its activity as an inhibitor against S-adenosyl-L-methionine-dependent methyltransferase reactions (Fuller et al., 1978; Paolantonacci et al., 1985; Pheulouzat et al., 1992; Zheng et al., 2006).

Further investigations were conducted to determine the antiviral activity of sinefungin against FHV-1 in detail. Sinefungin inhibited FHV-1 replication in a dose-dependent manner with an EC_{50} value of 25 μM (9.5 μg ml $^{-1}$) at 37 °C. Few reports on the comparison of antiviral drugs against FHV-1 are available. For example, acyclovir is typically used for the treatment of an ocular disease that is caused by FHV-1 infection with an EC_{50} of 250 μM. Adefovir inhibits herpesvirus infection, including those caused by human simplex virus (HSV) (De Clercq et al., 2013), cytomegalovirus (CMV) (Andrei et al., 1991) and varicella-zoster virus (Andrei et al., 1995); it can inhibit FHV-1 infection in vitro with an EC_{50} value of 73 μM. Foscarnet, which is administered for CMV in human retinitis or HSV infection (De Clercq et al., 2013), is also effective against FHV-1 infection with an EC_{50} value of 140 μM (van der Meulen et al., 2006).

Accordingly, sinefungin might be as effective and useful for the treatment of FHV-1 infection as acyclovir, adefovir and foscarnet, based on the comparable EC_{50} values revealed in our in vitro assay, and sinefungin shows no obvious cytotoxicity toward the host feline kidney cell line. A previous study reported that sinefungin was cytotoxic to NCTC clone 929 mouse cells of strain L, known as fibroblasts (Pugh et al., 1978). This toxicity might be due in part to the difference of the host and tissue from which the culture cells were derived. According to a recent database, sinefungin and acyclovir have similarly low toxicity; for sinefungin oral LD_{50} is 1 g/kg (mouse) and subcutaneous LD_{50} is 185 mg/kg (mouse) (Cayman Chemical Company., 2015). For acyclovir, oral LD_{50} is >10 mg/kg (mouse) and subcutaneous LD_{50} is 1,118 mg/kg (mouse) (Cayman Chemical Company., 2018).

2.6. Figure legends

Fig. 2-1 Antiviral effects of sinefungin toward FCV, FIPV, FHV-1, PRV and EHV-1. CRFK cells were propagated with FCV, FIPV, FHV-1 or PRV and FHK-Tcl3.1 cells were propagated with EHV-1 at an M.O.I. of 0.01 with/without sinefungin (100 μg ml^{-1}; 262 μM). *$p < 0.0001$ referenced to the plaque yield by FCV.

Fig. 2-2 Dose-dependent anti-FHV-1 activity of sinefungin and its cytotoxicity in CRFK cells. CRFK cells were inoculated with FHV-1 at an M.O.I. of 0.01 with different concentrations of sinefungin (0–100 μg ml^{-1}) at 37 °C (dark bar) or at 4 °C (white bar) for 1 h. After adsorption, the cells were incubated in the presence of twofold diluted sinefungin solution (0–100 μg ml^{-1}) at 37 °C and the virus titre was determined by plaque assay. The percentage of viable cells is represented by the white circle.

Fig. 2-3 Dose-dependent anti-FHV-1 activity of acyclovir in CRFK cells. The antiviral effect was tested under the same condition with different concentrations of acyclovir (0–100 μg ml^{-1}) at 37 °C (meshed bar).

