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ORIGINAL PAPER

A highly virulent canine distemper virus strain isolated from vaccinated mink in China

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Abstract

An outbreak of canine distemper in 2017 in mink breeding farms (Shandong province, China) caused severe pneumonia, hardened footpads, and death in more than 5000 vaccinated animals. Sequencing of the hemagglutinin and fusion protein genes from the WH2 canine distemper virus (CDV) strain we isolated from the infected minks were clustered into the recently isolated CDV Asia-1 genotype group. The WH2 strain was distinct from the current vaccine strains, containing a novel potential N-glycosylation site in its hemagglutinin protein. It also contained amino acid mutations in the fusion protein gene (I87N, T110P and L386I), and the T110P mutation results in N-glycosylation site silencing. WH2 was highly virulent in both unvaccinated and vaccinated animals in our pathogenesis experiments. Immunohistochemistry results revealed positive staining of different organs in unvaccinated and vaccinated animals. The serum in vitro neutralizing antibody titers for the vaccinated mink group and a dog were higher for the WH2 strain than those of the HNly150520B strain (isolated from a dog). These findings indicate that the current commercial vaccines provide incomplete protection against WH2 challenge infections. Thus, a new vaccine strain is urgently needed to protect against variant CDV strains.

Keywords Canine distemper virus · Pathogenesis · Virulence · N-glycosylation

Introduction

Canine distemper virus (CDV), an enveloped, non-segmented negative sense, single-stranded RNA virus, is a *Morbillivirus* genus member of the *Paramyxoviridae* family, which includes measles virus, peste des petits ruminants virus, and rinderpest virus [1–3]. CDV causes a fatal, highly infectious, multi-systemic disease in wild and domestic *Canidae* such as dogs, mink, and foxes [4, 5]. However, over the last three decades, natural infections with CDV have been reported in other animal species [6, 7]. Although CDV is mainly transmitted by respiratory excretions (e.g., aerosols from the upper respiratory tracts of infected individuals), urine and feces are also infectious. The clinical signs in different species include respiratory infection, oculonasal discharge, vomiting, fever, depression, foot pad hyperkeratosis, neurological complications, and even death [7]. Live attenuated vaccines have reduced the incidence of CDV infections. However, sporadic cases and large outbreaks of canine distemper in vaccinated dogs, mink, foxes, and raccoon dogs have been reported worldwide [8–12]. Antigenic differences in the hemagglutinin (H) protein between field-isolated and vaccine strains, which are responsible for virus neutralization, play a key role in immune response and possibly lead to vaccine failure [13].

CDV outbreaks with high mortality rates in vaccinated fur animal breeding farms have emerged in China. The first major outbreak in vaccinated breeding farms occurred between 2011–2013 in Shandong, Liaoning, Hebei, and Heilongjiang provinces of North China [8]. Frequent CDV outbreaks in 2017 occurred in vaccinated mink on breeding farms in Shandong Province, China. Approximately, 10,000

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minks (30–60% morbidity) in these outbreaks displayed the typical clinical signs of canine distemper, such as ocular discharge, appetite loss, and hardened footpads. The severity of the hardened footpads rendered some animals unable to stand. About 30–70% of the infected animals died from severe pneumonia and anorexia. All the minks, however, had been vaccinated with the Snyder Hill or Onderstepoort CDV vaccine strain two months before the outbreak. The CDV WH2 strain was isolated from moribund minks that showed the clinical signs of ocular discharge, anorexia, and hardened footpads. Herein, the pathogenicity and tissue distribution of the WH2 strain in vaccinated and unvaccinated mink groups after virus challenge were analyzed.

Materials and methods

Cells, viruses and sera

Vero/DogSLAM cells [14] and Chinese hamster ovary (CHO) cells were grown in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, USA) with 2% fetal bovine serum (Gibco). The CDV HNly150520B strain was isolated from a dog [14]. The dog serum samples collected were from dogs vaccinated with versican plus DHPPi (Zoetis Inc., Parsippany, NJ, USA) at a pet hospital.

Virus isolation and identification

Lung samples were obtained from vaccinated mink from a Shandong Province farm. Vero/DogSLAM cell monolayers were infected with CDV lung homogenates until a cytopathic effect (CPE) was observed. The isolated virus was identified by an indirect immunofluorescence assay. The Vero/DogSLAM cells were fixed with acetone for 1 h. After washing with phosphate-buffered saline three times, the cells were incubated with anti-CDV-N antibody (6D9) for 1 h before staining with FITC-conjugated anti-mouse IgG (Sigma) [14]. Fluorescence was observed with a fluorescent microscope (Olympus, Tokyo, Japan). The isolated virus was designated WH2.

Plasmids

Total RNA from WH2 and HNly150520B strains was extracted and reverse-transcribed using super script III reverse transcriptase (Invitrogen, USA) to generate cDNA. Hemagglutinin (H) and fusion (F) genes from both strains were amplified by polymerase chain reaction (PCR) using previously described primers [14]. Their amplicons were inserted into the pCAGGS mammalian expression vector to generate pCAG-WH2F, pCAG-HNly150520BF, pCAG-WH2H and pCAG-HNly150520BH plasmids. The pCAG-Dog signaling lymphocyte activation molecule (SLAM) was constructed as previously described [15].

