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Serotype, antigenicity, and pathogenicity of a naturally recombinant TW I genotype infectious bronchitis coronavirus in China

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ABSTRACT

Since 2009, strains of the naturally recombinant TW I genotype of infectious bronchitis virus (IBV) have caused considerable damage to the Chinese poultry industry. To better understand the antigenicity and pathogenesis of this genotype, the characteristics of the ck/CH/LDL/140520 strain were compared to those of four commercial IB vaccine strains that are used commonly in China, as well as four attenuated viruses that represent two types of IBV strains, which are believed to have originated in China and are the predominant IBV types circulating in chicken flocks in China and many other parts of the world. The results showed that all eight strains were genetically and serotypically different from the strain ck/CH/LDL/140520. Furthermore, neither the vaccine strains nor the attenuated viruses could provide complete respiratory protection of chickens against a challenge with the ck/CH/LDL/140520 strain, indicating that it is necessary to develop new live vaccines or to evaluate the use of established vaccines in combination to control naturally recombinant TW I-type IBV strains in the future. Our results showed that strain ck/CH/LDL/140520 is very pathogenic, and that it is able to cause cystic oviducts in a high percentage of birds, as well as mortality due to nephritis and respiratory distress with complete tracheal ciliostasis, especially in chickens infected at 1 day of age.

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1. Introduction

Being a coronavirus and, therefore, a single-stranded RNA virus, infectious bronchitis virus (IBV) has an enormous capacity to change by spontaneous mutation and genetic recombination. It is believed that spontaneous mutations and genetic recombination can occur randomly in the IBV genome; however, if these events occur in the spike (S) gene, especially in its hypervariable regions, these events are most likely to result in the emergence of many different antigenic or genotypic types, which are commonly referred to as variants. Therefore, IBV is ubiquitous in most parts of the world where poultry are reared, and it is able to spread very rapidly in non-protected chicken flocks, leading to heavy economic losses in poultry industries (Cavanagh and Gelb, 2008). In general, while many new variants are unable to replicate or survive for a long time, a few variants that are of economic importance have emerged worldwide or in restricted geographic areas. Therefore, effective surveillance, which is primarily based on the isolation and identification of the virus type causing disease, is of great importance.

Classification or typing of IBV strains is very important for implementing control measures, research purposes, and understanding the epidemiology and evolution of IBVs. Thus far, two major groups of classification systems, including functional tests that examine the biological functions of a virus (immunotypes, protectotypes, and serotypes) and non-functional tests that assess the viral genome (genotypes), are commonly used for IBV typing (de Wit, 2000). For a specific IBV strain, although evidence from some studies suggests that there is a high correlation between the genotype and serotype, other studies have presented conflicting data (de Wit, 2000; Zhang et al., 2015; Chen et al., 2015), which may lead to contradictory results. The disadvantages of each system are that they only analyze one or several characteristics of a virus strain. Hence, data from only one system has to be interpreted with caution, while a more objective and accurate conclusion can be drawn by completely analyzing the results from different systems, although it is suggested that the preferred typing system usually depends on the goal (e.g., selection of

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vaccination programs or epidemiological studies), available techniques, experience, and costs (de Wit, 2000).

A large number of IBV genotypes and variants have been isolated in China in recent years (Han et al., 2011), among which two genotypes, the LX4 and ck/CH/LDL/97I types (also known as QX- and Q1-like, respectively), were first isolated in China and subsequently have become widespread worldwide (Valastro et al., 2016). Among these IBV genotypes, the TW1 type was first isolated in 1992 in Taiwan, and it was considered to have a different genotype than all of the other IBVs (Wang and Tsai, 1996). The majority of TW1 IBVs isolated from Taiwan are nephropathogenic, and they have mortality rates ranging from 10 to 60% in 1-day-old specific-pathogen-free (SPF) chickens (Wang and Tsai, 1996). However, the naturally recombinant TW I (nrTW I) genotype was first isolated in 2009 in China, and it was thought to have originated from a natural recombination between LX4 and TW1 viruses (Xu et al., 2016). Despite a previous study that characterized the genetic characteristics of an nrTW I type virus and its nephropathogenicity in 7-day-old SPF chickens (Xu et al., 2016), there is no further information on this important IBV variant. To better understand the nrTW I type, a series of experiments was performed to investigate its antigenicity and pathogenicity in the oviducts of SPF layers, and to evaluate the protection provided by commercial vaccines and attenuated viruses.

2. Materials and methods

2.1. Virus strains

Nine IBV strains, including four vaccine strains (H120, LDT3-A, 4/91, and Connecticut (Conn)), four attenuated strains (ck/CH/LDL/091022 [LDL/091022], ck/CH/LSD/120720 [LSD/120720], ck/CH/LGX/100508 [LGX/100508], and ck/CH/LDL/97I [LDL/97I]) and the ck/CH/LDL/140520 (nrTW I) strain (Xu et al., 2016), were used in this study. The IBV strains LDL/091022 (Liu et al., 2013), LSD/120720, and LGX/100508 (Chen et al., 2015) were isolated in China and belong to the LX4 type. These three strains were attenuated by serially passaging them 120 (P120), 90 (P90), and 70 (P70) times, respectively, in 10-day-old SPF chicken eggs. The LDL/091022 P120, LSD/120720 P90, and LGX/100508 P70 strains were shown to be fully attenuated in SPF chickens (Liu et al., 2009a). Furthermore, vaccination with attenuated viruses provided complete protection against their virulent parental viruses (data not shown). The LDL/97I strain was also passaged 115 times (P115) in SPF chicken eggs (Liu et al., 2009b). Each of the virus stocks was prepared in 10-day-old embryonated SPF chicken eggs by the allantoic route of inoculation, and the infectious allantoic fluid was collected 48 h post-inoculation as previously described (Liu et al., 2009a). The titers of the viruses were determined as previously described (Chen et al., 2015), and the median embryo infectious dose (EID_{50}) was calculated using the method of Reed and Muench (1938).