Fig. 2-1

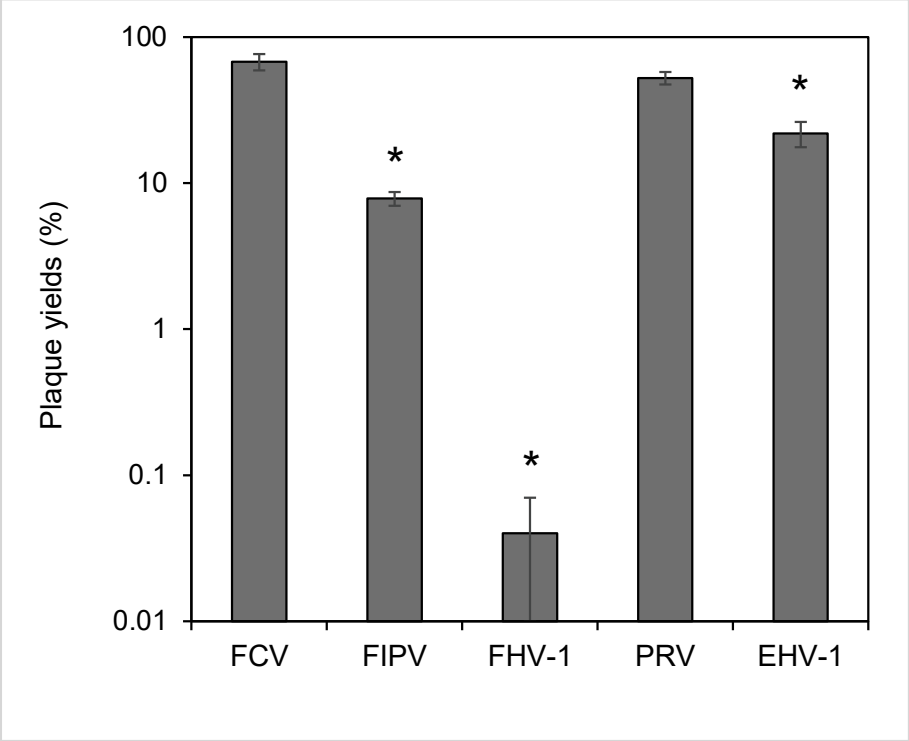


Fig. 2-2

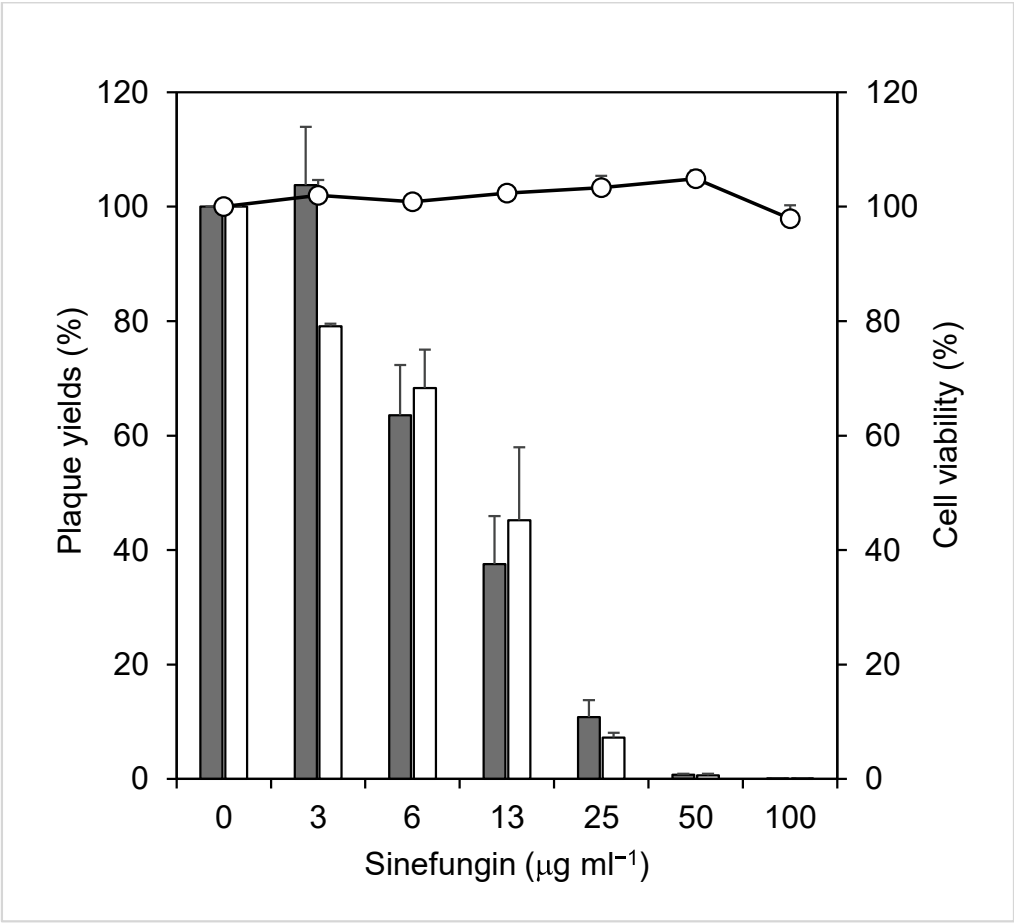
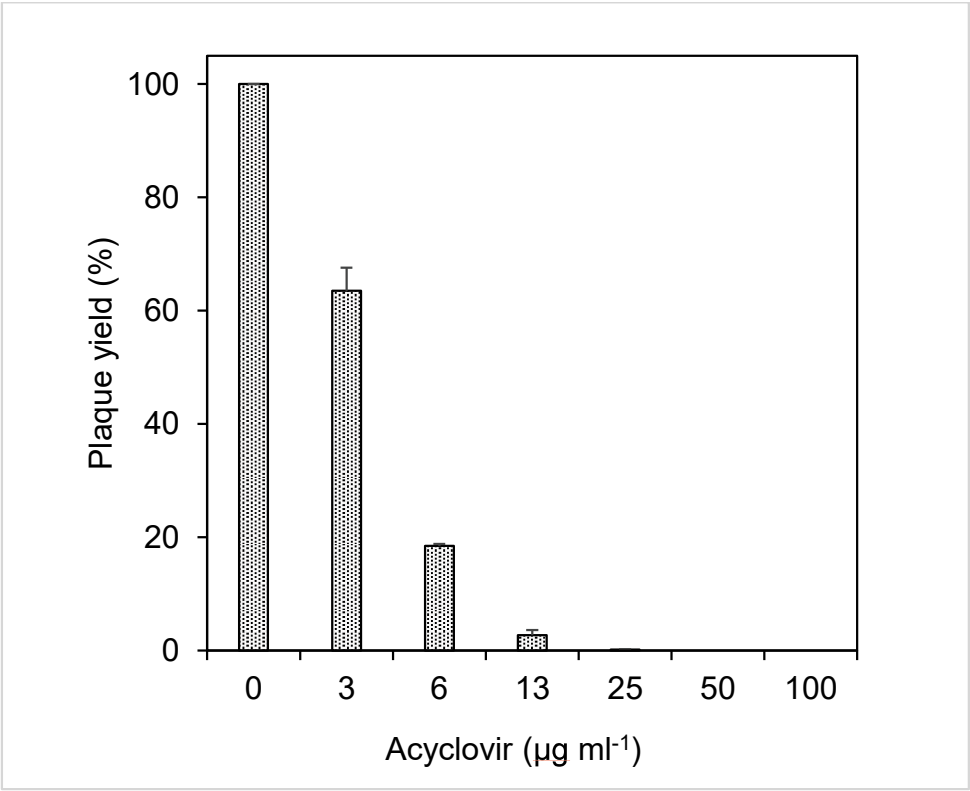