Viral growth kinetics

The CDV WH2 strain's growth kinetics were assessed and compared with those of the HNly150520B strain. Vero/DogSLAM cells were infected with either virus at a multiplicity of infection (MOI) of 0.01. Cells and supernatants were harvested at 6, 12, 24, 36, 48, 72, 96 and 120 h after infection (hpi) and stored at − 80 °C. The 50% tissue culture infectious dose ($TCID_{50}$) was used to calculate the amount of infectious virus. The growth kinetics of each virus were tested in triplicate and the resulting titers were averaged.

Sequencing and phylogenetic analysis of the WH2 strain

Total RNA from the WH2 strain was extracted using the Viral Nucleic Acid Extraction Kit II (Geneaid, China) in accordance with the manufacturer's instructions. Fifteen overlapping fragments covering the whole viral genome were amplified using the primers shown in Supplementary Table 1. The amplified segments were cloned into the pEASY-Blunt vector (TransGen Biotech, China) in accordance with the manufacturer's instructions and then sequenced (GENEWIZ, China). The phylogenetic and molecular analyses were evaluated using the neighbor-joining method in MEGA 5.10 [16].

Fusion experiments

Six different CHO cell groups were transfected with pCAG plasmids harboring the F gene and the H gene in various combinations. Group 1 CHO cells were transfected with pCAG-DogSLAM, pCAG-WH2F and pCAG-WH2H plasmids, which contain the SLAM gene, F gene and H gene from the CDV WH2 strain. As controls, CHO cells were transfected with pCAG-DogSLAM, pCAG-WH2F or pCAG-WH2H in groups 2 and 3. Similarly, CHO cells in groups 4–6 were transfected with the same or singular combinations of the above plasmids containing the corresponding genes from the CDV HNly150520B strain.

Animals and experimental design

Fifteen 5-month-old minks (free of CDV and mink enteritis virus, and antibody negative for CDV) were randomly divided into 3 groups ($n = 5$). To evaluate the virulence of the WH2 strain, group 1 animals were vaccinated with the commercial vaccine, and animals from groups 2 and 3 received Dulbecco's modified Eagle medium as the placebo. At 21 days post-vaccination (dpv), minks in groups 1 and 2

were challenged intranasally with the WH2 strain, and group 3 minks were the unchallenged controls. Their clinical signs and rectal temperatures were recorded daily until 21 days post-infection (dpi). All animals were humanely euthanized before autopsying.

Histology and immunohistochemistry

Lung, liver, cerebrum, spleen, tonsil, kidneys, intestinal tract, pancreas, submandibular lymph nodes and mesenteric lymph node tissue samples were collected from all the minks. The samples were fixed with 10% formaldehyde, processed into paraffin blocks, and cut into sections. The sections were stained with hematoxylin and eosin. CDV antigen in the infected minks' tissues was detected using a immunohistochemistry assay with an anti-CDV-N antibody (6D9), as previously described [14].

Neutralizing antibody measurements

Serum samples from the minks and dogs were collected from vaccinated animals and the neutralizing titers were tested using Vero/DogSLAM cells. Briefly, the twofold serial serum dilutions (starting from 1:2) added to 96-well microtiter plates were mixed with 10^2 $TCID_{50}$ units of CDV virus (HNly150520B strain or WH2 strain). After incubation (37 °C, 1 h), 2×10^4 Vero/DogSLAM cells were added. The plates were reincubated at 37 °C in a humidified atmosphere containing 5% CO_2 for 5–6 days. The Reed-Muench method was used for titer calculation.

Results

Virus isolation

Visually striking CPE could be observed at 30 hpi in the form of large syncytia in lung homogenate supernatants from the CDV-infected mink samples cultured in Vero/DogSLAM cells (Supplementary Fig. 1a). The CDV strain causing this effect was isolated and named WH2. As shown in Supplementary Fig. 1c, specific immunofluorescent signals were visualized in the WH2-infected cells but not in the mock-infected cells.

Sequence analysis of viral H and F genes and the complete genome of the WH2 strain

The complete WH2 genome, which is 15,246 nucleotides (nt) long, shares 97.1–99.4% nucleotide identity with other Asia-1 genotype strains, and 92.2% nucleotide identity with the vaccine strains (Snyder Hill, Onderstepoort, CDV-3). As shown in Fig. 1, WH2 clusters within the branch of isolates from fur animals (mink, fox and raccoon dog).

The WH2 strain's H gene was found to be 1,824 nt long and encode a 607 amino acid (aa) protein. As shown in Fig. 2a, the WH2 strain clusters into the Asia-1 genotype branch and shares 97.3–99.6% nucleotide identity with its members. WH2, however, shares 90.4–90.8% nucleotide identity and 89.5–90.6% aa identity with the vaccine strains (Snyder Hill, Onderstepoort, Lederle, Convac, CDV-3). While the aa sequence of the H gene encoded by the WH2 strain contains 10 potential N-glycosylation sites (Supplementary table 2), the protein contains a novel potential N-glycosylation site (aa 542–544) only found in the CDV isolated from vaccinated foxes, minks, and raccoon dogs in the Chinese disease outbreaks after 2011 [8]. Contrastingly, the Onderstepoort, Snyder Hill, Convac, and Lederle vaccine strains encode 4, 7, 7, and 6 potential N-glycosylation sites in the H protein, respectively (Supplementary Table 2).