2.2. Chickens and eggs

White Leghorn SPF layer chickens and fertile SPF chicken eggs were obtained from the Harbin Veterinary Research Institute. The birds were maintained in isolators with negative pressure, and food and water were provided ad libitum. All experimental procedures were approved by the Ethical and Animal Welfare Committee of Heilongjiang province, China.

2.3. Sequence analysis

The S1 genes of strains LSD/120720 P90 and LGX/100508 P70 were amplified and sequenced as previously described (Liu et al., 2013). The sequences have been submitted to GenBank, and they have been assigned accession numbers KT957547 and KT957548, respectively. The S1 gene sequences of the remaining eight viruses, including the H120, LDT3-A, 4/91, Conn vaccine, LDL/091022 P120, and LDL/97I P115, nrTW I and the TW I-type TW2575/98 strains, were selected from the GenBank database and used for S1 gene comparison. Both the sequences of amino acid and nucleotide were assembled and the similarities were calculated using the Clustal W method available in the BioEdit software package (version 7.0.3.0., available at: http://www.mbio.ncsu.edu/bioEdit/bioedit). In addition, 14 other reference strains in which the S1 subunit sequences were available in GenBank (www.ncbi.nlm.nih.gov/genbank/) were also selected for a phylogenetic analysis. The characteristics of the reference viruses are listed in Table 1. A phylogenetic tree based on the S1 gene was constructed from aligned amino acid

Table 1
Backgrounds of the IBV strains in this study.

Strain	Country (Province)[a]	Year	Genotype	Original description	Host	Accession number
LDL/091022	China (Liaoning)	2009	LX4	Liu et al. (2013)	Chicken	HM194640
LGX/100508	China (Guangxi)	2010	LX4	Gao et al. (2010), unpublished	Chicken	KT957548
LX4	China (Xinjiang)	1999	LX4	Liu and Kong (2004)	Chicken	AY189157
QXIBV	China (Shandong)	2011	LX4	Pan et al. (1999), unpublished	Chicken	AF193423
LSD/120720	China (Shandong)	2012	LX4	Gao et al. (2012), unpublished	Chicken	KT957547
LDT3-A	Vaccine strain		tl/CH/LDT3/03			KR608272
tl/CH/LDT3/03	China (Guangdong)	2003	tl/CH/LDT3/03	Liu et al. (2005)	Duck	AY702975
Partridge/GD/S14/2003	China (Guangdong)	2003	tl/CH/LDT3/03	Fu et al. (2003), unpublished	Partridge	AY646283
4/91	Vaccine strain		973/B			AF093793
4/91(UK)	UK	1991	973/B	Armesto et al. (2011)	Chicken	JN192154
TA03	China (Shandong)	2003	973/B	Yang et al. (2004), unpublished	Chicken	AY837465
Spain/92/185	Spain	1992	973/B	Dolz et al. (2008)	Chicken	DQ396092
H120	Vaccine strain		Massachusetts			FJ888351
SD/97/01	China (Shandong)		Massachusetts	Chen et al. (1999), unpublished	Chicken	AF208240
M41	USA		Massachusetts	Binns et al. (1986)	Chicken	X04722
Beaudette	USA		Massachusetts	Binns et al. (1985)	Chicken	NC_001451
Conn	Vaccine strain		Connecticut			KF696629
LDL/140520	China (Liaoning)	2014	TW I	Xu et al. (2016)	Chicken	KP790143
ck/CH/LHLJ/140756	China (Heilongjiang)	2014	TW I	Xu et al. (2016)	Chicken	KP790144
ck/CH/LHLJ/111043	China (Heilongjiang)	2011	TW I	Xu et al. (2016)	Chicken	JQ739314
LDL/97I	China (Liaoning)	1997	ck/CH/LDL/97I	Liu et al. (2006)	Chicken	DQ068701
Q1	NA	1996–1998	ck/CH/LDL/97I	Yu et al. (2001)	Chicken	AF286302
T3	NA	1996–1998	ck/CH/LDL/97I	Yu et al. (2001)	Chicken	AF227438

[a] Country (province) where the viruses were isolated.

sequences by the neighbor-joining method with 1000 bootstraps using the MEGA4 program (Tamura et al., 2007).

2.4. Virus cross-neutralization tests

Four IBV vaccine strains (LDT3-A, 4/91, H120 and Conn), the attenuated LDL/97I P115 and nrTW I were used for virus cross-neutralization test in this study. Because strains LDL/091022 P120, LSD/120720 P90 and LGX/100508 P70 belong to the same genotype (LX4-type or QX-like), hence, only the strains LDL/091022 P120 and LSD/120720 P90 were used. In addition, the IBV strain ck/CH/LSC/99I, which represents another important genotype in China (Han et al., 2011), was also used for virus cross-neutralization test in this study.

