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Research Note—

Identification of a Novel Nephropathogenic Infectious Bronchitis Virus in Israel

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SUMMARY. A novel infectious bronchitis variant, designated as IS/885/00, associated with nephritis, was isolated from outbreaks in 23 broiler farms in Israel. The virus was first identified by reverse transcriptase–polymerase chain reaction and showed a distinct restriction fragment length polymorphism pattern from previously described Israeli isolates. Sequence analysis of the S1 gene and the deduced amino acid sequence revealed 97.2% protein similarity to genotype IS/720/99 and 71.6% similarity to the vaccine strain H120, the only strain permitted for use in this country. A database search in GenBank revealed a closely related isolate from Egypt, Egypt/Beni-Seug/01, with 96.6% similarity. Other published nephropathogenic infectious bronchitis virus strains/isolates shared less than 77% similarity with IS/885/00. A vaccine protection test in specific-pathogen-free chicks indicated 91% protection to the trachea and only 25% protection to the kidneys in vaccinated birds challenged with IS/885/00.

RESUMEN. *Nota de Investigación*—Identificación de una nueva cepa nefropatogénica del virus de bronquitis infecciosa aviar.

Se aisló una nueva cepa variante del virus de bronquitis infecciosa asociada con síntomas de nefritis, identificada como IS/885/00, a partir de muestras tomadas en un brote de la enfermedad ocurrido en 23 granjas de pollos de engorde en Israel. El virus se identificó inicialmente mediante la prueba de transcripción reversa acoplada a la reacción en cadena por la polimerasa y la técnica de polimorfismo de la longitud de los fragmentos de restricción. El patrón de restricción observado fue diferente a los comunmente encontrados en las cepas del virus aisladas en Israel. El análisis de la secuencia de nucleótidos del gen S1, y la secuencia de aminoácidos deducida de la misma, revelaron un porcentaje de similitud de un 97.2% con la cepa IS/720/99 y un 71.6% de similitud con la cepa vacunal H120, la cual es la única cepa vacunal utilizada en el país. Una búsqueda en la base de datos del GeneBank mostró una relación cercana con una cepa aislada en Egipto, la cepa Egipto/Beni-Seug/01, con un 96.6% de similitud. Otras secuencias publicadas, obtenidas a partir de cepas nefropatogénicas del virus de bronquitis, presentan menos de un 77% de similitud con la cepa IS/885/00. Los niveles de protección observados en una prueba de protección vacunal realizada en pollos libres de patógenos específicos vacunados y luego desafiados con la cepa IS/885/00 fueron de un 91% en la tráquea y un 25% en el riñón.

Key words: broilers, infectious bronchitis virus, genotype, spike protein gene, sequence, vaccine

Abbreviations: ELISA = enzyme-linked immunosorbent assay; IBV = infectious bronchitis virus; NIBV = nephropathogenic infectious bronchitis virus; Mass = Massachusetts; PBS = phosphate-buffered saline; RFLP = restriction fragment length polymorphism; RT-PCR = reverse transcriptase–polymerase chain reaction

Infectious bronchitis virus (IBV) is prevalent in all countries with an intensive poultry industry. Respiratory disease is the most frequently observed syndrome caused by IBV (3,16). Nevertheless, this same virus has been implicated in nephritis, as reported from many countries (9,20,21). Vaccination is only partially successful because of the continual emergence of antigenic variants (10).

Although many countries share some common antigenic types, IBV strains are thought to be unique within a geographic region. Thus, several different vaccine strains are used, and vaccination programs may differ from one country to another. In Israel, the only permitted vaccines are the live, attenuated H120 strain or an inactivated Massachusetts (Mass) serotype. In studies conducted in Israel since 1995, four IBV genotypes have been identified and analyzed. A Mass field isolate, differing slightly from the vaccine strain H120, and two novel variants designated Variant I and Variant II were previously described (1,17). A fourth variant, IS/720/99, originally isolated from tracheal swabs from an infected broiler flock, was typed by reverse transcriptase–polymerase chain reaction–restriction fragment length polymorphism (RT-PCR-RFLP) by sequencing of the S1 gene and by a vaccine protection test (Meir et al., unpubl. data).