We found that the F gene from the WH2 strain, which is 1966 nt long and encodes 662 amino acids, clusters within the Asia-1 genotype branch, and shares 96.7–99.0% nucleotide identity and 96.4–98.9% aa identity with its branch members. It also shares 90.4–90.7% and 89.6–90.2% nucleotide identity and aa identity, respectively, with the vaccine strains (Fig. 2b). The F protein from WH2 also contains amino acid mutations (I87N, T110P and L386I) that are missing in the other isolates.

Growth properties of WH2

The growth properties of WH2 and HNly150520B were compared in Vero/DogSLAM cells. As shown in Supplementary Fig. 2, WH2 and HNly150520B display similar viral replication kinetics in the cell supernatants and cell debris.

Cell–cell fusion efficiency in a transient-expression assay

To investigate the fusion efficiency of the WH2 strain, CHO cells were transfected with different recombinant plasmids. As shown in Supplementary Fig. 3, CHO cells transfected with pCAG-DogSLAM, pCAG-WH2F and pCAG-WH2H plasmids displayed a striking CPE in the form of large syncytia. As with the WH2 strain, a similar CPE was also observed in CHO cells expressing the corresponding proteins from the HNly150520B strain. The fusion efficiency was virtually identical between the two CDV strains. As expected, the CHO cells co-transfected with the other plasmids lacked syncytia.

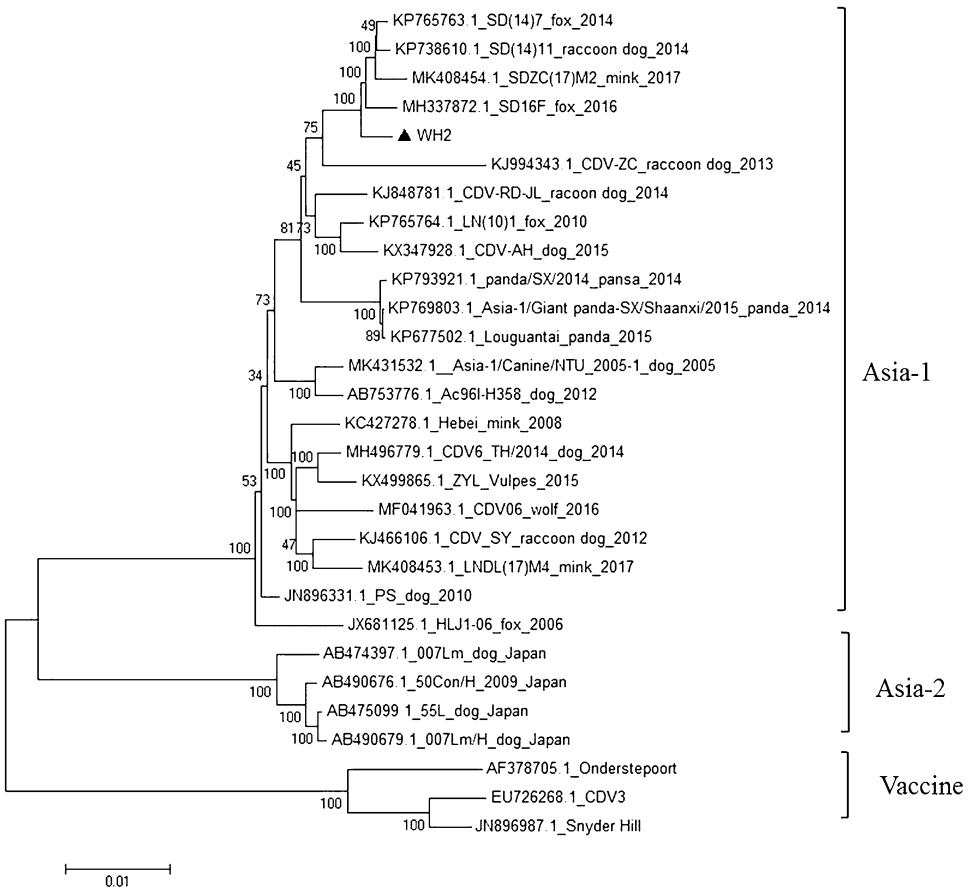

Fig. 1 Phylogenetic tree of the WH2 strain CDV and other CDV strains based on the full-length genome. The phylogenetic tree was constructed using a distance-based neighbor-joining method with 1000 bootstrap replicates in MEGA6. Numbers along the branches are bootstrap values. The black triangle denotes the WH2 strain. Scale bar indicates nucleotide substitution per site

Clinical presentations

Five unvaccinated minks infected with WH2 developed fever from 3 dpi. Three vaccinated minks also developed fever but had a reduced fever period (Fig. 3, Supplementary Fig. 4). As expected, the temperatures of the control animals were normal. Clinical signs including ocular discharge, dry snout, appetite loss, and hardened footpads (hyperkeratosis) were observed in the unvaccinated mink group post challenge, whereas only two animals in the vaccinated group displayed clinical signs. Clinical signs in the unvaccinated animals were severer than those of their vaccinated counterparts. The hardened footpads of the group 2 minks were similar to those of the dead mink from breeding farms in Shandong Province (Fig. 4). Two minks in the vaccinated group behaved as normal. One dying mink in the unvaccinated group was humanely euthanized and the other minks survived the study's endpoint.