Sera against these IBV strains were prepared as previously described (Guo et al., 2014). Briefly, 20-day-old SPF chickens were inoculated by a combined intraocular and intranasal route using a total dose of 10^6 EID_{50} of each virus per bird, respectively. After 2 weeks, a booster dose of each virus with 10^6 EID_{50} was administered by intravenous inoculation to the bird. Birds were exsanguinated 1 week after the last inoculation and the separated sera from chickens inoculated with the same virus were pooled. All sera were inactivated at 56 °C for 30 min and stored in 2.0 ml aliquots at −80 °C until required. For virus neutralization, sera were serially diluted two-fold with sterile phosphate-buffered saline (PBS) and mixed with 200 EID_{50} of the IBV strains. After incubation for 1 h at 37 °C, virus-serum mixtures were inoculated into the allantoic cavity of SPF chicken embryos, which were observed for 7 d. The end-point titer of each serum sample was calculated using the method of Reed and Muench (1938).

2.5. Pathogenicity test

Fifty-five 1-day-old SPF layer chickens were separated into four groups, and they were provided with food and water ad libitum. Groups 1–3 included 15 birds, and they were challenged with the nrTW I strain when they were 1, 15, and 30 days old, respectively. The challenge strain (10^6 EID_{50} in 0.1 ml of diluent per bird) was applied by the intranasal and ocular routes. Group 4, which served as a negative control, included 10 birds that were not challenged. The chicks were examined daily for clinical signs of infection, such as tracheal rales, nasal discharge, watery eyes, and wheezing. The clinical signs from all of the birds in each group were counted by three people over a 2-min period. Morbidity and mortality were recorded daily. Gross lesions were also carefully examined from the dead chickens, and the kidneys of the dead chickens were subjected to immunohistochemistry (IHC) using monoclonal antibody 6D10 (Han et al., 2013), which is directed against the nucleoprotein, as previously described (de Wit et al., 2011; Xu et al., 2016). Blood samples were collected on 4, 8, 12, 16, 20, and 24 d post-challenge (dpc) from all birds, and they were examined for the presence of antibodies against IBV using a commercial enzyme-linked immunosorbent assay (ELISA) kit (IDEXX, Portland, ME, USA) according to the manufacturer's instructions. At 4 months of age, the birds were killed humanely using carbon dioxide/oxygen, followed by exsanguination. A post-mortem examination was performed, and special attention was paid to abnormalities in the oviducts and kidneys. Meanwhile, samples from the kidney were collected to detect the presence of IBV by IHC.

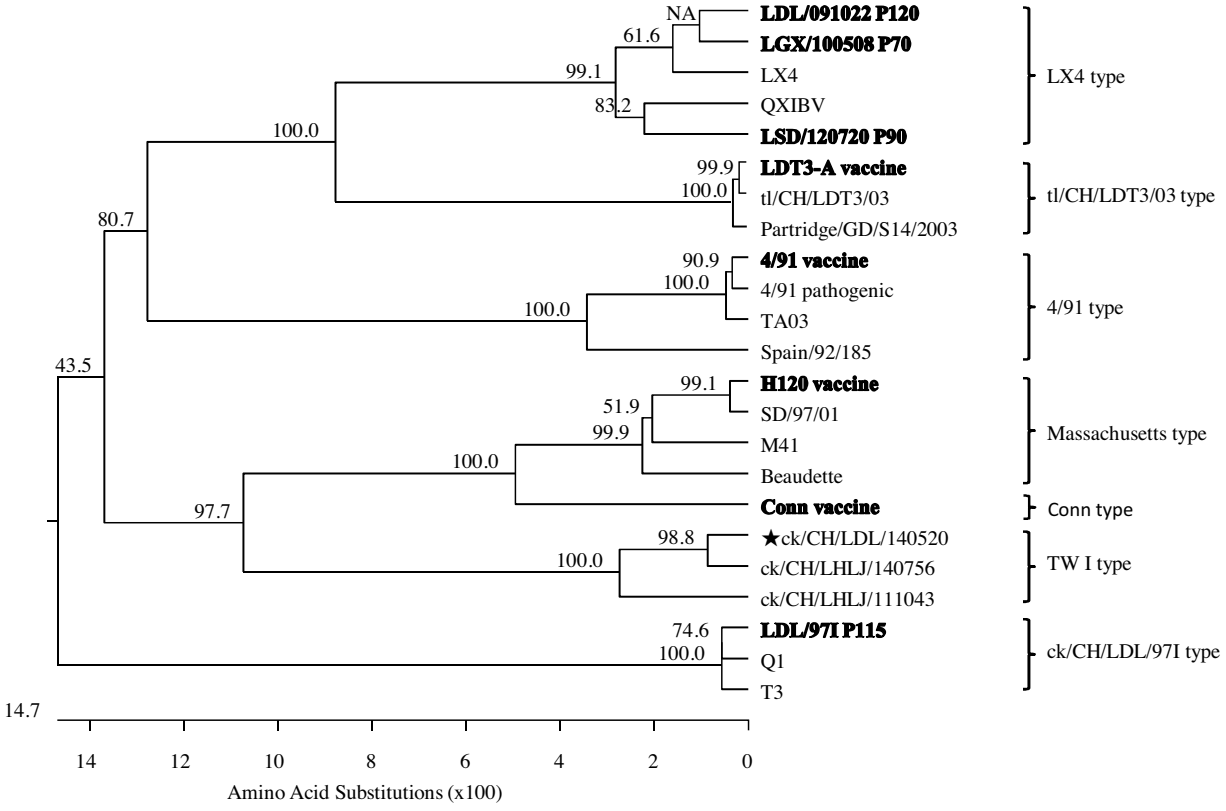

Fig. 1. Phylogenetic trees constructed from the nucleotide sequences of the S1 subunit gene of the four vaccine strains, four attenuated viruses, and 14 reference IBV strains. The trees were computed using the neighbor-joining method. The significance of the tree topology was assessed by 1000 bootstrapping calculations. GenBank accession numbers are indicated in Table 1.