In the winter of 2000 a severe outbreak of renal disease occurred in several broiler farms, causing great economic loss to the Israeli broiler industry as a result of mortality and poor weight gain of the birds. The causative agent was determined to be a nephropathogenic infectious bronchitis virus (NIBV) by virus isolation from kidneys, RT-PCR, and by elimination of other possible pathogens. In this study, molecular characterization of the isolate, designated IS/885/00, was carried out by analyzing the genetic relatedness of the S1 gene sequence, compared to previous Israeli genotypes as well as published sequence data of closely related IBV strains, other NIBV isolates, and vaccine strains. Special emphasis was put on vaccine strain 4/91 in the genetic analysis because of its proved ability to protect against kidney damage by NIBV (4) and its possible future introduction in Israel.

We conducted serologic and antigenic tests designed to evaluate the H120 vaccine protection efficiency against this virus.

MATERIALS AND METHODS

Case history. Acute renal disease was observed in 23 broiler farms located in Israel. The disease signs included severe morbidity, diarrhea, dehydration, swollen kidneys, and mortality, ranging from 15% to 25% during the winter months. First outbreaks of the disease were recorded during the second half of 2000 and continued through the fall and winter of 2003. All flocks were vaccinated at 1 day of age by spray with a Massachusetts (H120) serotype live, attenuated vaccine. IBV was isolated from birds in the age range of 21 to 43 days.

Virus isolation and propagation. Nine-to-eleven-day-old embryonating specific-pathogen-free (SPF) chicken eggs (Charles Rivers SPAFAS, North Franklin, CT) were used for isolation, propagation, and titration of IBV field isolates from kidney samples and virus reisolation from tracheal swabs and kidney samples in the vaccine protection test.

RNA extraction and RT-PCR-RFLP. In order to increase RNA yield, pretreatment of allantoic fluid collected 48 hr postinfection was carried out as follows: 0.5 ml of phenol/chloroform with isoamyl alcohol (pH 5.2) (Amresco, Cleveland, OH) was added to an equal volume of allantoic fluid collected 48 hr postinfection. The mixture was vortexed and centrifuged for 2 min. The aqueous layer was collected and the same procedure was repeated. Total RNA was extracted from the aqueous layer using the QIAamp Viral RNA Mini Kit (QIAGEN, Santa Clarita, CA), according to the manufacturer's instructions. RT-PCR-RFLP was performed as described by Kwon *et al.* (11) using the primers Degenerate3′ and NewS1OLIGO5′ (13). The amplification product was digested with the restriction endonucleases *BstY*I, *Hae*III, and *Xcm*I (New England BioLabs, Beverly, MA), and RFLPs were visualized by agarose gel electrophoresis (11). Restriction band sizes were calculated by visual comparison to the molecular weight marker made from pBR328 DNA digested with *Bg*lI and *Hinf* I (Roche, Mannheim, Germany) and supported by gel documentation and analysis software (GeneTool, SYNGENE, Cambridge, United Kingdom). The S1 gene sequence was mapped for the relevant restriction sites using the Webcutter 2.0 software (Max Heiman) and compared to the recorded data.

Viruses. Two field isolates were used in the protection trial. IS/665 was isolated from the trachea of 14-day-old broiler chicks with a respiratory disease. This isolate was shown to have a Mass-like genotype by RFLP and by sequence analysis of the S1 gene and the deduced amino acid sequence. It was selected as positive control because of its relatedness to the vaccine strain (99.3% protein similarity to H120) (Gelb *et al.,* unpubl. data). IS/885/00 was isolated from kidneys of a 36-day-old broiler from a flock with severe renal disease. Both viruses were passaged three times in embryonated eggs, and the allantoic fluid was tested for the presence of paramixo and orthomixo viruses by the hemagglutination test and IBDV and adenoviruses by specific PCR tests.