Gross pathology assessments and histopathological assay

Histopathology and immunohistochemistry were performed after virus challenge. As shown in Fig. 5, lymphocyte, macrophage and mononuclear cell infiltrates and eosinophilic inclusion bodies were observed in the lungs, and eosinophilic inclusion bodies were observed in the bronchiolar epithelial cells and alveolar epithelial cells from the vaccinated mink group. Eosinophilic inclusion bodies and fatty degeneration were found in the kidneys. By contrast, pathological changes were less common and severe in the vaccinated groups than in the unvaccinated group. No gross pathological changes occurred in the control group minks. The immunohistochemistry results showed strong positive staining in the tonsils, submandibular lymph nodes, mesenteric lymph glands, lungs, spleen, liver, kidneys, intestinal tract, pancreas and cerebrum from the unvaccinated mink group (Fig. 6). The vaccinated group showed weaker positive staining of these tissues than the unvaccinated group. The tissue staining intensities in the different mink animals is summarized in Supplementary Table 3. No positive staining was detected in the mock controls.

In vitro neutralizing antibody titers

The CDV neutralizing titers in the mink and dog sera at 21 dpv were determined for HNly150520B and WH2 strains.

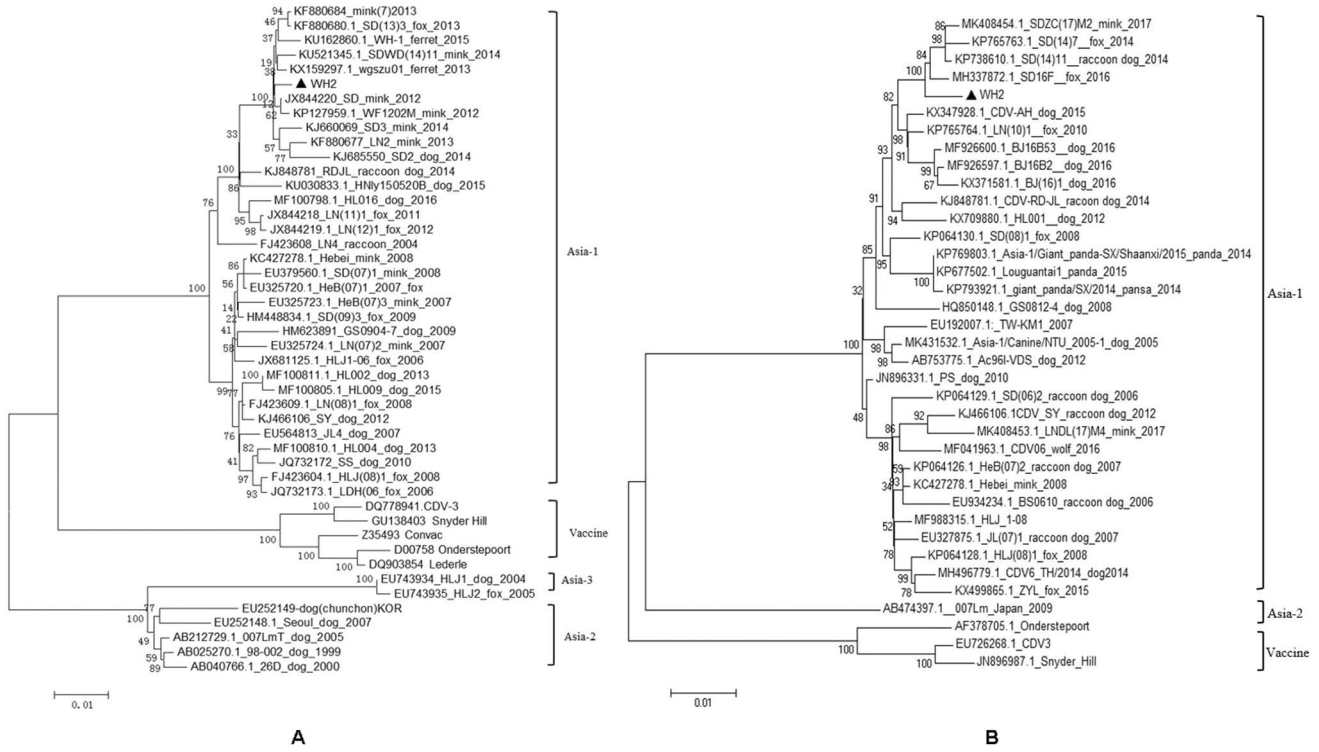

Fig. 2 Phylogenetic tree of WH2 strain CDV and other reported viruses based on full-length sequence of H gene and F gene. The phylogenetic tree of H gene (**a**) and F gene (**b**) was constructed using a distance-based neighbor-joining method with 1000 bootstrap replicates in MEGA6. Numbers along the branches are bootstrap values. The black triangle denotes the WH2 strain. Scale bar indicates nucleotide substitution per site