120
100
80
60
40
20
0
Plaque yields (%)
Cell viability (%)
0
3
6
13
25
50
100
Sinefungin (μg ml⁻¹)

Fig. 2-3



3. CHAPTER 2 Influenza A virus infection in domestic ferrets

3.1. Abstract

Ferrets are animals that are known to be susceptible to influenza A virus (IAV) infection. To evaluate the risk of IAV transmission from diseased ferrets to humans, we performed a serosurvey to detect specific antibodies against the H1, H3, H5, and H7 subtypes of IAV. We found a high positive rate of the H1 (24.1%) and H3 (5.2%) subtypes in pet ferrets by using an enzyme-linked immunosorbent assay for hemagglutinin proteins. The results were confirmed by the virus-neutralization test for representative antibody-positive serum samples. We also detected hemagglutinin and neuraminidase genes in two ferrets showing acute respiratory illness and whose owner was diagnosed with IAV infection; a human H1N1pdm virus was isolated from one of these ferrets. Our findings suggest that attention should be paid for IAV infection from humans to ferrets, and vice versa.

3.2. Introduction

Influenza A viruses (IAVs) are segmented, negative-sense, single-stranded RNA viruses of the family Orthomyxoviridae. They infect a wide range of host species in addition to humans (Shao et al., 2017). Domestic animals, such as dogs and cats, which live in relatively closer contact with humans than other animals, are susceptible to IAVs, and can be an accidental source of IAV infection in humans (Chen et al., 2018; Horimoto et al., 2015; Marinova-Petkova et al., 2017). Therefore, epidemiological surveys of IAV infection among pet animals are needed to assess the risk of IAV transmission from animals. Ferrets are highly susceptible to IAV infection, and infected ferrets exhibit sneezing, nasal discharge, and high fever, and are an experimentally useful model of IAV infection (Haga et al., 2010). However, no epidemiological survey of IAV in pet ferrets has been performed to date.

We established an enzyme-linked immunosorbent assay (ELISA) using extracts from HEK-293T cells transfected with plasmids expressing the hemagglutinin (HA) of A/California/04/2009 (H1N1), A/duck/Mongolia/301/2001 (H3N2), A/chicken/Yamaguchi/7/2004 (H5N1), and A/seal/Massachusetts/1/1980 (H7N7) to examine the seroprevalence of IAV infections in domestic ferrets. In a previous study, we used a similar method to successfully establish an ELISA for the detection of antibodies against hepatitis E virus infection in numerous mammalian species (Yonemitsu et al., 2016). In the present study, ELISA was performed using 79 serum and/or plasma samples collected from pet ferrets in animal hospitals throughout Japan between August 2012 and January 2018. Although the samples were collected for the diagnosis of ferret coronavirus infection, many of them were collected regardless of their clinical signs (Terada et al., 2014; Minami et al., 2016).

3.3. Materials and Methods

3.3.1. Samples

Plasma or serum samples were collected from 79 domestic ferrets by veterinary clinicians in Japan from 2012 to 2018 for the seroepidemiological research.

Pharyngeal swabs of two diseased ferrets were collected by veterinary clinicians two days after the respiratory diseases appeared.

3.3.2. Cells

For the plasmid transfection, human embryonic kidney 293T (HEK-293T) cells were cultured in Dulbecco's Modified Eagle Medium (Thermo Fisher Scientific) with 10% fetal calf serum (GE Healthcare Japan), 100 U/ml penicillin and 100 μg/ml streptomycin at 37◦C in 5% CO_2.

MDCK and AX-4 cells were maintained in Modified Eagle Medium (MEM) (Thermo Fisher Scientific) with 5% fetal calf serum (FCS) (GE Healthcare Japan), 100 U/ml penicillin and 100 μg/ml streptomycin at 37◦C in 5% CO_2.

3.3.3. Expression plasmids

The plasmids, pCAGGS, expressing the hemagglutinin (HA) of A/California/04/2009 (H1N1), A/duck/Mongolia/301/2001 (H3N2), A/chicken/Yamaguchi/7/2004 (H5N1), and A/seal/Massachusetts/1/1980 (H7N7) were kindly provided by Professor Taisuke Horimoto in the University of Tokyo and Professor Ayato Takada in Hokkaido University.