S1 gene sequence analysis. The PCR products of two field isolates were excised from an agarose gel and purified with the QIAquick Gel Extraction Kit (QIAGEN). Direct sequencing of both strands was performed with the primers Degenerate3′ and NewS1 OLIGO 5′. In addition, we used an internal primer B06 (5′-GTGGCTGGA-CCTATAACTTA-3′) at position 575, downstream of the S1 gene start codon, designed by the use of GeneFisher software (6).

Sequencing was performed at the DNA Sequencing Unit, The Weizmann Institute (Rehovot, Israel), utilizing the Applied Biosystems DNA Sequencer with a BigDye Terminator Cycle Sequencing Kit from ABI (Applied Biosystems, Foster City, CA).

A database search for related IBV strains/isolates was performed using the network BLAST server (15). Comparative analysis of the deduced amino acid sequences was performed with the CLUSTAL V (7) package of DNAStar, (MegAlign, Version 5.0, 2001; DNAStar, Inc., Madison, WI).

Nucleotide sequence accession numbers. The S1 gene and amino acid sequence of IS/885/00, based on two identical field isolates, were deposited with GenBank (accession No. AY279533). For sequence analysis we included the S1 gene sequence data of previously isolated Israeli IBV genetic variants: Variant I, AF093795; Variant II, AF093794; IS/720/99, AY091552. In addition we analyzed the S1 sequence of vaccine strain H120 (accession No. J04329) and seven NIBV strains, as follows: 4/91 (AAD19888); B1648 (X87238); SC021202 (AY237817); W93 (AY427818); X (AY427819); PA/Wolgemuth/98 (AF305595); and PA/171/99 (AF419314). The sequence of a closely related IBV isolate from Egypt, Egypt/Beni-Seuf/01, was retrieved from GenBank (accession No. AF395531) and was included in the genetic analysis. This strain was isolated in 2001, and partial S1 gene sequence was submitted by Gelb *et al.* (unpubl. data).

Vaccine protection test. The experimental design is summarized in Table 1. Two groups of 12 one-day-old SPF chickens each were placed in individual positive-pressure Horsfal–Bauer isolators. In addition, 12 chickens were assigned as unvaccinated controls (six per group) and placed in a separate isolator located in a different room. At 2 wk of age, serum samples were collected from 12 birds, and the two test groups were inoculated with a dose/bird of the H120 vaccine (Bio Vac, Or Akiva, Israel) via eyedrop. Four weeks later, serum samples were collected from the control birds and from nine birds, randomly selected, from each vaccinated group. Six control (unvaccinated) birds were placed in the group 1 isolator, and all 18 birds were challenged with IS/665. The remaining six control birds were placed in the isolator with group 2, and all birds in that group were challenged with IS/885/00. Challenge virus was administered via eyedrop with a titer of LD_{50} $10^{4.5}$ per bird. Five days postchallenge, tracheal swabs were collected from all birds and placed in virus isolation medium (50% glycerol; 50% phosphate-buffered saline [PBS] penicillin, 1000 I.U./ml; streptomycin sulfate, 400 μg/ml; and gentamycin sulfate, 160 μg/ml). The trial was terminated 9 days postchallenge. This time was selected based on a published study in which most kidney lesions were seen between 7 and 10 days postinoculation (4).

Table 1. Experimental design of vaccine protection trial.