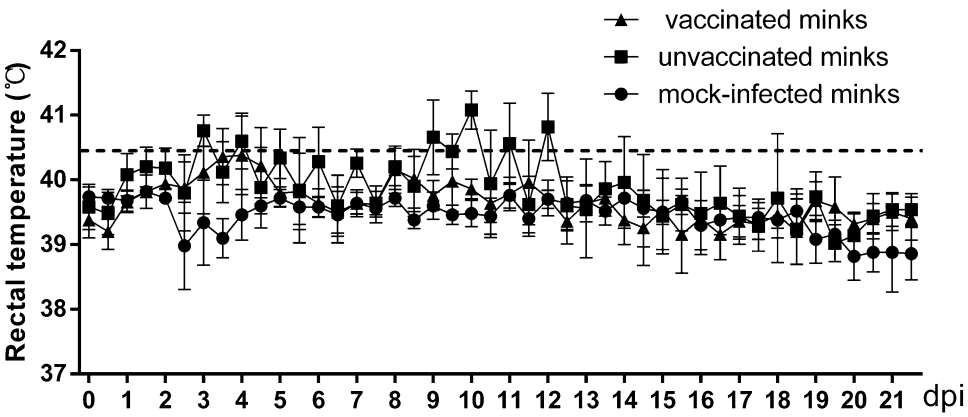

Fig. 3 Body temperature of minks infected with the WH2 isolate. Body temperature of vaccinated minks and unvaccinated minks after challenge with the WH2 strain and mock-infected minks. Clinical fever was defined as body temperature 40.5 °C or higher

As shown in Supplementary Fig. 5, the mink serum titers against strain WH2 ranged from 1:15.8 to 1:128.8 (average titer, 1:58.1). Contrastingly, the titers against the HNly150520B ranged from 1:51.3 to 1:182 (average titer, 1:115.6). The titers against the WH2 strain in dogs ranged from 1:20 to 1:40.7 (average titer, 1:29.6), whereas those against the HNly150520B strain ranged from 1:64.6 to 1:182 (average titer, 1:119.1).

Discussion

Despite the vaccination procedures adopted in China over the last decades, CDV remains a serious threat to wild animals and domestic dogs, and is responsible for significant economic losses in the fur industry. CDV infections were previously found in animals that had been vaccinated with CDV-3 or Onderstepoort CDV vaccines in China's