3.3.4. Transfection in HEK-293T

HEK-293T cells were transfected with the HA-expression plasmids and, as a control, empty plasmids. 16 μg of plasmids were mixed with 40 μl of PEI (2 mg/ml) and the mixture was added into HEK-293T cells in 90mm dish. After the incubation for three days at 37◦C, the transfected HEK-293T cells were collected with a cell scraper (Techno Plastic Products AG) and washed twice with phosphate-buffered saline (PBS). The cells were treated with 0.5 ml of RIPA buffer (1% sodium deoxycholate, 1% Triton X-100, 10 mM Tris-HCl pH 7.4, 150 mM sodium chloride, 0.5 mM ethylene diamine tetra acetic acid) for 1 h at 4◦C, and the supernatant was collected after centrifugation at 20630 x g for 30 min at 4◦C. The extracts were stored at -80◦C until use.

3.3.5. Enzyme-linked immunosorbent assay (ELISA)

The extracts from HEK-293T cells transfected with the HA-expression plasmids or empty plasmids were diluted into 5 μg/ml with coating buffer (0.05 M carbonate-bicarbonate buffer, pH 9.6), and distributed at 100 μl per well into 96-well microplates (Maxisorp; Nunc, Roskilde, Denmark). After the incubation at 37◦C for 2 h, the 96-well microplates were placed at 4◦C overnight. Wells in the plates were washed three times with PBS containing 0.05% Tween 20 (PBS-T), and then incubated with 200 μl of 1% Block Ace (DS Pharma Biomedical Co., Ltd) in PBS at 37◦C for 30 min. After three times washing with PBS-T, 100-fold diluted sera or plasma samples with PBS-T containing 0.4% Block Ace were added to duplicate wells as the 1st antibody. After incubation at 37◦C for 30 min, wells were washed three times with PBS-T and incubated at 37◦C for 30 min with 100 μl per well of HRP conjugated protein A/G (Thermo Fisher Scientific) diluted with PBS-T containing 0.4% Block Ace. After three

times washing with PBS-T, 100 µl of substrate reagent (ABTS Microwell Peroxidase Substrate, KPL, MA, USA) was added and the plates were shaken gently for 30 min. The enzymatic reaction was stopped by adding 100 µl of 1% SDS. Absorbance was measured using a spectrophotometer (iMarkTM; BIO-RAD) at a wavelength of 405 nm. Absorbance of wells coated with extracts from mock-transfected cells was subtracted from those from pCAGGS-HAs transfected cells. The ELISA absorbance greater than 0.5 was tentatively considered to be positive.

3.3.6. Virus isolation

Oral swabs in PBS were centrifuged, and the supernatants were collected and diluted with MEM containing 0.3% FCS. The dilutions were inoculated into MDCK or AX-4 cells in 6-well plates for 1hr at 37◦C. Then, the cells were washed with MEM twice, and cultured in MEM with 0.3% FCS. The CPE appeared only in AX-4 cells, not in MDCK cells.

3.3.7. Virus-neutralization (VN) test

To compare the results between ELISA and VN test, ELISA-positive (n=9) and -negative ferret samples (n=7) against HA(H1) was examined by VN test using human IAVs, former seasonal H1N1 virus (A/Kawasaki/UTK4/09), seasonal H3N2 virus (A/Kawasaki/UTK20/08), and H1N1pdm virus (A/Osaka/369/09). 100 $TCID_{50}$ (50% tissue culture infective dose) of these virus strains were pre-incubated with two-fold serial dilutions of serum samples after receptor-destroying enzyme (RDE II, Denka-seiken Co., Ltd, Tokyo, Japan) to remove non-specific inhibitors, and incubated for 30

min on MDCK cells. The neutralizing activity of the test sera was determined by observation of these cytopathic effects a few days after infection.

3.3.8. RT-PCR

The pharyngeal swabs of two diseased ferrets were mixed with PBS and centrifuged at 2,000 x g for 5 min at 4 °C. For RT-PCR, RNA was extracted from the supernatants using QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany).

The genotyping RT-PCR for HA gene was carried out using the primer sets, Type A/M30F2/08 (5'- ATG AGY CTT YTA ACC GAG GTC GAA ACG-3') and Type A/M264R3/08 (5'- TGG ACA AAN CGT CTA CGC TGC AG -3')for IAV consensus RT-PCR, NIID-swH1 ConvPCR Primer-F1 (5'- TGC ATT TGG GTA AAT GTA ACA TTG -3') and NIID-swH1 ConvPCR Primer-R1 (5'- AAT GTA GGA TTT RCT GAK CTT TGG-3') for H1pdm09, H3HA1-BIGIN (5'- AGC AAA AGC AGG GGA TAA TTC-3') and H3HA1-END (5'- TGC CTG AAA CCG TAC CAA CC-3') for H3 HA and H1 HA1-BEGIN (5'- AGC AAA AGC AGG GGA AAA TAA-3') and H1 R10 (5'- GCT ATT TCT GGG GTG AAT CT-3') for H1 former seasonal HA with QIAGEN OneStep RT-PCR Kit (QIAGEN, Hilden, Germany) (Nakamura et al., 2019).

HA and NA genes were amplified using specific primers for complete nucleotide analysis. All primer sets are available in previous report by Nakamura and corroborators (Nakamura et al., 2019).

3.3.9. Sequence analysis

To determine the sequence of PCR amplicons, after the purification using MinElute PCR Purification Kit or MinElute Gel Extraction Kit (QIAGEN, Hilden,

Germany), the sanger sequencing was performed using the BigDye Terminator Ver 3.1 Cycle Sequencing Kit (Thermo Fisher Scientific) according to the manufacturer's instructions.