2.6. Vaccination-challenge tests

One hundred 1-day-old SPF chickens were separated into 10 experimental groups, each containing 10 birds. Birds in groups 1–8 were vaccinated with 10^4 EID_{50} of the H120, LDT3-A, 4/91, and Conn vaccines strains, and the LDL/091022 P120, LSD/120720 P90, LGX/100508 P70, and LDL/97I P115 attenuated strains, respectively, in a total of 0.1 ml of PBS per bird, by the intranasal and ocular routes. Birds in groups 9 and 10 received 100 μl of sterile PBS via the same routes. Blood samples were collected at 4, 8, 12, 16, and 20 d post-vaccination (dpv). At 20 dpv, birds in groups 1–9 were challenged with 10^6 EID_{50} of the virulent nrTW I strain, in a total of 100 μl of PBS per bird, by the intranasal and ocular routes. Birds in group 10 were not exposed to virus and served as a negative control. Nasopharyngeal and blood samples were collected from all of the birds in each group at 4, 8, 12, 16, and 20 dpc.

Nasopharyngeal swabs were used to recover viruses from 10-day-old SPF chicken eggs, and viruses were subsequently detected by reverse transcription polymerase chain reaction (RT-PCR) as previously described (Liu et al., 2009a). The serum was stored at −70 °C for detecting antibodies against IBV using the aforementioned ELISA kit as previously described (Liu et al., 2009a).

3. Results

3.1. Strain nrTW I differs genetically from the vaccine and attenuated strains

As illustrated in Fig. 1, the four commercial vaccine strains (H120, LDT3-A, 4/91, and Conn), which represent different serotypes (Massachusetts (Mass), tl/CH/LDT3/03, 793/B, and Connecticut, respectively) of IBV, were genetically different from the nrTW I strain, as indicated by an analysis of their S1 genes. Of the four attenuated strains, the LDL/091022 P120, LSD/120720 P90, and LGX/100508 P70 strains belonged to the LX4 genotype, while the LDL/97I P115 strain belonged to the ck/CH/LDL/97 genotype. These two genotypes were also genetically different from those of the nrTW I strain and the four vaccine strains. In accordance with these results, strain nrTW I did not share more than 82% nucleotide and amino acid similarities with the S1 genes and S1 proteins of the vaccine and attenuated virus strains in this study (Table 2). In addition, similar to previous results (Xu et al., 2016), strain nrTW I shared the highest nucleotide (98.5%) and amino acid (97.4%) similarities with the TW I prototype strain TW2575/98.

3.2. Strain nrTW I represents a novel IBV serotype

The results of the two-way cross-neutralization tests using the IBV strain nrTW I and the antisera against the four vaccine strains, which represented the Mass, tl/CH/LDT3/03, 793/B (4/91), and Conn serotypes, showed that the strains belonged to different serotypes (Table 3). The results using the nrTW I strain and the antisera against the deduced parental virus types, the LDL/091022 P120, LSD/120720 P90, LGX/100508 P70, and LDL/97I P115 strains, also showed non-detectable levels of cross-neutralization.

Table 3
Results of the reciprocal β virus neutralization tests using the ck/CH/LDL/14520 and other IBV strains (serum dilution using a constant amount of virus).

Virus	Serum								
	1	2	3	4	5	6	7	8	9
1. nrTW I	1260[a]	42	<2	<2	<2	<2	<2	<2	6
2. LDT3-A	10	904	–	–	–	–	–	–	–
3. 4/91	<2	–	832	–	–	–	–	–	–
4. H120	<2	–	–	892	–	–	–	–	–
5. Conn	10	–	–	–	628	–	–	–	–
6. LDL/97I	<2	–	–	–	–	648	–	–	–
7. ck/CH/LSC/99I	<2	–	–	–	–	–	1448	–	–
8. LSD/120720	<2	–	–	–	–	–	–	494	–
9. LDL/091022	12	–	–	–	–	–	–	–	294

–, Not tested.
[a] Reciprocal titer.

