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Relationship between serotypes and genotypes based on the hypervariable region of the S1 gene of infectious bronchitis virus

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Summary. To group infectious bronchitis virus (IBV) isolates, a genetic grouping method based on hypervariable region 1 (HVR 1, nucleotides 168 to 197) was compared with that based on the whole S1 gene. Both methods resulted in the same grouping data. So the grouping method based on HVR 1 could represent the grouping method based on the whole S1 gene. Taiwan isolates could not be placed within the existing groups. In order to test the correlation between genotype and serotype, a one-way neutralization test was used to compare 9 Taiwan isolates selected from different genotypes with Massachusetts (Mass) (H120) and Connecticut (Conn) standard strains. In addition, a two-way cross-neutralization test was performed in embryonated eggs with the β method (constant-virus, diluted-serum) and the reciprocal neutralization titers were calculated to give the relatedness (r) values. The results of two kinds of neutralizing tests showed that the serotypes of 9 isolates were different from Mass or Conn. Based on the r-values, 9 isolates were divided into two serotypes which were correlated with their genotypes. From pathogenicity tests, IBV Taiwan isolates could be divided into high, intermediate, and low pathogenicity according to their pathogenicity indexes. However, no relationship exists between pathotype and genotype. In conclusion, the genetic typing method based on HVR 1 can be used for typing IBV isolates.

Introduction

Infectious bronchitis virus (IBV) causes a highly contagious disease in chickens. Various IBV isolates have been identified in different parts of the world [4]. The major IBVs isolated in Taiwan are mostly nephrotropic strains [18]. The infected chickens show signs of depression, dehydration, polyuria and death, and their kidneys are swollen with severe urate deposition. Outbreaks of IB occur frequently in spite of intensive vaccination. Infection with the field viruses was not prevented by the available vaccines, due to the serotype differences [19].

Extensive antigenic variation is a feature of IBV. Several classification methods based on restriction fragment length polymorphism have been published [1, 12].

The most antigenic of the virus neutralization antibody-inducing epitopes is formed by a few amino acids that occur in the first and third quarters of the S1 subunit, especially in the hypervariable regions (HVR) [4]. A polymerase chain reaction and restriction fragment length polymorphism analysis based on HVR 1 was reported [20]. Those primers were used to amplify a 5′ part of the S1 gene that encodes the virus-coated protein which provide the antigenic determinations [11]. The PCR product is shorter and easier to be obtained than those from any other comparable methods. However, there are no reports on the relationship between serotype and genotype based on HVR 1. The two purposes of this experiment are to compare the genotypes obtained from HVR 1 and the whole S1 gene and the relationship among serotypes, pathotypes and genotypes based on HVR 1.

Materials and methods

Genetic grouping of published IBV strains

Grouping IBV strains based on the whole S1 gene and on HVR 1 in S1 gene (C2U-C3L region) [20] was compared. The sequences and locations of C2U and C3L primers were C2U: 5′-TGGTT GGCAT TTACA CGGGG-3′ (114–133) and C3L: 5′-CAATG GGTAA CAAAC AC-3′ (341– 325). The C2U-C3L region was at the 5′ terminal of the S1 gene. For grouping IBV strains, the sequences of known IBV strains obtained from GenBank + EMBL and IBV Iowa 609 from Dr. Collisson (Texas A and M University, College Station, Texas, USA) were analyzed. For comparing Taiwan isolates with the published IBV strains, the sequences of Taiwan isolates [20] were included in the analysis based on HVR 1. The sequence of the C2U-C3L region in the S1 gene of a new Taiwan isolate, A2296, was included for analysis. By using the Jukes-Cantor distance correction method in the Web-based sequence analysis program (Seq-Web, GCG, USA), the evolutionary phylogenetic tree was generated.

Viruses

The IBV Taiwan isolates, The A1246, A1960, A1211, A1928, A1449, A1927, A1171, and A2012 were described elsewhere [20]. The isolate A2296 was isolated from Yiang-Mei, Taiwan in September 1996. They had been purified by three consecutive limit dilutions and were in the 6^{th} to 10^{th} egg passage. The H120 was from a vaccine strain (Intervet), and the Connecticut strain was from Taiwan Animal Health Research Institute with unknown passage histories.