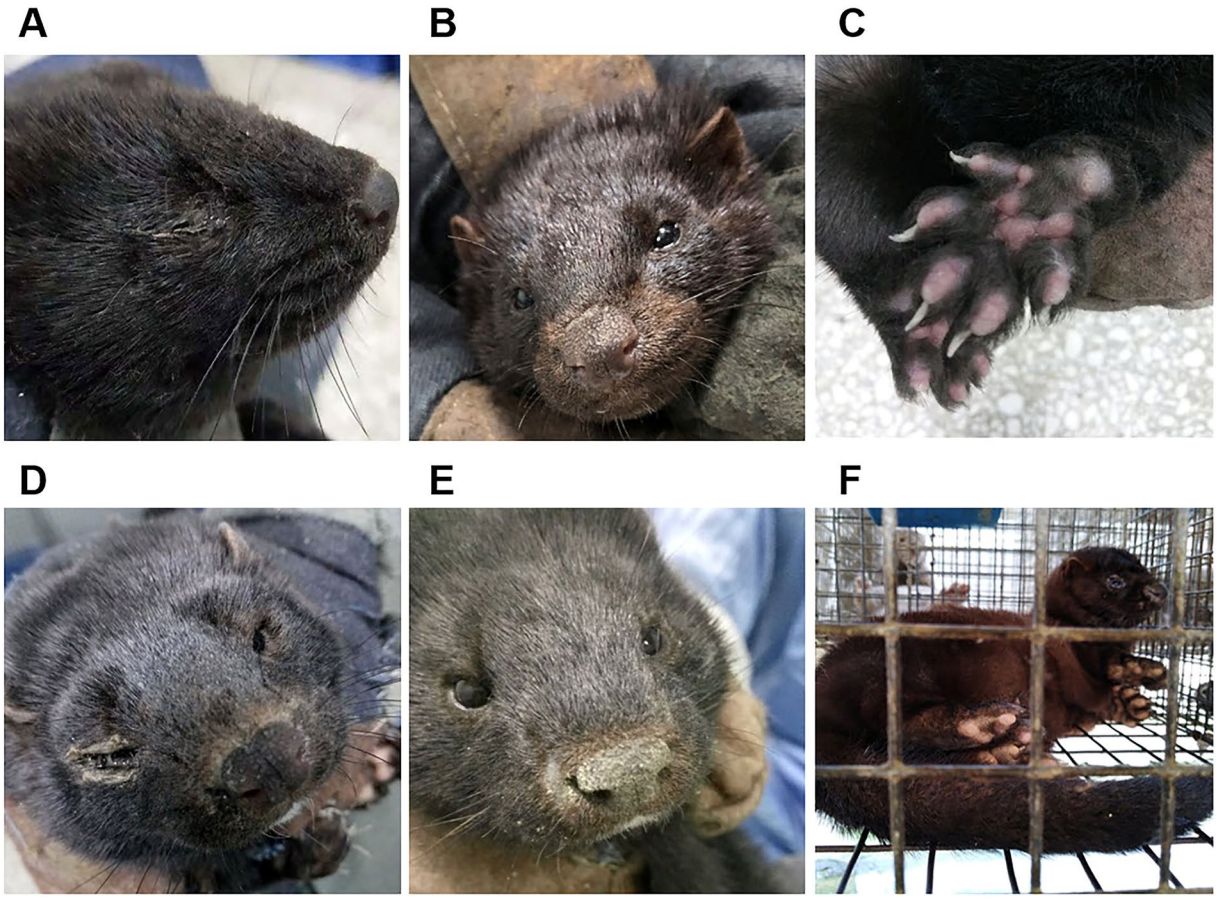

Fig. 4 Clinical signs in WH2-challenged minks. Vaccinated (**a**, **b**) and unvaccinated (**c**, **d**, **e**) minks after challenge with the WH2 CDV isolate and one CDV case **(f)** from a mink farm. **a**, **d** Ocular discharge. **b**, **e** Nasal mirror drying. **c** Hardened footpad. **f** Hardened footpads of a CDV infection case mink from a farm in Shandong Province (The mink could not stand due to severely affected footpads)

Northeast region from 2011 to 2013. Mink breeding farms in Shandong Province that were recently found to contain infected animals with CDV experienced high mortality (30–70%). Subsequently, the WH2 CDV strain was isolated from the lungs of dead mink. The WH2 H gene shares 97.1 to ~ 99.6% sequence identity with the strains isolated from Northeast China between 2011 and 2013; hence, this gene differs from those in the viruses isolated in Northeast China during that time. However, one study reported that minks remain sufficiently protected from challenge with the virulent CDV LNM strain (at 100 ID50) [17], possibly because the challenge strain differs from the wild-type strain. The CDV infection characteristics in the vaccinated mink group indicate that the current commercial vaccines cannot provide complete protection to the circulating viral strains in the field.

There are many reasons for immune failure after vaccination, such as a poor immune response, maternal antibody interference, vaccine quality, and genetic diversity in non-vaccine strains. Genetic diversity in the viral population, which can be a leading factor, can generate more pathogenic variants [18] and the abovementioned factors may explain why the CDV outbreak occurred. Phylogenetic analysis of the CDV H gene, a key CDV protein, is regularly used for investigating geographically distinct lineages of CDV strains [13, 18–21]. The WH2 strain, which clusters with the Asia-1 genotype, appears to dominate the Chinese territories and infect different animal species [19]. The WH2 strain contains 10 potential glycosylation sites, whereas the glycosylation sites in the Onderstepoort, Lederle, Snyder Hill, and Convac vaccine strains contain 4, 6, 7, and 7, respectively. Other studies have reported on differences in the H gene between the wild-type CDVs and the vaccine strains [22–26]. Antigenic epitopes, which are shielded by sugar moieties in the wild-type strains and contain more potential glycosylation sites, may create the vaccine escape mechanisms that may hinder neutralizing antibody binding to the H protein [8]. Accordingly, circulation of the 10 potential N-glycosylation site-containing WH2 strain might explain why CDV vaccines have failed. Thus, a new vaccine to control the recently prevalent CDV strains is urgently needed. Such strains could be attenuated

