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Influenza virus vaccine for neglected hosts: horses and dogs

This study provides information regarding vaccine research and the epidemiology of influenza virus in neglected hosts (horses and dogs). Equine influenza virus (EIV) causes a highly contagious disease in horses and other equids, and outbreaks have occurred worldwide. EIV has resulted in costly damage to the horse industry and has the ability of cross the host species barrier from horses to dogs. Canine influenza is a virus of equine or avian origin and infects companion animals that live in close contact with humans; this results in possible exposure to the seasonal epizootic influenza virus. There have been case reports of genetic reassortment between human and canine influenza viruses, which results in high virulence and the ability of transmission to ferrets. This emphasizes the need for vaccine research on neglected hosts to update knowledge on current strains and to advance technology for controlling influenza outbreaks for public health.

Keywords: Influenza A virus, H3N8 subtype, H3N2 subtype, Disease transmission, Infectious, Influenza vaccine

Introduction

Equine influenza virus (EIV) causes common respiratory diseases in horses, donkeys, and mules. The horse population of North America suffered an epidemic of respiratory illnesses in 1872, recorded as “The Great Epizootic of 1872,” the cause of which was likely influenza A virus [1]. Equine respiratory diseases were first identified through the isolation of influenza A virus from horses in 1956 [2]. To date, two immunologically distinct influenza A viruses have been isolated from horses, specifically H7N7 and H3N8 subtypes [3]. The H7N7 EIV has not been identified in a clinical setting since 1980 [4]. Serological assays showed that the virus continued to circulate in horses in Asia and Eastern Europe in the 1990s, although it is thought to have become extinct in the natural environment. In contrast, H3N8 EIV has become endemic to many countries since its recognition in Florida in 1963 [5]. H3N8 EIV evolved as a single lineage for several decades before splitting into two genetically and antigenically distinct lineages during the 1980s [6]. One is a Eurasian lineage that circulates in Europe, whereas the other is the American lineage, which is further classified into Argentina, Kentucky, and Florida sublineages. The Floridian H3N8 virus has spread to other countries (e.g., China, Japan, and Australia) [7,8].

Dogs were not considered a natural host of the influenza virus until H3N8 canine influenza, which originated from equine influenza, and was identified in racing greyhounds in 2004 [9]. The virus continued to affect racing greyhounds and spread to pet dogs, becoming epidemiologically independent from the EIV and endemic to North America [10]. In Thailand in 2006, a dog died after eating ducks infected with highly pathogenic H5N1 avian influenza virus [11], and previous studies showed that dogs are susceptible to subclinical H5N1 infection [12]. The outbreak was thought to be temporary, because sustained dog-to-dog transmission was not reported. In South Korea in 2007, a large number of dogs suffered from influenza-like illness in kennels and clinics; phylogenetic analysis indicated that the causative agent was H3N2 influenza virus, which originated from birds [13]. Transmission to dogs likely occurred through the animals' consumption of infected poultry near a live animal market. Sustaining virological and serological properties through dog-to-dog transmission [14], the H3N2 canine influenza virus (CIV) spread to China and Thailand [15,16] and became the world's second subtype of CIV. In 2015, over 1,000 cases of the H3N2 canine influenza infection were confirmed spreading to 25 states in the United States. Furthermore, a novel H3N1 CIV resulting from genome reassortment was isolated from a dog co-infected with pandemic H1N1 (pdm H1N1) and canine H3N2 influenza viruses [17] in 2011. This event revealed the possibility that dogs are susceptible to human influenza viruses and suggested that a dog could be a vessel for the recombination of new human influenza viruses.