3.3.10. Phylogenetic analysis

Phylogenetic analysis of HA and NA genes was constructed using a maximum likelihood with 1000 bootstrap replications in MEGA 7.0 software. All sequences of other strains were deposited in GISAID.

3.4. Results

3.4.1. Seroprevalence of IAV among domestic ferrets

The results of the ELISA indicated that 19 of 79 (24.1%) and four of 77 (5.2%) ferrets had antibodies against HA(H1) and HA(H3), respectively. In contrast, no ferret had antibodies against HA(H5) or HA(H7) (Fig. 3-1).

To confirm the IAV infection in representative ELISA-positive ferrets, we performed a VN test to detect antibodies against the former seasonal H1N1 virus (A/Kawasaki/UTK4/09), seasonal H3N2 virus (A/Kawasaki/UTK20/08), and H1N1pdm virus (A/Osaka/369/09) using MDCK cells (Table 3-1). Due to the limited volume of serum samples, not all samples were applied for VN test. All nine HA(H1)-positive ferrets possessed high levels of VN antibodies against the H1N1pdm virus, but not against the former seasonal H1N1 virus. None of the seven HA(H1)-negative ferrets had VN antibodies against the H1N1pdm virus or former seasonal H1N1 virus. One of two HA(H3)-positive ferrets had VN antibodies against the seasonal H3N2 virus. Interestingly, two HA(H3)-negative ferrets were positive for VN antibodies against the seasonal H3N2 virus.

3.4.2. Identification of two diseased ferrets

We collected clinical samples from two ferrets exhibiting acute respiratory illness in an animal hospital in Tokyo, Japan, in 2019. They had been kept in their owner’s house as pets, and their owner was diagnosed with influenza at a hospital just 1 day before the ferrets exhibited the clinical signs. One ferret exhibited sneezing and nasal discharge, and recovered from the illness 6 days after the first clinical sign. The

other ferret exhibited not only sneezing and nasal discharge, but also diarrhea, lethargy, anorexia, and open-mouth breathing. Reverse transcription polymerase chain reaction was performed with the nasal discharge samples from the two ferrets (Nakamura et al., 2019), and the complete sequences of the HA and neuraminidase (NA) genes were determined. Both the HA and NA nucleotide sequences were completely identical between the two ferrets, and were highly homologous to those of H1pdm and N1, respectively. Phylogenetic analysis indicated that these HA genes were classified into clade 6B.1 (Fig. 3-2).

Virus isolation was also performed using AX-4 cells (Hatakeyama et al., 2005), and the IAV A/ferret/Tokyo/358/2019 was isolated from the nasal swab of the ferret that recovered earlier. The HA and NA nucleotide sequences of the isolate had 100% identity to those of the clinical sample (DDBJ accession numbers: LC635746 for HA and LC635747 for NA).

3.5. Discussion

In this study, the sequence of the influenza virus that infected the owner of the diseased pet ferrets could not be obtained. However, these ferrets had been kept in the owner's house and did not go outside, and their clinical signs were observed just after the owner's influenza infection. In addition, the two pet ferrets developed respiratory diseases at the same time, and the obtained sequences were identical, indicating that they were infected from the same source. Therefore, although there is no clear evidence for direct transmission from the owner to the ferrets, the epidemiological evidence indicates that the ferrets were infected from their owner.

IAV was isolated from the nasal swabs of the infected ferrets, which suggested that contact with IAV-infected ferrets may be associated with a risk of IAV infection for veterinarians and animal handlers in veterinary hospitals, pet shops, and breeding farms. We observed that 24.1% of ferrets were seropositive for HA(H1) and had VN antibodies against H1N1pdm virus (Fig. 3-1 and Table 3-1). Therefore, pet ferrets may be infected with H1N1pdm virus at a relatively high rate. Two HA(H3)-negative ferrets were positive for VN antibodies against the seasonal H3N2 virus. This result may have been influenced by the differences in antigenicity among the H3 subtypes.

In conclusion, we found that around 20% of pet ferrets have been infected with IAV. This finding indicates that reverse zoonotic infection of IAV from humans to animals may occur frequently, and IAV infection from diseased animals to humans should be surveyed for further assessment of the risk.

3.6. Figure legends

Fig.3-1 Seroprevalence of influenza A virus infection in domestic ferrets. ELISA was performed using serum samples (diluted 1:100) from pet ferrets. Each dot represents a single sample that was examined. In the ELISA, an absorbance value greater than 0.5 was tentatively defined as seropositive for each HA subtype.

Fig. 3-2 Phylogenetic tree of HA genes using maximum likelihood method. Bootstrap values were calculated from 1000 bootstrap replications. Accession numbers of GISAID were followed by the name of IAV strain. Red indicates the sequences which we have determined in this study.