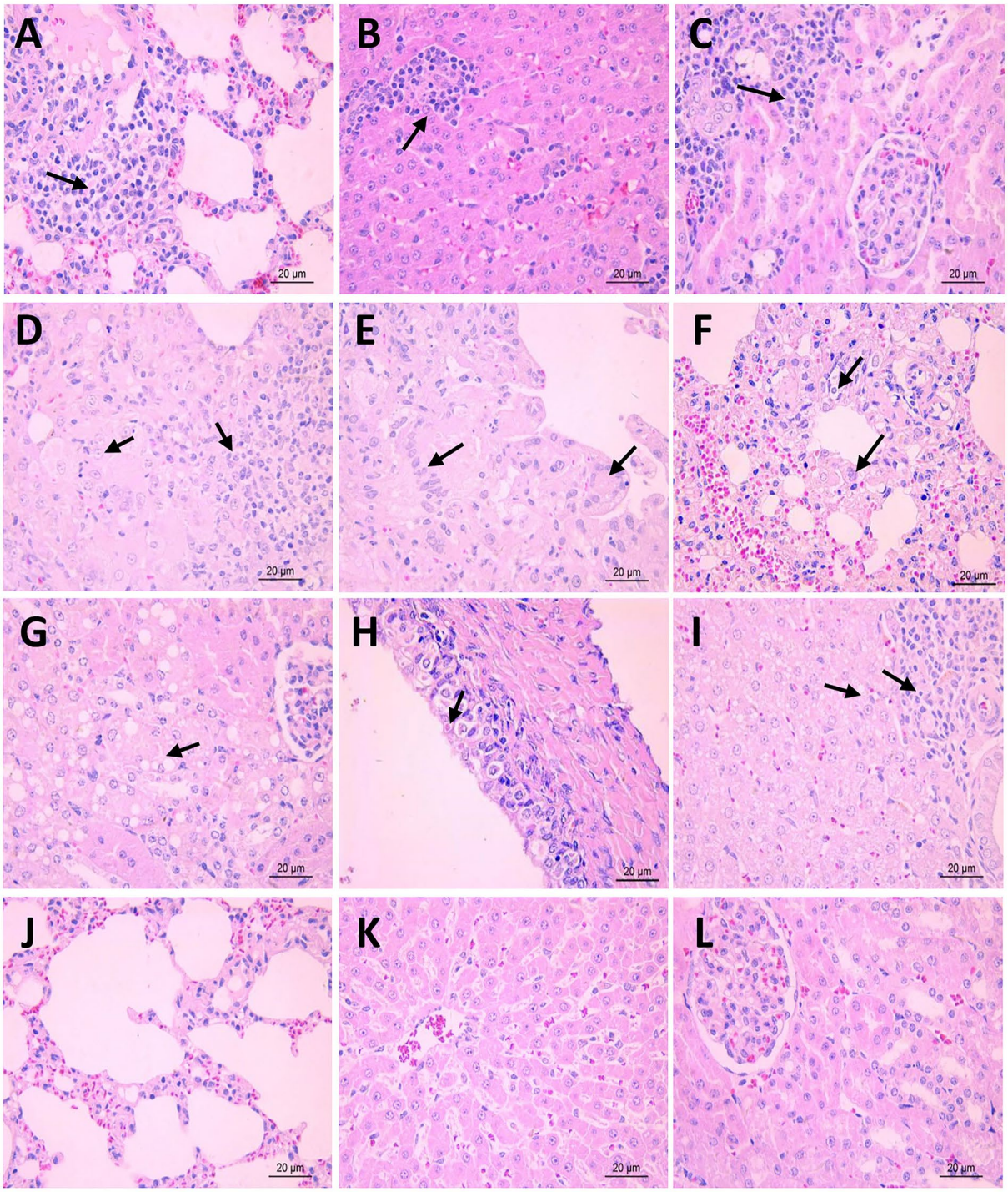

Fig. 5 Histological findings of lungs, liver, and kidneys in minks challenged with the WH2 strain CDV (**a–i**) and in mock-infected minks (**j–l**). **a–c** Infiltration of inflammatory cells in lung (**a**), liver (**b**), and kidney (**c**) of vaccinated and challenged animals. **d–f** Lung tissues of unvaccinated, challenged minks, showing infiltration of lymphocytes and macrophages (**d**), mononuclear cells (**e**), and eosinophilic inclusion bodies (**f**). **g–i** Infiltration of inflammatory cells and fatty degeneration in the liver (**g**), and eosinophilic inclusion bodies (**h**) and fatty degeneration (**i**) in the kidney of unvaccinated, challenged minks. **j–l** Lung (**j**), liver (**k**), and kidney (**l**) of mock-infected minks