Treatment group	Vaccination (2 wk of age)	Challenge virus (6 wk of age)	Number of birds
1	H120	IS/665	12
	None	IS/665	6
2	H120	IS/885	12
	None	IS/885	6

All birds were bled and euthanatized. Kidney samples were collected for virus reisolation and histology tests. Virus reisolation was attempted from individual tracheal swabs and kidney homogenate (30% w/v in PBS containing penicillin, 200,000 I.U./ml, and streptomycin sulfate, 20 mg/ml) by injection of five SPF embryonating eggs per sample. Eggs were incubated at 37 C. At 48 hr, two eggs were removed from incubation and placed overnight at 4 C, after which the allantoic fluid was collected for RNA extraction. The remaining three eggs were incubated for a total of 7 days postinoculation. A result was considered positive for IBV infection when typical lesions or mortality of at least one injected embryo was recorded at more than 48 hr postinoculation (14). RT-PCR test was performed as described above, with primers Degenerate3′ and New S1OLIGO5′.

Serologic test. An enzyme-linked immunosorbent assay (ELISA) (FlockChek®, IDEXX Laboratory, Inc., Westbrook, ME) was used to monitor antibody levels generated by IBV in both challenge groups.

RESULTS

RFLP analysis. RFLP analysis was conducted to differentiate IS/885/00 from previously identified Israeli field isolates. The IS/885/00 isolate had a distinct RFLP pattern when digested by *BstY*I, *Hae*III, and *Xcm*I, compared to Variant I, Variant II, and Mass (data not shown). IS/720/99 and IS/885/00 had identical fragment patterns when digested by *Hae*III (band sizes of 1200 base pairs [bp] 378 bp, and 290 bp) and *XcmI* (band sizes 1000 bp and 650 bp). When digested by *BstY*I IS/720/99 yielded one band of approximately 1100 bp and two overlapping bands of approximately 340 bp (Fig. 1), whereas digestion of IS/885/00 by *BstY*I yielded only two bands, approximately 1100 bp and 670 bp in size. Results were confirmed by restriction enzyme mapping of the S1 gene sequence.

Sequence analysis. Comparative analysis of the S1 glycoprotein deduced amino acid sequence

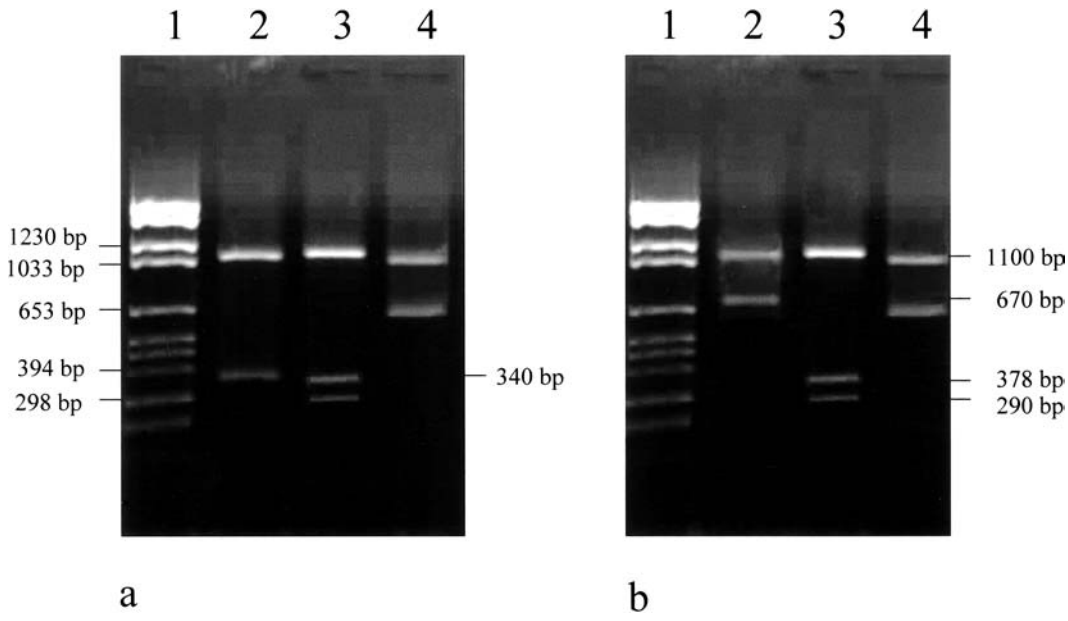

Fig. 1. RFLP patterns of the S1 gene of (a) IS/720/99 and (b) IS/885/00, digested with *BstY*I (lane 2), *Hae*III (lane 3), and *XcM*I (lane 4). Lane 1 = Molecular weight marker pBR328 DNA digested with *Bg*lI and *Hinf*I. Marker band sizes are marked on the right side of panel a. Restriction fragment sizes are marked on the left side of panels a and b.