Fig. 3-1

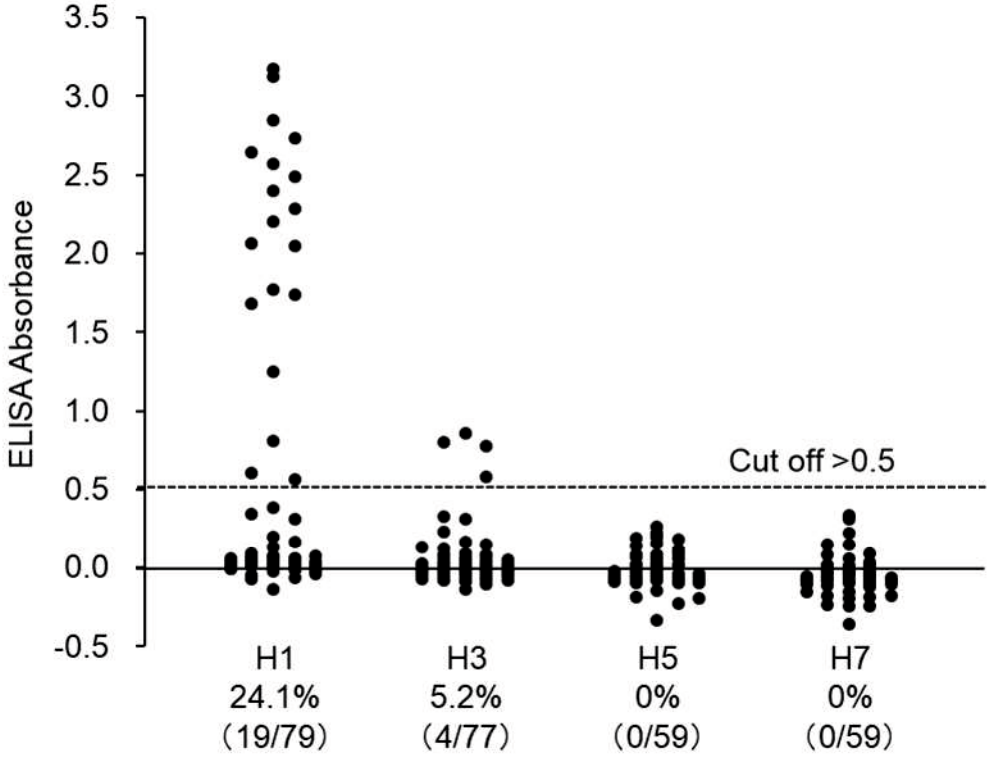

3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
ELISA Absorbance
Cut off >0.5
H1
24.1%
(19/79)
H3
5.2%
(4/77)
H5
0%
(0/59)
H7
0%
(0/59)