PCR and direct sequencing

RT-PCR using C2U-C3L as a primer set and sequencing [20] were performed for the isolate A2296. The sequence was analyzed with Taiwan isolates described above by the same program.

Production of antisera

The antisera to the Taiwan isolates were made in SPF chickens hatched and reared in isolators kept in separate rooms. Each three-week-old chicken was inoculated intratracheally with $10^5 EID_{50}$ of IBV. The chickens were reinoculated by intravenous injection of $10^6 EID_{50}$ of IBV at the ages of 5 weeks and 8 weeks, respectively. Blood was obtained two weeks after the last inoculation, and the collected serum was heat-inactivated and stored at $-20\,^{\circ}C$.

One-direction virus neutralization test

Constant-virus constant-serum procedure developed by Cowen and Hitchner was used for serotyping in SPF chicken embryos [7]. One hundred EID_{50} of the virus isolates were reacted with 20 units of anti- H120 and anti-Conn antisera, respectively. Viruses were considered to be of the same serotype if the serum protected 50% or more of the embryos.

Cross VN test

The β VN method with constant virus and diluted serum was employed in SPF chicken embryos for serotyping. Serial four-fold dilutions of serum were reacted with one hundred EID_{50} of IBV at room temperature for one hour. Virus-serum mixtures were inoculated into the allantoic cavity of SPF chicken embryos. The embryos were observed for 7 days. Eight days after inoculation, the eggs were opened and examined for lesions regarded as typical for infection with IBV. The r-values were calculated by the formula of Archetti and Horsfall [2]. The viruses were regarded as being serotypically different if their r-value was below 5% [9].

Pathogenicity test

One hundred one-day-old SPF chicks (Chi-Din Branch, Taiwan Animal Health Research Institute, Chi-Din, Taiwan) were divided into 10 groups with 10 chicks in each group. They were placed in positive pressure isolation units in different rooms and fed *ad libitum*. The antibody to IBV for the sera from one-day-old were determined to be free by the enzyme- linked immunosorbent assay system using KPL kits (Kirkegaard and Perry Labs, Inc., Gaithersburg, MD). Groups of 10 chicks were inoculated at one day of age by the intranasal route with $10^{4.3} \sim 10^{6.0}$ EID_{50} of the nine IBV isolates. The same amount of tryptose phosphate broth was inoculated into control chicks. After inoculation, the chicks were observed daily for 14 days, and clinical signs and mortality were recorded. At the end of the experiments, the survivors were killed humanely. The dead chicks and the chicks killed were necropsied. The clinical sign scores, lesion scores of infected chickens, as well as the pathogenicity of IBV isolates were calculated and interpreted according to the methods described by Avellaneda et al. [3] with small modifications. Briefly, the clinical signs system used was as follows: 0 = no clinical signs; 1 = lacrimation, slight head shaking and watery feces; 2 = lacrimation, presence of nasal exudate, depression, watery feces; 3 = same as 2 but stronger, severe watery feces. Lesions in trachea were evaluated as follows: 0 = no lesions; 1 = slight increase of mucin; 2 = large increase of mucin; 3 = large increase of mucin and mucosal congestion. Lesions in the kidney were evaluated as follows: 0 = no lesions; 1 = swelling, urate visible only under stereomicroscopy; 2 = swelling with urate; 3 = same as 2 with large amount of urate deposit in kidney. The pathogenicity index = no. of chicks with lesion score $\geq 1 +$ 1 point for every 10% mortality. Based on the values of these indexes, the isolates were divided into three pathogenicity groups: high ($\geq$19), intermediate (10$\sim$18), and low (1$\sim$9). Analysis of variance and least significant different method were used to test the differences in interval data [16] and Wilcoxon rank sum test was used for scoring data [17].