was performed to assess the relatedness among Israeli variants (Variant I, Variant II, IS720/99, IS/885/00) and to find similarities between IS/885/00 and the vaccine strains H120, 4/91, and other published IBV strains, especially NIBV strains. Pairwise similarity and divergence is presented in Fig. 2, and a phylogenetic analysis is presented in Fig. 3. IS/885/00 and IS/720/99 shared 97.2% similarity with each other and 81% and 77.1% similarity with Variant II, respectively. Variant I and the vaccine strain H120 shared 71.6% similarity with IS/885/00. A comparison of IS/885/00 to other NIBV strains showed 74% similarity with 4/91 and between 71.9% and 76.4% similarity with the other six NIBV included in this study. BLAST analysis of the GenBank data revealed a closely related isolate from Egypt, Egypt/Beni-Seuf/01, sharing 96.6% amino acid similarity with IS/885/

% Similarity

% Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13		
1		71.6	76	92	68.6	73.8	71.9	71.6	72.5	69.4	72.4	68.6	71.6	**1**	Variant I
2	31.9		73.3	72.1	70.5	71.1	72.7	98.3	74.7	70.2	71.8	69.1	71.6	**2**	H120
3	29	29.8		76.5	73.6	77.1	71.9	73	74.7	71.9	80.4	77.1	81	**3**	Variant II
4	8.5	31.2	27.5		71	75.7	73.2	72.4	74	72.1	74	71.2	74	**4**	4/91
5	35.6	29.8	28.8	31.8		74.2	68.9	73.4	71.8	89.5	71.8	69.9	73.6	**5**	PA/Wolgemuth/98
6	29.4	30.1	25.5	26.7	27.8		71.2	73.7	75.2	75.5	71.5	65.3	76.4	**6**	B1648
7	31.8	30.7	32.7	29.4	34.9	31.1		73	79.8	70	69.9	66.1	71.9	**7**	X
8	32.4	1.7	30.7	31.2	28.2	28.4	31.3		76.7	73.6	71.8	69.5	74.5	**8**	W93
9	31	27.5	27.7	28.2	30.3	26.3	22.2	25.6		72.2	72.4	66.5	75.7	**9**	SC021202
10	34.4	31.2	31	30.2	10.5	26.2	32.9	28.6	29.5		72.7	70.8	74.9	**10**	PA/171/99
11	33.7	31.2	21.7	30.9	30.6	32.9	35.4	31.6	30.7	30.2		97.5	97.2	**11**	IS/720/99
12	39.8	33.1	25.5	35.7	32.9	42.2	39.8	33.1	37.6	32.4	2.6		96.6	**12**	Egypt/Beni-Seuf/01
13	34.6	31.5	20.6	30.9	29.4	26.9	33.1	28.8	27	28.1	2.8	3.5		**13**	IS/885/00
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**		

Fig. 2. S1 gene deduced amino acid sequence similarities (%) and divergence (%) among four Israeli field isolates, the vaccine strain H120, and eight IBV strains/isolates from outside Israel.