Molecular Epidemiology of EIV and CIV

EIV has been isolated as two subtypes, H7N7 and H3N8. H7N7, or A/equine/Prague/56, was isolated in 1956 [2] and H3N8 was isolated in 1963 [3] and has been circulating since its discovery. Hemagglutinin (HA) sequence analysis revealed a single lineage that has diverged into two genetically and antigenically distinct lineages [6,18,19]. The first is the Eurasian lineage that is circulating on the European continent, and continues to form a single clade. The other is the American lineage, which comprises the majority of EIV and is distributed as Argentina, Kentucky, and Florida sub-lineages [20]. The Florida strain has evolved into two clades. Florida clade 1 has been isolated in North America since 2003 (A/equine/Wisconsin/1/03) and clade 2 has spread to Europe (A/equine/Newmarket/5/03). In 2007, EIV belonging to the Florida sub-

lineage clade 1 was associated with an outbreak in Australia and Japan, whereas clade 2 was isolated from China, Mongolia, and India during the same period [21]. However, recent phylogenetic analysis showed that the Central Asia EIV belongs to a distinct clade separate from the Florida clade 2, indicating the continuing evolution of EIV [8].

CIV is epizootic and distinguished by two subtypes, H3N8 in North America and H3N2 in East Asia [10,13]. Both CIVs represent monophyletic clades. The H3N8 subtype, which originated from equine H3N8, has shown gene flow, in comparison to Florida isolates obtained between 2003 and 2005; however, these viruses maintain a common ancestor [22]. Since its first report in South Korea in 2007 [13], neighboring countries have continued to report outbreaks of H3N2. In China, phylogenetic analysis of the recently isolated A/canine/Liaoning/27/2012 and A/canine/Liaoning/H6/2012 strains indicated their close relationship to avian-origin H3N2 subtype CIVs, derived from the same ancestor of the classic Korean CIV [23]. The A/canine/Thailand/CU-DC5299/12 isolate also belongs to the same clade as the classic Korean CIV [16]. Thus, the two CIV subtypes, H3N8 of North America and H3N2 of Korea, have not diverged into sub-lineages.

Pathogenesis of EIV and CIV

EIV and CIV can be primary pathogens and cause respiratory disease by themselves. However, infection by the virus can lead to synergistic pathogenesis when co-infection occurs with prevalent respiratory pathogens such as equine herpesviruses and *Streptococcus equi zooepidemicus* in horse, and *Bordetella bronchiseptica* in dogs. Within 1-3 days of infection, symptoms develop, including fever (up to 41°C), depression, anorexia, and weakness, which clinically differentiates influenza from other viral infections of the upper respiratory tract. Local respiratory symptoms can also occur, such as serous nasal discharge, submandibular lymphadenopathy, and a dry, harsh, nonproductive cough. Cough develops early during the course of infection and may persist for several weeks. Nasal discharge, although scant and serous initially, can become mucopurulent due to secondary bacterial infection [24]. Clinically, primary influenza viral pneumonia begins like typical uncomplicated influenza disease in the upper respiratory tract, but the acute illness rapidly progresses with signs of lower respiratory tract disease, including cough, dyspnea, and hypoxemia. The respiratory tract epithelium takes 3 weeks to recover, and thus mammals are susceptible to sec-

ondary bacterial complications such as pneumonia, pleuropneumonia, chronic bronchitis, recrudescence of fever, dyspnea, and cough with sputum, which signals the onset of bacterial pneumonia [25].

Influenza A virus commonly induces a cascade of immune responses. Viral proteins evoke immune responses by eliciting production of antibodies and activation of T-lymphocytes. The HA and neuraminidase (NA) surface proteins induce neutralizing antibodies through which CD4 T lymphocytes help B lymphocytes to generate anti-HA and anti-NA antibodies that mediate protective immunity [26]. Anti-HA antibody production is the first line of defense, preventing viral attachment, whereas anti-NA antibodies inhibit the enzymatic activity of NA to reduce viral budding. At the onset of infection, the innate immune response is activated, resulting in the release of cytokines and recruitment of granulocytes to the site of infection; at the same time, antigen-specific humoral and cellular adaptive immune responses are initiated and begin to take effect after a few days. To overcome host defenses, the influenza virus expresses the non-structural proteins NS1 and 2, which are generally believed to sequester viral dsRNA to inactivate the protein kinase R, thereby antagonizing the host interferon (IFN α/β) system [27].