3.3. Protection is provided by vaccination with different viruses

None of the chickens that were vaccinated with the LDT3-A vaccine strain or the attenuated LDL/091022 and LDL/97I viruses showed clinical signs or mortality when challenged with the IBV strain nrTW I, thereby demonstrating that vaccination with these three viruses provided a high degree of clinical protection. In contrast, respiratory signs (sneezing, nasal discharge, and tracheal rales) developed at 3–4 dpc and lasted until 10 dpc in some of the chickens in the groups that were vaccinated with the H120, 4/91, and Conn vaccines, and the attenuated LSD/120720 and LGX/100508 viruses when challenged with the nrTW I IBV strain at 20 days of age. In addition, some of the chickens died in these groups, indicating that vaccination with these viruses could not provide complete protection against the nrTW I strain. No birds died during the experiment and no clinical signs were observed in the negative control group. As listed in Table 4, the nrTW I challenge virus was re-isolated from the tracheas of nearly all of the birds in the eight vaccinated groups at 4 dpc, as well as from non-vaccinated birds that were challenged. As expected, none of the chickens in the negative control group tested positive for virus isolation. The serological responses induced by the IBV vaccines and the challenge virus are presented in Table 4. All of the birds in each of the vaccinated groups seroconverted at 20 dpv, as well as at 12 dpc with the nrTW I strain. None of the birds in the negative control group seroconverted during the experiment.

Table 2
Nucleotide and amino acid similarities of the S1 gene and protein among strain nrTW I and the eight vaccine/attenuated strains.[a]

Strain		Amino acid identity (%)									
		1	2	3	4	5	6	7	8	9	10
1	nrTW I		78.5	78.8	77.9	81.4	75.7	80.8	80.5	75.3	97.4
2	LDL/091022	78.0		98.0	94.2	84.6	78.7	76.7	75.8	75.7	79.6
3	LGX/100508	78.1	97.6		94.4	84.2	78.7	77.1	75.7	76.1	79.4
4	LSD/120720	78.5	94.1	94.4		84.8	77.3	76.7	75.6	75.1	78.8
5	LDT3-A	81.4	86.7	86.7	86.8		75.4	79.8	78.7	73.9	81.8
6	4/91	76.1	78.4	78.3	77.8	77.8		74.9	75.1	75.9	76.8
7	H120	79.8	77.8	77.8	76.9	80.6	77.3		91.8	74.5	81.6
8	Conn	79.5	76.7	76.5	76.3	79.7	76.9	94.1		75.1	81.6
9	LDL/97I	75.8	75.8	75.6	75.6	76.2	77.6	76.3	75.8		76.6
10	TW2575/98	98.5	77.8	78.0	78.6	82.2	76.6	81.1	80.8	76.7	
		Nucleotide identity (%)									

[a] The first 1632 nucleotides, starting at the AUG translation start codon, of the S gene were compared.

Table 4
Results of the vaccination-challenge tests (groups of chickens were vaccinated with IBV vaccine or attenuated strains, and challenged with the nrTW I strain).

Group*	Morbidity	Mortality	Antibody response[a]										Virus recovery after challenge[b]				
			Vaccinated					Challenged					4 d[c]	8 d	12 d	16 d	20 d
			4 d	8 d	12 d	16 d	20 d	4 d	8 d	12 d	16 d	20 d					
H120	10/10	4/10	0/10	0/10	7/10	10/10	10/10	10/10	6/6	6/6	6/6	6/6	10/10	5/6	4/6	2/6	1/6
LDT3-A	0/10	0/10	0/10	2/10	6/10	9/10	10/10	10/10	10/10	10/10	10/10	10/10	10/10	8/10	1/10	1/10	0/10
4/91	1/10	1/10	0/10	0/10	2/10	8/10	10/10	10/10	10/10	9/9	9/9	9/9	10/10	9/10	5/9	3/9	0/9
Conn	4/10	1/10	0/10	4/10	7/10	10/10	10/10	10/10	9/9	9/9	9/9	9/9	9/10	6/9	4/9	0/9	–
LDL/091022 P120	0/10	0/10	0/10	4/10	10/10	10/10	10/10	10/10	10/10	10/10	10/10	10/10	8/10	10/10	2/10	0/10	–
LSD/120720 P90	3/10	2/10	0/10	0/10	3/10	7/10	10/10	10/10	10/10	8/8	8/8	8/8	10/10	8/10	4/8	0/8	–
LGX/100508 P70	2/10	2/10	0/10	0/10	3/10	8/10	10/10	10/10	10/10	9/9	8/8	8/8	9/10	3/10	2/9	0/8	–
LDL/97I P115	0/10	0/10	0/10	2/10	6/10	10/10	10/10	10/10	10/10	10/10	10/10	10/10	10/10	8/10	1/10	1/10	0/10
nrTW I[d]	10/10	7/10	0/10	6/10	10/10	10/10	10/10	0/10	3/3	3/3	3/3	3/3	10/10	3/3	1/3	0/3	–
Negative control	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10

[a] Number of chickens that seroconverted/number inoculated.
[b] Two procedures, including virus isolation and subsequent RT-PCR, were used for virus recovery after challenge as described previously (Liu et al., 2008). The results from the two procedures were identical. Number of chickens that were positive for virus recovery/number challenged.
[c] Days after challenge.
[d] The chickens in this group were not vaccinated with the vaccine or attenuated viruses, but were challenged at 20 days of age with the IBV nrTW I strain.

3.4. Pathogenicity of the nrTW I strain

All of the chickens in the 1- and 15-day-old groups that were challenged with the nrTW I strain exhibited the aforementioned respiratory signs at 3–4 dpc, and nine and two chickens, respectively, died at 4–9 dpc. Similar to the negative control group, no birds died and no clinical signs were observed in the 30-day-old group (Table 5).