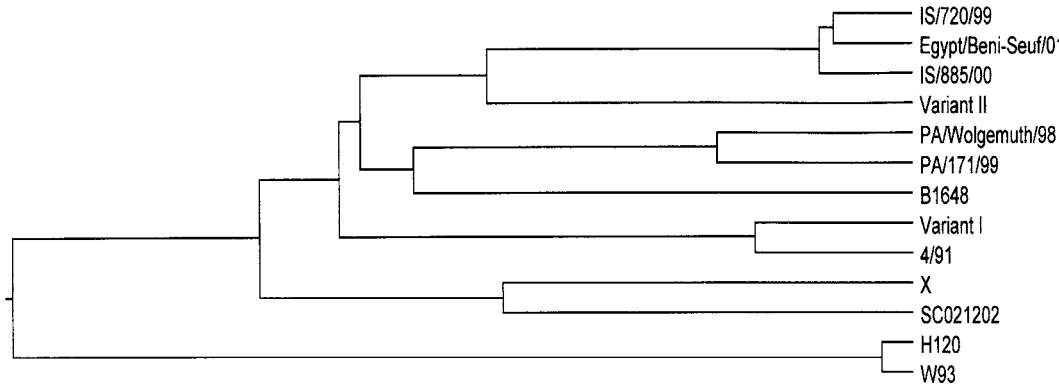

Fig. 3. Phylogenetic tree of deduced amino acid sequences from the IBV S1 gene of Israeli field isolates and IBV strains/isolates from outside Israel.

00 and grouped within the same genotypic cluster as isolates IS/720/99, IS/885/00, and Variant II (Fig. 3). The next closest isolate from outside Israel was strain B from New Zealand (submitted to GenBank by McFarlane *et al.,* accession No. AF151954), sharing 76.4% amino acid similarity with IS/885/00 (data not shown).

Vaccine efficacy test. Vaccine protection of the upper respiratory tract was defined by virus reisolation in embryonated SPF eggs from tracheal swabs sampled 5 days postchallenge. Virus identification was performed by RT-PCR and by observing typical IBV embryo lesions (Table 2). In the vaccinated birds, IBV was reisolated from 2 out of 12 birds when challenged with the homologous serotype, indicating 83% protection. When challenged with IS/885/00, IBV was reisolated from one bird out of 12, thus indicating 92% protection of the trachea. IBV was reisolated from four out of six unvaccinated birds challenged with IS/665 and from six out of six birds challenged with IS/885/00, indicating 34% and 0% protection, respectively. Renal protection was defined by virus reisolation from kidneys harvested 9 days postchallenge by the two above methods and by histopathology (Table 3). The number of infected birds was determined by demonstrating presence of virus by at least one method. Vaccination provided 92% protection against challenge with the homologous virus and 25% protection when challenged with IS/885/00. The unvaccinated birds showed 34% and 0% protection, respectively.

ELISA test. Antibody levels against IBV were measured prior to vaccination and 4 wk postvaccination by a commercial ELISA kit. Initial antibody levels were insignificant, and a rise of 0.1 $\log_{10}$ in titer was recorded postvaccination (data not shown). Postchallenge antibody levels are presented in Table 3. Nine days postchallenge with the homologous serotype, a titer of 1949 was detected in sera from the vaccinated birds and a titer of 4166 in the unvaccinated birds. Challenge with IS/885/00 gave a titer of 2392 in the unvaccinated birds and a titer of 13,810 in the vaccinated birds, a value that is approximately seven-fold higher than the titer obtained following challenge with a homologous serotype.

DISCUSSION

Twenty-three field isolates from outbreaks of acute renal disease were identified as NIBV by the RT-PCR-RFLP method. Based on a unique RFLP pattern shared by all isolates, IS/885/00 was designated as

Table 2. Efficacy of H120 vaccination in chickens challenged with field isolates IS/665 and IS/885/00 tested in tracheal swabs sampled 5 days postchallenge.

Challenge virus	IS/665 (positive/ total birds)		IS/885 (positive/ total birds)	
Vaccine	H120	None	H120	None
Virus re-isolation[A]	2/12	6/6	1/12	3/6
Virus re-isolation[B]	2/12	4/6	0/12	5/6
Number infected[C]	2/12	6/6	1/12	5/6
% Protection	83	0	92	17

[A]Embryo IBV lesions and mortality.
[B]Virus identification by RT-PCR of allantoic fluid harvested 48 hr postinoculation.
[C]Number positive for IBV presence by one or more methods.