Fig. 3-2

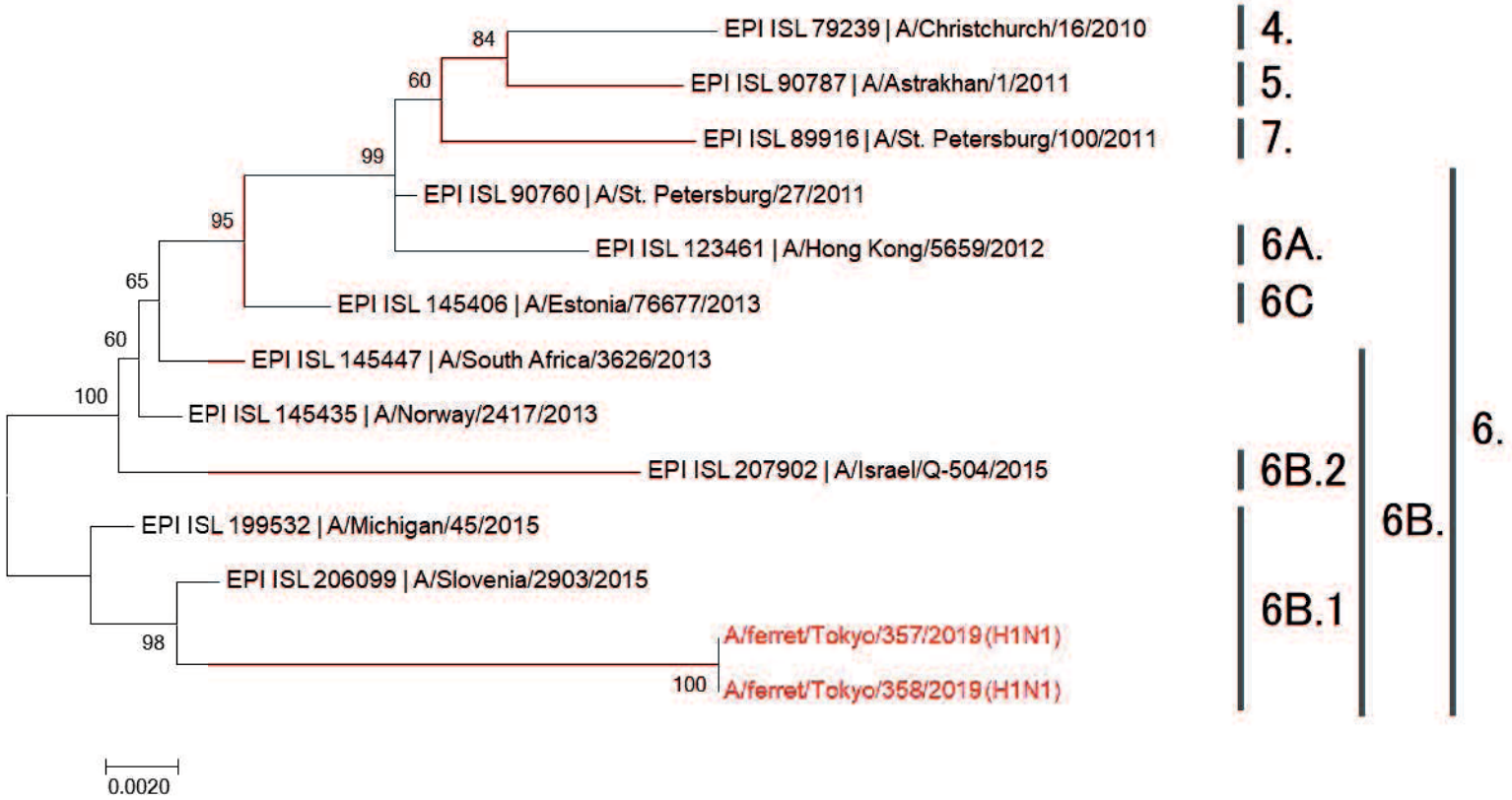

EPI ISL 79239 | A/Christchurch/16/2010
EPI ISL 90787 | A/Astrakhan/1/2011
EPI ISL 89916 | A/St. Petersburg/100/2011
EPI ISL 90760 | A/St. Petersburg/27/2011
EPI ISL 123461 | A/Hong Kong/5659/2012
EPI ISL 145406 | A/Estonia/76677/2013
EPI ISL 145447 | A/South Africa/3626/2013
EPI ISL 145435 | A/Norway/2417/2013
EPI ISL 207902 | A/Israel/Q-504/2015
EPI ISL 199532 | A/Michigan/45/2015
EPI ISL 206099 | A/Slovenia/2903/2015
A/ferret/Tokyo/357/2019(H1N1)
A/ferret/Tokyo/358/2019(H1N1)
84
60
99
95
65
60
100
98
100
0.0020
4.
5.
7.
6A.
6C
6B.2
6B.1
6B.
6.

Table. 3-1 The results of the virus-neutralization (VN) test and enzyme-linked immunosorbent assay (ELISA) using representative sera

Sample ID	ELISA absorbance		VN titer		
	H1	H3	H1N1pdm	H1N1	H3N2
1	**2.29***	-0.02	**1:512**	<1:8	<1:8
2	**1.74**	0.05	**1:32**	<1:8	<1:8
5	**2.40**	**0.86**	**1:512**	<1:8	**1:64**
7	**3.13**	0.23	**1:1024**	<1:8	**1:32**
13	**1.68**	0.15	**1:512**	<1:8	<1:8
32	**2.07**	-0.01	**1:512**	<1:8	<1:8
33	**0.81**	0.01	**1:128**	<1:8	<1:8
34	**1.77**	0.00	**1:128**	<1:8	<1:8
41	**2.85**	-0.09	**1:512**	<1:8	<1:8
3	0.05	**0.78**	<1:8	<1:8	<1:8
4	-0.00	-0.06	<1:8	<1:8	<1:8
6	0.02	-0.04	<1:8	<1:8	<1:8
10	0.03	-0.02	<1:8	<1:8	<1:8
17	0.02	0.33	<1:8	<1:8	<1:8
18	0.05	0.03	<1:8	<1:8	**1:64**
35	-0.07	-0.08	<1:8	<1:8	<1:8

*Bold letters indicate positive results.

4. General conclusion

Recently, many kinds of animals were kept as pets by human beings. The animal-to-human transmission of zoonotic diseases occurs and becomes a problem for public health. Domestic animals are infected with the pathogens from not only other animals or the environment but also human beings, as reverse zoonosis. “One health” approach has been considered as a key concept to maintain human and animal health. Therefore, treatment and the surveillance of infectious diseases among companion animals are required to achieve “One Health”.

In this study, I discovered a new possible candidate of treatment to FHV-1, established a diagnostic system of IAV among ferrets, and conducted serosurvey of IAV infection.

In CHAPTER 1, The antiviral activity of sinefungin, a nucleoside antibiotic, against FHV-1 was analyzed *in vitro*.

Previous reports indicated that sinefungin can inhibit the growth of bacteria, fungus, parasite and herpesvirus. Therefore, to evaluate efficiency of sinefungin to animal viruses, I compared the viral titers of FCV, FIPV, FHV-1, PRV and EHV-1 under treatment with sinefungin. As the result, I found that sinefungin inhibited FHV-1 infection significantly by blocking viral replication, but not viral entry. In addition, the antiviral activity of sinefungin against FHV-1 was in dose-dependent manner without the cytotoxicity and the 50% effective concentration of sinefungin was similar to that of acyclovir, which known as antiviral agents of herpesviruses. Thus, sinefungin or this analog would lead to alternative effective treatment of FHV-1 infection.

In CHAPTER 2, IAV infection was observed in diseased ferrets and serologically surveyed in domestic ferrets.

Ferrets are susceptible to IAV infection and used for animal experiments. To survey the seroprevalence of IAV among domestic ferrets, I performed ELISA using HA-expressing plasmids and 79 serum and/or plasma samples collected from August 2012 to January 2018. The positive rate of the H1 and H3 subtypes was 24.1% and 5.2%, respectively. The result of VN test indicated that domestic ferrets would be infected with H1N1pdm09.