Obvious hyperemia of the tracheal mucosa with catarrhal exudation was observed in all of the dead chickens. The most remarkable lesions were detected in the kidneys of the dead chickens. The affected kidneys were enlarged and pale, and urate deposition was observed in the tubules and ureters (Fig. 2A). Similar to our previous results (Xu et al., 2016), viral antigen was detected by IHC in the kidneys of all of the dead birds. Antigen was found in the cytoplasm of the tubular epithelial cells and in the mucous membrane of the ureters and collecting ducts (Fig. 2B). Interestingly, neither gross lesions nor IHC-positive cells were observed in the kidneys of the surviving chickens at the end of the experiment. In addition, characteristic dilatation of the oviduct developed in most (4/6) of the birds in the 1-day-old group. In contrast, one and two of the birds in the 15- and 30-day-old groups, respectively, exhibited cystic oviducts (Fig. 3; Table 5). Surprisingly, the dilatation and serous fluid accumulation in the oviducts were observed not only in the chickens in the 1- and 15-day-old groups, but also in chickens in the 30-day-old group, although only 2/15 of the birds in the 30-day-old group showed these lesions. As expected, no such lesions were observed in the birds of the negative control group.

All of the birds in the 15- and 30-day-old groups seroconverted at 12 and 8 dpc, respectively, with strain nrTW I, which was earlier than that in the 1-day-old group, in which all of the birds seroconverted at 16 days of age (Table 5). We did not observe seroconversion in any chickens of the negative control group.

4. Discussion

In this study, most IB vaccines, including two types of government-approved vaccines (H120 and LDT3-A) and two types of non-government-approved vaccines (4/91 and Conn) that are used commonly in China, as well as the nrTW I type strains, were selected for genotyping, an S1 gene comparison, serotyping, and vaccination-challenge tests. In addition, we also selected four attenuated strains representing two types (the LX4 type strains LDL/091022, LSD/120720, and LGX/100508, and the ck/CH/LDL/97I type strain LDL/97I) of IBV strains that are believed to have originated in China and are the predominant IBV types circulating in chicken flocks in China and many other parts of the world (Valastro et al., 2016). A phylogenetic analysis and an S1 gene comparison showed that the aforementioned six types of viruses are genetically different from each other and from strain nrTW I. This is in accordance with the results of the cross-neutralization tests that showed that the serotype of strain nrTW I differs from those of the selected six serotypes in this study and likely represents a novel serotype. The cross-neutralization tests did not include the TW I prototype strain TW2575/98 from Taiwan, China, which was unavailable for the comparison.

Table 5
Results of the challenge test using the nrTW I to infect SPF chickens of different ages.

Group	Morbidity	Mortality	Lesions[a]		IHC[b]		Antibody Response[c]					
			Nephritis	Cystic oviducts	Kidney		4	8	12	16	20	24
					Dead	Survived						
1-day-old	15/15	9/15	9/9	4/6	9/9	0/6	0/15	0/7	4/6	6/6	6/6	6/6
15-day-old	15/15	2/15	2/2	1/13	2/2	0/13	0/15	0/13	13/13	13/13	13/13	13/13
30-day-old	0/15	0/15	–	2/15	–	0/15	0/15	15/15	15/15	15/15	15/15	15/15
Control	0/10	0/10	–	0/10	–	0/10	0/10	0/10	0/10	0/10	0/10	0/10

[a] The post-mortem examinations were only conducted for the chickens that died from a challenge with the nrTW I strain, as well as for the chickens that survived 120 days post-challenge. All of the dead chickens showed nephritis. The surviving chickens were examined for oviduct abnormalities.
[b] The presence of IBV antigen in the kidneys of the dead and surviving chickens were investigated using IHC.
[c] The antibody responses against IBV were only examined from 4 to 24 days post-challenge.