Transmission of EIV into Dogs

Influenza A virus can be transmitted from one species to another, causing multiple viral genome reassortments to occur [28-30]. Previous studies have shown that the H3N8 subtype was introduced into horses more than 40 years ago; since then, there has been very little genetic exchange between this subtype and viruses from other species [31], suggesting horses were an endpoint until the H3N8 CIV was identified as being of equine origin.

H3N8 CIV was first isolated from racing greyhounds that had died of hemorrhagic pneumonia; the dogs had been kept near H3N8 EIV-infected horses in Florida, in January of 2004 [9]. EIV was identified as the cause of the outbreak upon isolation from a dead host, and EIV-specific antibodies were found in other dogs. Sequence and phylogenetic analyses revealed that the virus was highly homologous to the H3N8 EIV, which was in circulation at that time. The transmitted H3N8 EIV evolved into the H3N8 CIV, which spread among pet dogs and became enzootic to the United States. The H3N8 CIVs seem to form a distinct monophyletic group, although the eight genes of CIV share 96% sequence identity with those of

the 2002 and 2003 Florida sub-lineages of equine influenza [10]. In the U.K., interspecies transmission of H3N8 influenza from horses to dogs was revealed through serology and immunohistochemistry during a respiratory outbreak among English foxhounds in 2002 [32]. In an Australian outbreak of H3N8 equine influenza in 2007, several dogs presented influenza-like symptoms, and H3N8 CIV was identified [33].

Emerging Reassortment of CIV

Dogs are susceptible to a variety of influenza viruses. The first case of interspecies transmission in dogs was the incidence of EIV in racing greyhounds in 2004, in Florida [9]. Avian influenza virus was transmitted to dogs in Asia when the highly pathogenic H5N1 avian influenza virus was transmitted to dogs that ingested infected duck carcasses [34]. This was reproducible through experimental infection of dogs with HPAI H5N1, thus demonstrating that dogs are susceptible to H5N1 subclinical infection [12,35]. In South Korea, H3N2 viruses were isolated from dogs with severe respiratory disease symptoms. The isolates shared high nucleotide sequence identity with the H3N2 avian influenza virus, suggesting that the viruses were transmitted directly from infected birds to dogs through consumption of infected poultry products or through aerosol transmission [13]. Recent evidence has identified pdm H1N1 and seasonal human H3N2 influenza virus infections in dogs in Korean and China, which was demonstrated by experimental inoculations and serological evidence of pdm H1N1 infection alone and in combination with canine H3N2 infection [36,37]. Furthermore, routine surveillance revealed another reassortant H3N2 CIV carrying the M gene of the pdm H1N1 virus in South Korea; this virus was transmissible to ferrets and produced symptoms typical of respiratory disease [38]. Thus, dogs are susceptible to a variety of influenza viruses, including equine, avian, and human influenza A. These cases raise concerns that dogs could play a role in virus adaptation and genome mixing before spread to other species.

Purpose of CIV and EIV Vaccination

Dogs are a primary companion animal that can be infected with human influenza viruses owing to their ecophysiological characteristics and the fact that they possess receptors of both avian and human influenza viruses, SAa 2,3-gal and SAa 2,6-gal, respectively [13]. Dogs live in close contact with humans, and thus there are many opportunities for contact with