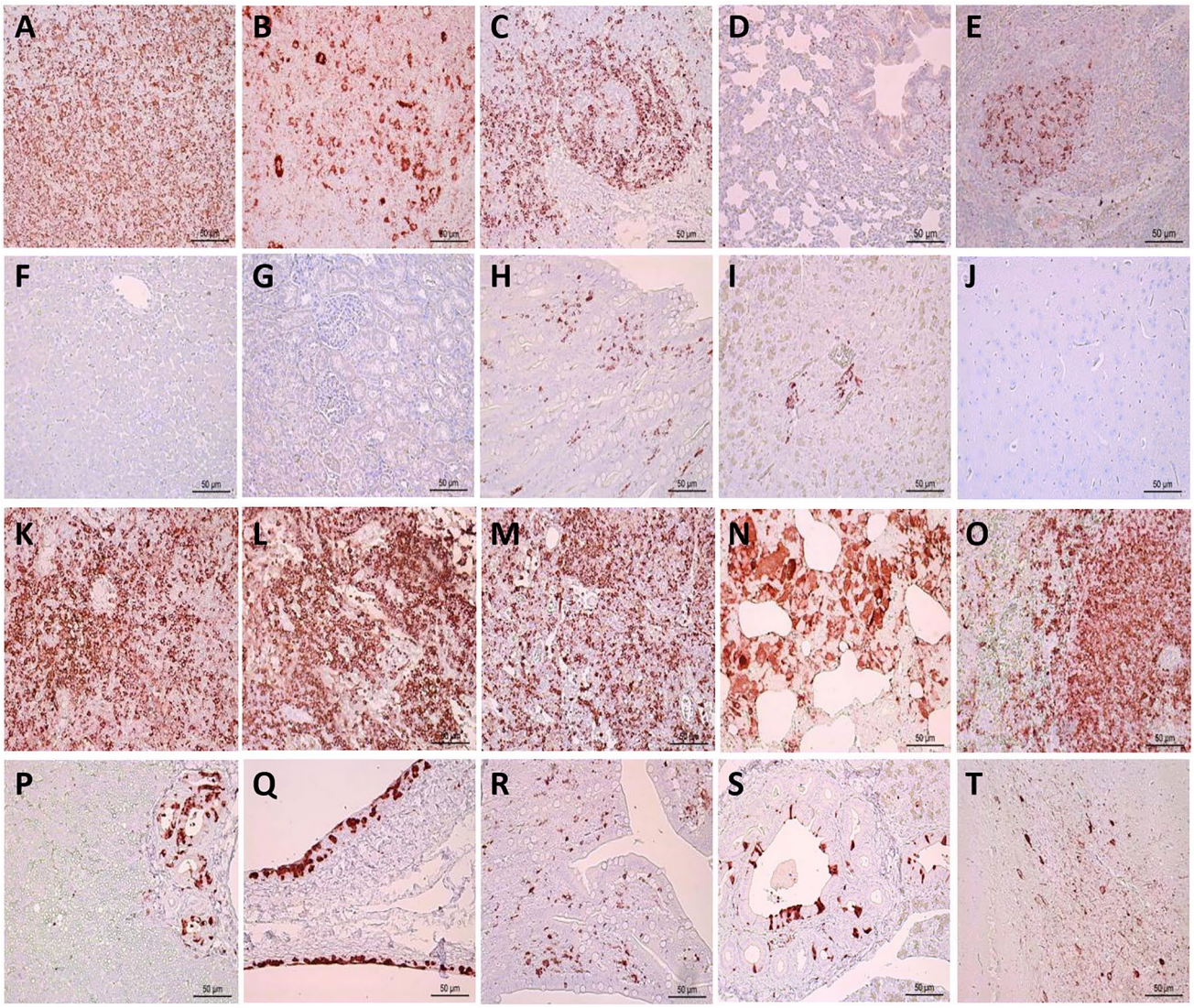

Fig. 6 Immunochemistry results of vaccinated (**a**–**j**) and unvaccinated (**k**–**t**) minks challenged with the WH2 strain CDV. Positive staining was identified in Tonsil (**a**), submandibular lymph node (**b**), mesenteric lymph node (**c**), lung (**d**), spleen (**e**), intestinal tract (**h**), and pancreas (**i**) of vaccinated minks. No positive staining was detected in the liver (**f**), kidney (**g**), cerebrum (**j**) of vaccinated minks. The unvaccinated minks showed stronger staining in Tonsil (**k**), submandibular lymph node (**l**), mesenteric lymph node (**m**), lung (**n**), spleen (**o**), liver (**p**), kidney (**q**), duodenum(**r**), pancreas (**s**), and cerebrum (**t**)

to become potential vaccine candidates against the currently circulating wild-type strains.

The pre-peptide of the F protein spans residues 1–135 and the F1 and F2 subunits span residues 225–662 and 136–224, respectively. F protein from WH2 contains aa mutations in residues I87N, T110P and L386I. Unlike the frequent genetic variation seen in the first 135 amino acids, less variation is reportedly observed in the F1 subunit, and the F2 subunit is highly conserved [27]. Variation in the pre-peptide region affects viral fusion, thereby potentially contributing to broader neurovirulence [27, 28]. Herein, mink in the challenge groups experienced systemic infections and CDV-positive antigen cells were detected in their brains, contradicting a previous report in which experimental CDV infections in mink animals did not produce CDV-positive antigen cells in the brain [29]. This disparity could be related to the different virulence characteristics of the different CDV strains because although the WH2 strain was highly virulent, its fusion efficiency was not affected in this study (Supplementary Fig. 3). Although the 108–110 (N-A-P) N-glycosylation site is also missing in the WH2 strain, its presence is vital to its life cycle and virulence mechanisms [30, 31]. F and/or H gene variation has resulted in vaccine failure during CDV outbreaks [13, 32, 33].

The proficiency of syncytium formation in CDV varies among strains and the H and F proteins determine the extent

of cell–cell fusion [34]. Because the novel H protein requires better characterization, we performed a fusion experiment to understand CDV vaccine failure in vitro. The WH2's growth features and its efficiency of cell–cell fusion were similar to those of strain HNly150520B.

The neutralizing antibody titers of the post-vaccination dog and mink sera were also examined using the WH2 and HNly150520B strains. The HNly150520B strain was isolated from a dog whose H protein lacks the residue 542 glycosylation site. Based on the lower neutralizing titers for WH2 (with aa542 glycosylation) than for HNly150520B (no glycosylation at aa 542), it is possible that the vaccine used in the Shandong mink farm may not prevent infection by CDV strains with glycosylation at aa 542. The potential N-linked glycosylation site differences in the H proteins of CDVs may affect neutralization-related sites by disrupting important epitopes [19]. Hence, the vaccine may not prevent infections of other strains lacking the residue 542 glycosylation site. One study reported that de-glycosylation has little effect on neutralization [35], a finding warranting further investigation.

In conclusion, the H and F gene sequences of the WH2 strain isolated from a CDV-infected mink had important differences from CDV strains of vaccines. In particular, the isolated WH2 strain has 10 N-glycosylation sites and a T110P mutation, which is different from the current vaccine strains. Our study indicated that current CDV vaccines could not provide complete protection against wild strains probably due to genetic differences of the H protein of circulating CDVs from vaccine strains.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11262-021-01837-w.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This study was approved by the Animal Ethics Committee of National Research Center for Veterinary Medicine (Permit 20180310024). Dormicum and domitor were used in all surgery and all the mink were euthanized followed by exsanguination at the end of the experiment.

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