Accession numbers of standard IBV strains

The standard IBV strains included D1466 (accession number; M21971, J04329), V1397 (M21968, J04329), Ark99 (M99482), GAV-92 (U16157), CU-T2 (U04739), PP14 (M99483), Gray (L14069), JMK (L14070), SE17 (M99484), Holte (L18988), H120 (M21970, J04329), KB8523 (M21515), Beaudette (M95169, M27569), M41 (M21883), D3896 (X52084), UK/123/82 (X58067), D207 (M21969, J04329), 6–82 (X04723), UK/142/86 (X58066) and UK/167/84 (X58065). The Taiwan isolates and their accession numbers were A1211

(U38677), A1960 (U38678), A1171 (U38679), A1953 (U38680), A1955 (U38681), A2054 (U38683), A1967 (U38684), and A2012 (U38686).

Results

Grouping IBV stains

Similar results were obtained in the grouping based on the whole S1 gene (Fig. 1A) and on C2U-C3L region (Fig. 1B). In other words, the grouping based on C2U-C3L region could represent the grouping based on the whole S1 gene. The sequence of C2U-C3L region of S1 gene of IBV isolate A2296 is shown in Fig. 2, while sequences of other Taiwan isolates have been published previously [20]. When compared with the standard strains based on HVR 1, however, all Taiwan isolates were outside of all the standard strains. Taiwan isolates A1246, A1960, A1211, A1928, A1449, A1927, and A1171 belonged to Taiwan Group I (TW I), while A2012 and A2296 belonged to Taiwan Group II (TW II) (Fig. 3).

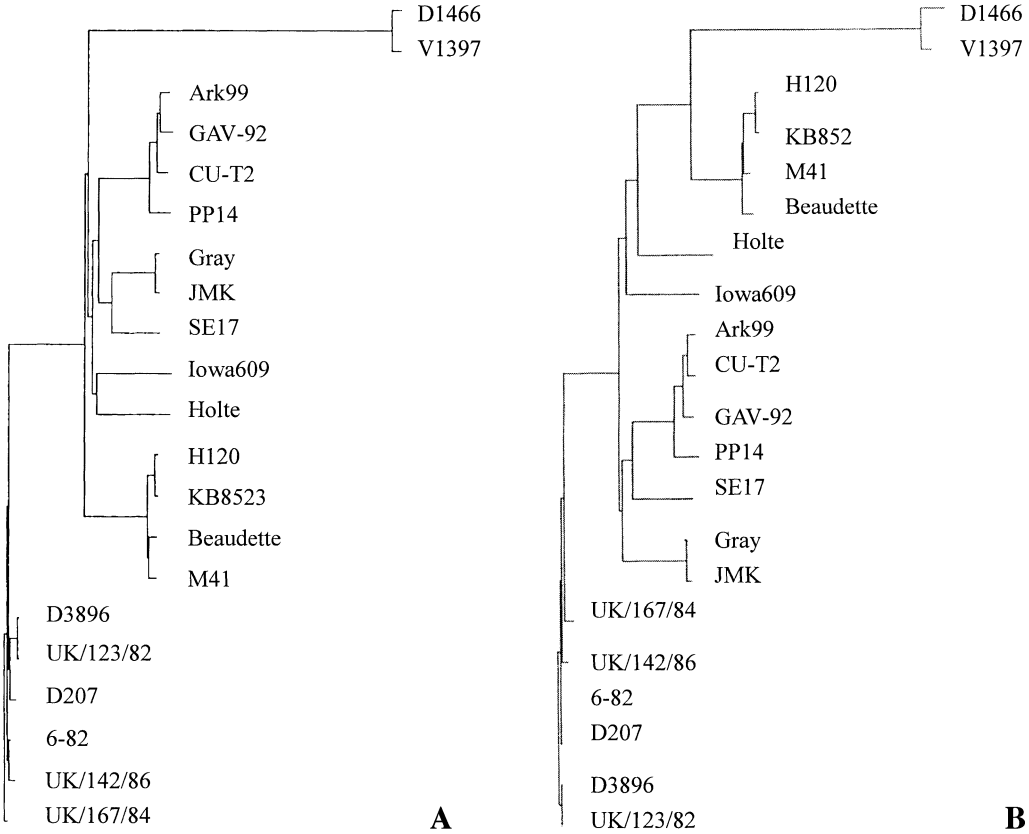

Fig. 1. Phylogenetic trees generated from the nucleotide sequences of the S1 genes (**A**) and HVR1 sequences (**B**) of published IBV strains obtained from GenBank + EMBL and IBV Iowa 609. Similar results were obtained from both grouping methods