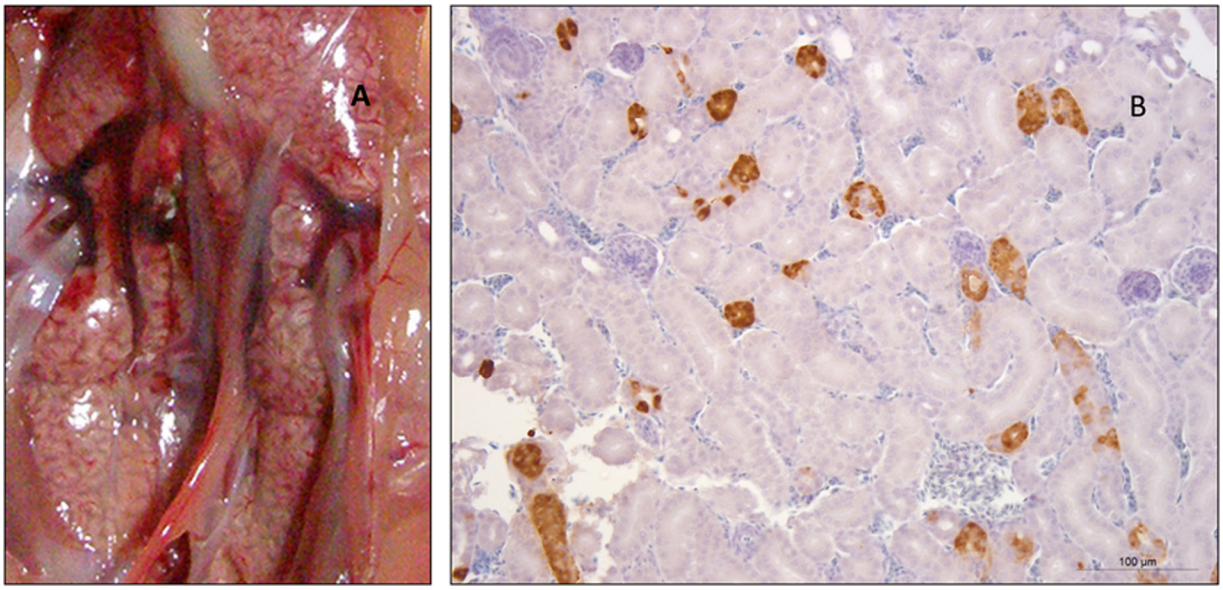

Fig. 2. Lesions in the kidneys of SPF chickens infected with the nrTW I strain. The renal lesions of the kidney caused by inoculation with the nrTW I strain (A). Kidneys are swollen, tubules and ureters are distended, and uric acid crystals are present. Representation of the type of immunohistochemical staining of IBV seen in the kidneys of infected chickens (B). Images were taken at 100× magnification.

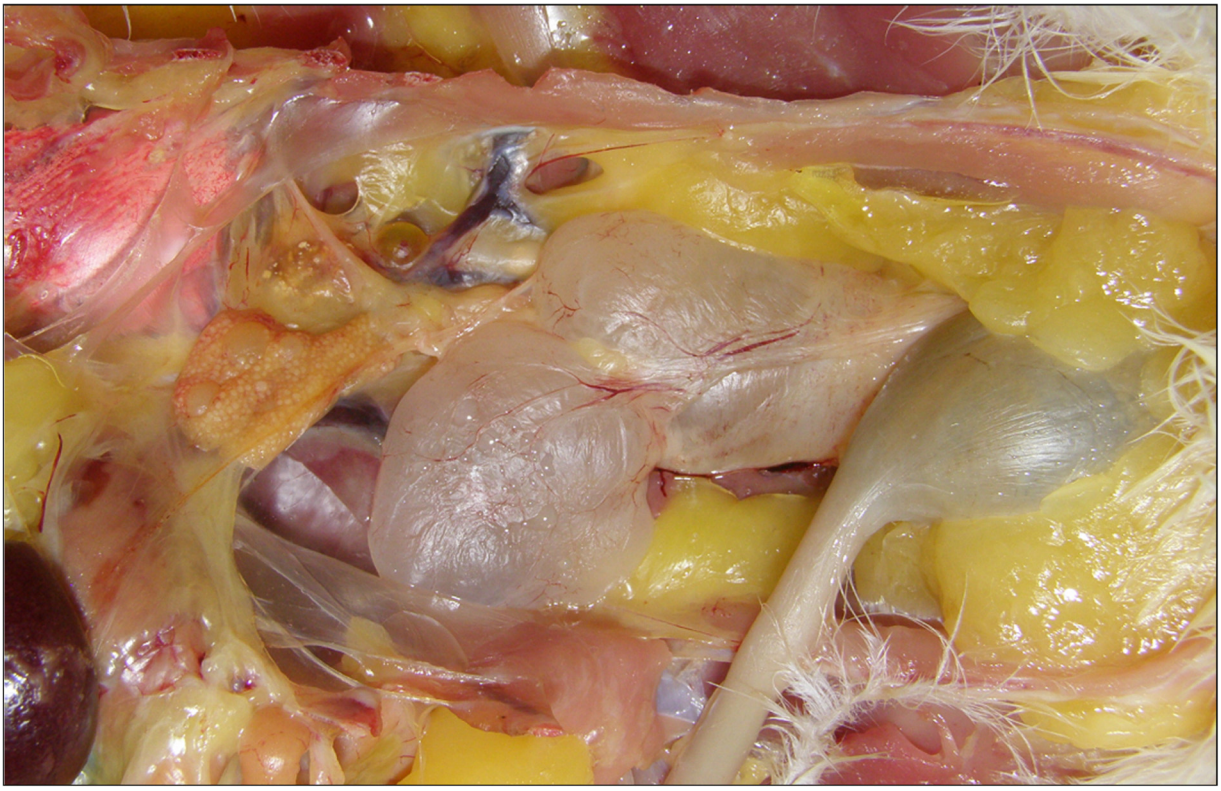

Fig. 3. Cystic dilution of the oviduct in SPF chickens that were infected with the nrTW I strain.

The nrTW I strain was isolated from chickens that had been vaccinated against IBV with the commercial live attenuated H120 vaccine at 1 day of age and then boosted at 14 days of age (Xu et al., 2016), possibly indicating that the vaccine does not provide sufficient protection against a challenge with strain nrTW I. Thus, further examination of the protection conferred by commercially available IBV vaccines and attenuated viruses against challenge with the novel serotype nrTW I strain was important for evaluating the extent of protection against distinct antigenic types. Comparatively, the vaccine strain LDT3-A and two attenuated viruses, LDL/091022 P120 and LDL/97I P115, provided good clinical protection against challenge with the nrTW I strain, although all the strains analyzed in this study have actually percentages of similarity with nrTW I that are of almost the same value (they ranged from 76.1 to 82% of similarity at a nucleotide level, and from 75.3 to 81.4% of similarity at an amino acid level). Some antigenic epitopes that