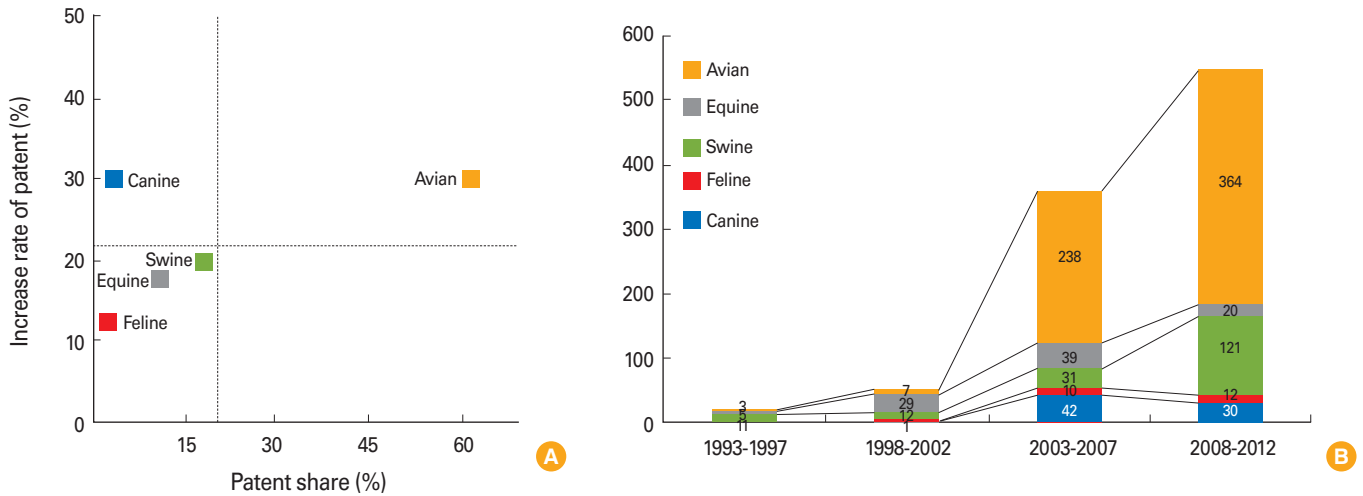


Fig. 1. Increased number influenza vaccine patents according to each animal species from 1993 to 2012. Patents for canine influenza virus occupied less than 5% of major veterinary influenza vaccines (A), and showed the highest rate of increase from 2002 (B). Equine influenza virus vaccines presented a downward trend from 2007 (B).

seasonal epizootic influenza viruses. This poses an important public health concern, because pre-existing CIV may recombine or reassort with human influenza viruses and give rise to novel viruses that could in turn lead to a unique pandemic. Recently, 23 distinct viral genotypes of influenza reassortants were isolated from a dog co-infected with pdm H1N1 and H3N2 CIV. Reassorted CIVs presented higher virulence in mice compared to that with classical H3N2 CIV and were even transmitted to ferrets by contact exposure [39]. These findings suggest that dogs might support genome mixing or serve as a source of novel influenza A viruses in humans. In this sense, vaccination and intensive monitoring of influenza infections in companion animals are necessary to control the potential for emergence of novel human influenza strains.

EIVs are capable of binding to receptors on canine respiratory epithelial cells [32], and H3N8 EIVs of American lineage could indeed be transmitted to dogs, after which mutated H3N8 CIVs were created [9,32,33]. In addition, it was experimentally confirmed that dogs exhibited seroconversion and viral shedding through close contact with EIV infected horses [40]. If EIV circulates in the horse population and transmits to dogs that are susceptible to human influenza viruses, there is a possibility that a co-infection with the EIV, CIV, and human influenza virus could occur in dogs, which would generate a new triple reassortant virus comprised of EIV, CIV, and human influenza virus genes. This emphasizes the need for horses to be vaccinated with updated circulating EIV strains to control mutant influenza viruses and secure public health.

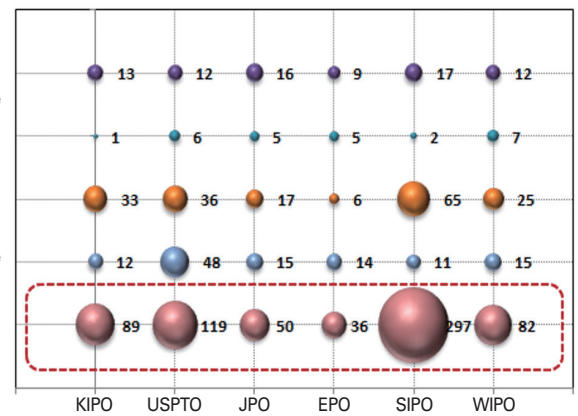


Fig. 2. Ratio of patents for influenza vaccine technology according to each animal species in six major intellectual property offices. United States leads in applications for patents for equine influenza virus vaccines. China and Japan had a high number of patents for equine influenza virus vaccines. KIPO, Korean Intellectual Property Office; USPTO, United States Patent and Trademark Office; JPO, Japan Patent Office; EPO, European Patent Office; SIPO, State Intellectual Property Office; WIPO, The World Intellectual Property Organization.