Furthermore, two ferrets, whose owner was diagnosed as IAV infection, exhibited respiratory diseases. RT-PCR and sequence analysis showed they were infected with H1N1pdm. IAV in these ferrets belonged to clade 6B.1 which is circulating among humans, indicating the possibility of IAV human-to-animal transmission. Finally, the success of virus isolation showed the risk for the spread of IAV from infected ferrets to human.

In CHAPTER 1, the discovery of the antiviral effect of sinefungin will contribute to new antiviral agents against many pathogens. In CHAPTER 2, I succeeded in establishment of ELISA system for the detection of antibodies against IAV infection from many animals. The results of ELISA using sera of domestic ferrets indicated that many events of transmission of IAV from human to ferret occurred in Japan.

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学位論文要旨

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Studies on treatment and diagnostic method for infectious diseases among domestic animals

(愛玩動物における感染症の治療法と診断法に関する研究)

近年、イヌ・ネコ・フェレットといった様々な動物が愛玩動物として飼育されており、ヒトと同様に多様な感染症に罹ることが知られている。愛玩動物における感染症の治療法や診断法を確立することは One Health の観点でも重要で、ヒトの健康の維持に直結するような公衆衛生上重要な課題である。ネコヘルペスウイルス１（FHV-1）は猫ウイルス性鼻気管炎の原因となるヘルペスウイルスとして知られ、上部呼吸器症状や流産などを引き起こす。インフルエンザ A ウイルス（IAV）はヒトにおいて呼吸器症状を引き起こす季節性のウイルスとして知られ、イヌ・ネコ・ブタといった様々な動物に感染することが知られている。季節性の IAV 以外にも、動物の体内で生まれる遺伝子再集合体のパンデミックも問題となる。本研究では、愛玩動物に感染が認められる上記の 2 種類のウイルスを解析し、FHV-1 の新規治療薬の提案と IAV の診断法の確立を目的とし、全 2 章で構成された。

第 1 章　　核酸系抗生物質シネフンギンの in vitro における抗 FHV-1 活性

シネフンギンは、ウイルス・細菌・原虫・真菌など様々な病原体に効果のある核酸

系抗生物質である。そこで我々はいくつかの動物ウイルスに対する抗ウイルス活性を検討した。

猫において主要なウイルスであるネコカリシウイルスと猫伝染性腹膜炎ウイルスとFHV-1、FHV-1と同じバリセロウイルス属である仮性狂犬病ウイルスとウマヘルペスウイルス1を用いたところ、FHV-1にのみ特異的な抗ウイルス活性が認められた。また、この抗ウイルス活性は顕著な細胞毒性なしでシネフンギンの濃度依存的に見られ、ウイルスの侵入後に作用することがわかった。その50%効果濃度（EC_{50}）は、局所投与でFHV-1に対して効果があるとされる抗ヘルペスウイルス薬のアシクロビルと同程度であることが分かった（シネフンギンのEC_{50}：9.5 $\mu g\ ml^{-1}$、アシクロビルのEC_{50}：18 $\mu g\ ml^{-1}$）。

第2章　　飼育フェレットにおけるIAV感染の疫学調査

フェレットはIAV感染に高感受性動物で、症状もヒトの場合と似ていることからモデル動物として用いられている。飼育フェレットにおける自然感染例はいくつか報告されているが、血清疫学調査の報告はない。したがって、ELISAによる抗HA抗体検出系を確立し、抗体保有率を調べた。そうしたところH1亜型に対して79頭中19頭（24.1%）、H3亜型に対して77頭中4頭（5.2%）が抗体陽性であった。しかし、H5及びH7亜型に抗体陽性となる個体はいなかった。次に、中和試験によって中和抗体の検出を試みた結果、H1亜型陽性9検体はすべてH1N1pdmに対する中和抗体陽性となり、飼育フェレットはH1N1pdmに感染していたことが強く示唆された。H3亜型に関してはELISAと中和試験に相違が認められる個体があったが、H3亜型内の抗原性の違いを反映していると考えられた。

また、飼い主がインフルエンザAウイルス陽性と診断され、くしゃみなどの呼吸器症状を呈したフェレット2頭からHA遺伝子とNA遺伝子のほぼ全長の塩基配列を決定した。2頭由来のHA及びNA遺伝子の塩基配列は完全に一致し、HA遺伝子の系統解析の結

果、これらの配列はヒトで流行が見られていたクレード 6B.1 に属していた。したがって、これらのフェレットは、飼い主から IAV に感染したことが強く疑われた。さらに、これらのフェレットのうち 1 頭のスワブからは IAV の分離に成功した。このことは、IAV 感染フェレットが感染性のあるウイルスを排出していることを示唆し、獣医医療関係者への感染リスクとなりえることを表している。

本研究では、FHV-1 や IAV といった愛玩動物に病原性のあるウイルスの治療法や診断法確立に貢献した。シネフンギンの抗 FHV-1 効果の発見のような新規治療薬候補の探求は、他薬剤との併用や類似化合物の開発によるさらなる効果的な治療法の提供を可能にするであろう。また、IAV のようなウイルス感染症の診断法の確立は、動物に対する感染リスクだけではなく、ヒトに対する感染リスクを評価するために非常に重要であるといえる。