Table 3. Efficacy of H120 vaccination in chickens challenged with field isolates IS/665 and IS/885/00 tested in kidneys harvested 9 days postchallenge.

Challenge virus	IS/665 (positive/ total birds		IS/885 (positive/ total birds)	
Vaccine	H120	None	H120	None
ELISA titer[A]	1949 (1161)	4166 (942)	13,810 (4248)	2392 (2455)
Histopathology[B]	0/12	2/6	5/12	3/6
Virus re-isolation[C]	1/12	3/6	9/12	4/6
Virus re-isolation[D]	0/12	0/6	5/12	5/6
Number infected[E]	1/12	4/6	9/12	6/6
% Protection	92	34	25	0

[A]ELISA mean titer (standard deviation) 9 days postchallenge.
[B]Mononuclear infiltration in kidney.
[C]Embryo IBV lesions and mortality.
[D]Virus identification by RT-PCR of allantoic fluid harvested 48 hr postinoculation.
[E]Number positive for IBV presence by one or more methods.

a novel IBV genotype easily distinguished from other Israeli variants. Although the difference in RFLP patterns was more pronounced when IS/885/00 was compared to H120, Variant I, and Variant II, a difference of only one band was observed when it was compared to isolate IS/720/99.

A vaccine protection test was performed to evaluate the antigenic significance of these genetic characteristics. The strong immune response of H120-vaccinated birds after challenge with IS/885/00, compared to that of birds challenged with the homologous virus, indicated that IS/885/00 was a different serotype.

IBV infection was evaluated by the embryo lesions/mortality method and confirmed by the RT-PCR method. Contrary to what might be expected, RT-PCR of allantoic fluid gave a lower proportion of infected birds than did observation of embryo lesions/mortality. This apparent contradiction may be explained by variation between individual embryonated eggs in each test and the sampling method used. A result was considered positive when lesions/mortality were recorded in at least one of three injected eggs, whereas RT-PCR was performed on the pooled allantoic fluid harvested from two different eggs. At low virus levels, as may occur in the vaccinated/challenged chickens, there may be an uneven particle distribution in the injected eggs.

Virus reisolation from kidneys of vaccinated birds challenged by IS/885/00 and lack of reisolation from tracheal swabs from the same birds indicated that the immune response provided protection to the upper respiratory tract, whereas the high antibody level did not provide renal protection against this virus. The Office International des Epizooties requirements for live, attenuated IBV vaccines are that 80% or more of the challenged vaccinated birds show no evidence of IBV in their trachea (18). No specifications with respect to a vaccine efficacy are given for kidney protection. Studies conducted in Belgium and Italy showed that vaccines of the Mass serotype protected poorly against NIBV isolates (2,12,19). Besides the heterogeneity of the vaccine and challenge viruses, insufficient renal protection can be attributed to the high level of attenuation of H120, resulting in a mild vaccine, sufficient to protect the respiratory tract but not sufficient to protect the kidney (5). A recent study conducted by Cook *et al.* (4) showed that the use of a combination of two vaccines, Ma5 (Mass) and 4/91, was highly prophylactic against kidney damage caused by the homologous Belgian NIBV isolate B1648. This combination was not tested with other NIBV isolates.

For the purpose of this study, the S1 gene of two isolates was sequenced and compared to previously published sequence data for IBV. No genetic linkage was found between IS/885/00 and commercial vaccine strains; thus, introduction of IBV vaccines foreign to this region is not advisable. Since the use of a more virulent vaccine has its drawbacks (8), development of a homologous vaccine to protect against IS/885/00 and its genetic variants should be considered. Further studies are necessary to evaluate the most efficient type of such a vaccine and the effect of combined or successive vaccination with H120.

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