Status of Vaccine Research

Although EIV vaccines have a comparatively low market share, which is on a downward trend (Fig. 1A, B), the EIV vaccine market was estimated to be \$11.7 million in 2012 and is expected to reach \$20.5 million by 2018 at a compound annual growth rate of 9.2% from 2013 to 2018. Among the major countries that develop the EIV vaccine, United States leads the market with 48 patents (42%), followed by Japan (13%), E.U. (12%), Korea (10%), and China (10%) (Fig. 2). Currently, the Univer-

Table 1. Current status of vaccine development for horses and dogs

Species	Company	Technology	Vaccine	Antigens	Vaccine strain
Dogs	MERCK	Whole inactivated sub-unit	Nobivac Canine Flu H3N8	Whole virus	Not assigned
	Zoetis		VANGUARD CIV H3N8	Whole virus	A/canine/Iowa/9A1/B5/08/D12 (H3N8)
	Zoetis MERCK		Canine influenza vaccine, H3N2 (conditionally licensed)	Whole virus	A/canine/Illinois/12191/2015 (H3N2)
Horses	Elanco	Whole inactivated sub-unit	Duvaxyn IE Plus	Whole virus	Newmarket/1/93 (H3N8) Suffolk/89 (H3N8) Prague/56 (H7N7)
	Boehringer Ingelheim Animal Health		Calvenza-03 EIV	Whole virus	Newmarket/2/93 (H3N8) Kentucky/2/95 (H3N8) Ohio/03 (H3N8)
	MSD Animal Health		Equilis Prequenza (updated 2013)	Whole virus	Newmarket/2/93 (H3N8) South Africa/4/03 (H3N8)
	MSD Animal Health	Modified live EIV	Equilis Prequenza	Sub-unit HA	Prague/56 (H7N7) Newmarket/1/93 (H3N8) Newmarket/2/93 (H3N8)
	Pfizer Ltd.		Equip F	Sub-unit mainly HA and NA	Newmarket/77 (H7N7) Borlänge/91 (H3N8) Kentucky/98 (H3N8)
	Intervet/Schering-Plough Animal Health (USA)		Flu Avert I.N	Whole virus	Attenuated, cold adapted EIV: Kentucky/91 (H3N8)
	Merial Animal Health Ltd.	Viral-vector based	PROTEQ FLU	HA	Ohio/03 (H3N8) Newmarket/2/93 (H3N8)
	Merial Animal Health Ltd.		PROTEQ FLU (updated 2014)	HA	Ohio/03 (H3N8) Richmond/1/07 (H3N8)

Modified from Paillot R. *Vaccines (Basel)* 2014;2:797-831 [41], according to the Creative Commons License. EIV, equine influenza virus; HA, hemagglutinin; NA, neuraminidase.

sity of Pittsburgh is concentrating on applying for an EIV vaccine patent.

Commercialized EIV vaccines are classified into three different groups according to their main technology, including whole inactivated subunit, live attenuated, and viral vector based (Table 1) [41]. Whole inactivated and subunit EIV vaccines were first developed and have been the most widely used in recent decades. These vaccines are produced using eggs or cell culture followed by chemical inactivation. Live attenuated vaccines are beneficial in terms of immunization because they stimulate a long lasting immune response. However, this type of vaccine is associated with the risk that it can infect the host or another person because it retains immunogenicity. Current commercialized EIV vaccines have been successfully tested for efficacy and there have been no reports that commercialized EIV vaccines cause infection [42]. Some vaccines use reverse genetics technology to make recombinant EIV viruses. For example, infection of recombinant EIV with a truncated NS1 gene shows no clinical sign of disease and produces only a small amount of virus shedding. This re-

sult supports the promising development of genetically manipulated vaccines. The canarypox vector vaccine uses recombinant canarypoxvirus, which contains a segment of an EIV gene (HA) that is expressed after injection. Because avian poxvirus undergoes an incomplete replication in mammalian cells, canarypoxvirus is a safe vector for EIV vaccination. This vaccine shows significantly reduced clinical signs and virus shedding during early onset [43]. The characterization of rapid onset immunization could prove that this vaccine is highly useful in an emergency. In addition, this vaccine shows long-term protection based on a strong antibody response and the secondary response of IFN γ and interleukin 2 stimulation [44].