have roles in the protection have also been identified on the S2 and N proteins of IBV (Ignjatovic and Sapats, 2005) and may attribute to the difference of the cross-protection. Further studies are needed to better understand the relationship between antigenic epitopes on S (both S1 and S2) and N proteins and the level of protection provided in challenge studies. As illustrated in Table 4, the currently available vaccines and attenuated viruses did not provide sufficient respiratory protection against nrTW I challenge, which is in agreement with the genotyping and serotyping results. The results showed that the challenge virus was shed by some chickens in all of the vaccinated groups for at least 12 dpc. Additionally, the high rates of challenge virus isolation (less than 80% at 4 dpc) from chickens that were vaccinated with the heterologous vaccines indicated that none of these vaccines and attenuated viruses provided sufficient protection against the IBV nrTW I strain, especially if a reduction of IBV transmission is considered to be important. These results revealed that it is necessary to develop new live vaccines or evaluate the use of established vaccines in combination to control nrTW I-type IBV strains in the future.

Over the past 20 years, nephropathogenic IBV strains have emerged as the most predominant IBV strains in the poultry industry, and they are responsible for many outbreaks of kidney disease on chicken farms (de Wit et al., 2011). Generally, the nephropathogenic IBV strains have a tropism for the epithelial cells of the respiratory tract, which leads to clinical signs such as excessive water consumption and wet droppings, followed by a severe renal infection that leads to increased mortality, as shown in the case for the nrTW I strain in our previous study (Xu et al., 2016), which was corroborated in the present study. Our results also confirmed that the morbidity and mortality caused by nrTW I infection was age-dependent (Cavanagh and Gelb, 2008). It was shown that many of the infections in young chickens caused by IBV strains result in permanent damage to the oviduct (Broadfoot et al., 1954, 1956), which leads to a significant reduction in egg production and quality upon sexual maturity. The effect of IBV on the oviduct of chickens is extremely variable. There are at least four major factors that influence the severity of oviduct lesions following IBV infection, as well as the outcome regarding the induction of false layers: the IBV strain that is involved, the age at infection, the presence of strain-specific, virus-neutralizing, maternally-derived antibodies at the time of infection, and the early protection induced by vaccinating young chickens (Crinion and Hofstad, 1972; de Wit et al., 2011). Infections with different strains of LX4 type (QX-like) viruses can induce cystic oviducts with water-like fluid accumulation in some chickens that survive the infection, and cystic oviducts were observed at different ages after challenge (Benyeda et al., 2009; de Wit et al., 2011), whereas no lesions could be observed in the oviducts after infection with the pathogenic 4/91 strain (Benyeda et al., 2009; de Wit et al., 2011). For the Mass serotype viruses, it appears that the induction of cystic lesions/false layers differs among different strains (Crinion, 1972; Jones and Jordan, 1972; Benyeda et al., 2009). These differences are not related to virulence because it was shown that a live attenuated strain, H52, was capable of producing similar pathological changes (Duff et al., 1971). A recent study showed that cystic oviducts were also found in some SPF female chicks that were infected with an IS/885/00-like virus (Awad et al., 2016), which was first detected in Israel (Meir et al., 2004) and later found in other parts of the world (Awad et al., 2016). In this study, we found that the nrTW I strain could induce cystic oviducts in SPF chickens of different ages within 1 month post-challenge. The nrTW I strain emerged recently in China, and it originated from recombination events between TW I- and LX4-like (QX) viruses (Xu et al., 2016). One of the parental viruses, the TW1 IBV strain, was first isolated in 1992 in Taiwan, and it is a nephropathogenic IBV strain; however, it was unknown whether it could induce cystic oviducts (Wang and Tsai, 1996). In contrast, it was clearly shown that another parental virus (the LX4 type or QX-like) could induce both nephritis and cystic oviducts in chickens that survived infection (Benyeda et al., 2009; de Wit et al., 2011). The nrTW I strain acquired the 3′ sequences of the Nsp16 and S1 genes from a TW I-like virus, and the rest of its genome from an LX4-like virus, although the tissue tropism and pathogenicity determinants of IBV remain unknown (Xu et al., 2016). In addition, our results also confirmed that as they age, chickens become more resistant to oviduct damage caused by nrTW I infections (Cavanagh and Gelb, 2008). Similar to other reports (Benyeda et al., 2009), our results showed that viral antigens in the kidneys and oviducts of the surviving chickens became undetectable immunohistochemically by the end of the experiment; this is in agreement with our field experience, which showed that IBVs could not be isolated (in most cases) from chickens exhibiting cystic oviducts.

In conclusion, these experiments confirmed previous observations that showed that the nrTW I strain has a novel genotype, and that it is a pathogenic IBV strain that can result in high mortality rates by causing nephritis in susceptible birds. Our results also showed that nrTW I infection induced age-dependent cystic oviducts. In addition, a cross-neutralization test showed that the nrTW I strain of the nrTW I genotype has a novel serotype that differs from those of the other strains used in this study. A vaccination-challenge test using different heterologous live vaccines and attenuated viruses in 1-day-old chickens did not induce high levels of protection against challenge at 20 days of age, which suggests that it is necessary to develop new live vaccines or evaluate the use of established vaccines in combination to control nrTW I type IBV strains in future.

Competing interests

The authors declare that they have no competing interests.

Acknowledgements

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