According to shares of influenza vaccine patents and their increasing rate, Pfizer Inc. concentrates the most on CIV vaccine development (Fig. 3). Currently, there are two commercialized H3N8 CIV vaccines and one H3N2 CIV vaccine is conditionally approved by the U.S. Department of Agriculture, which has been supplied to veterinarians to help address outbreaks. It is not clear whether the H3N8 CIV vaccine protects

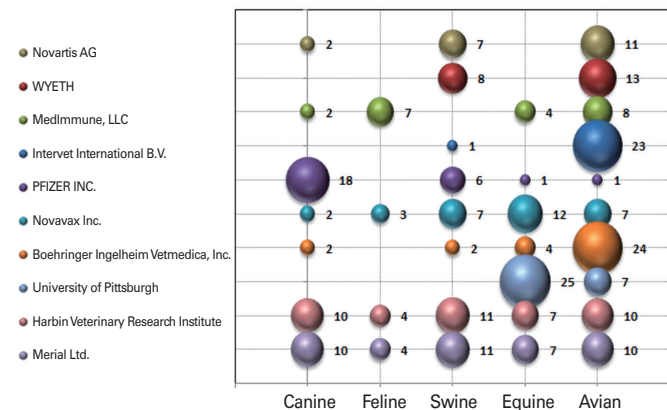


Fig. 3. Distribution of patent applicants of animal influenza vaccines. Merial Ltd. presented balanced patent applications for all veterinary species. The University of Pittsburgh and Pfizer Inc. applied for the highest number of patents for equine influenza virus and canine influenza virus vaccines, respectively.

against heterologous H3N2 CIV; however, trials demonstrated that it reduced the severity and duration of clinical illness, and abbreviated the shedding viral load [45,46]. Vaccination of H3N2 CIV is highly recommended, because the H3N2 CIV can spread by direct contact with respiratory discharge from infected dogs, and produce 10 times more virus than H3N8 CIV, making it more contagious [47]. An inactivated whole H3N2 CIV vaccine was developed in South Korea for the first time using A/canine/Korea/01/07H3N2 as a vaccine strain, and this vaccine was highly efficient in relieving clinical symptoms and decreasing viral shedding [48]. Further studies of H3N2 CIV vaccines are needed to elucidate vaccine efficacy and to determine if they provide any cross-protection against H3N8 CIV.

Conclusion

Equine and canine influenza cause highly infectious diseases that can spread worldwide, and EIV is capable of inter-species transmission from horses to dogs. This evokes major concern for public health and for controlling influenza virus outbreaks. In addition, dogs have closer contact with humans than pigs do, and are thus exposed to human influenza viruses such as pdm H1N1 and seasonal H3N2. Recent studies have identified seropositive samples against human influenza viruses in historical canine sera [37], and these viruses can transmit through contact exposure causing pneumonia and nasal viral shedding. This implies that vaccination of dogs is necessary not only with CIV H3N2, but also with human influenza virus

strains. Furthermore, same vaccines should be considered in horses to protect these animals from possible human influenza virus infection.

Vaccination is the top priority to prevent EIV and CIV; however, continuous antigenic variation causes mismatch between vaccine and outbreak influenza virus strains, as current commercialized vaccines are manufactured mainly with inactivated whole virus or subunit HA proteins. In this sense, active surveillance studies are essential to update vaccine strains according to the evolution of EIV and CIV. In addition, promising vaccine technologies, developed based on human research, must be applied for EIV and CIV vaccine design. In addition, evaluation of the efficacy and safety of virus-like particles, DNA vaccines, and modified live vaccines should be considered, as these are the next generation of vaccine types.

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