TGGTTGGCAA TTACACGGGG GTGCTTATGC AGTAGTCAAT GTTACTACTC AATTTAACAA TGCAGGCAAT GCGTCTGTCT GTATTGGTGG
TAGTATTCAA GGCGGTTATG TATTTAATGC TTCTTCTGTA GCTATAACCG CTCCGAATAA TGGTATGACT TGGTCTACAC CACAATTTTG
TACTGCACAC TGCAATTTTT CGGACTTTAC AGTGTTTGTT ACCCATTG

Fig. 2. Sequence of PCR product of IBV, A2296. The first 20 nucleotides and the last 17 nucleotides were the primer set, C2U-C3L

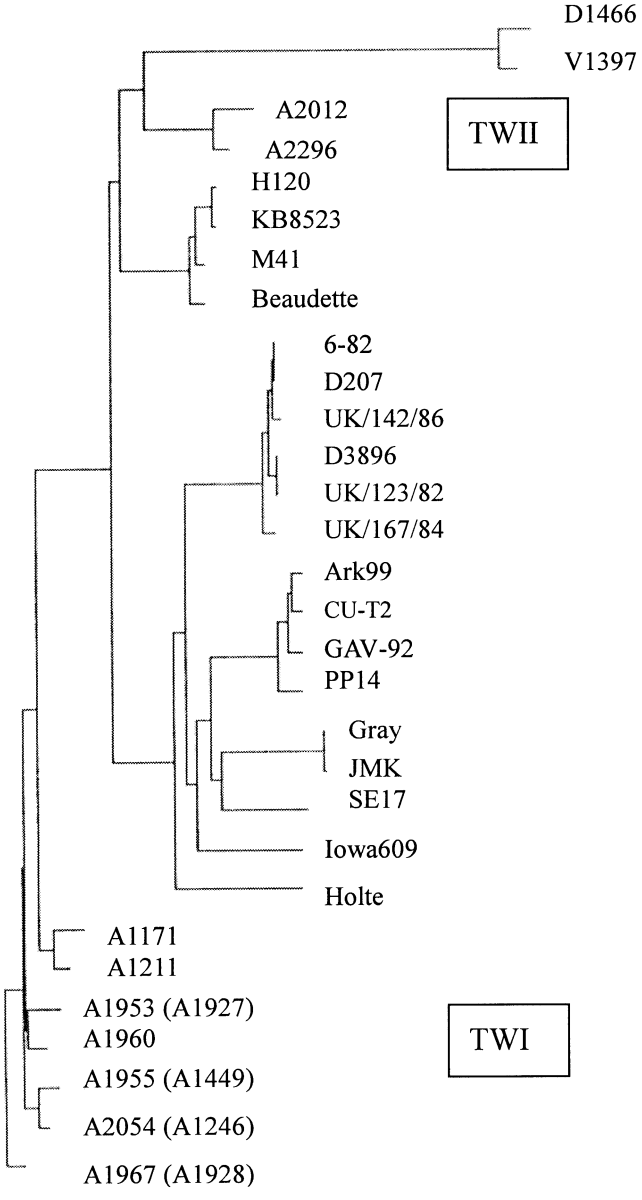

Fig. 3. Phylogenetic tree generated from comparing HVR1 sequences of Taiwan isolates and standard IBV strains. Taiwan isolates were outside of all the standard strains and divide into two distinct genetic groups, TW I and TW II. The HVR1 region of the isolates inside brackets had not been sequenced, but showed the same RFLP patterns with their corresponding isolates, which were showed in front, respectively

Table 1. Cross neutralization titers between IBV Taiwan isolates, Mass and Conn strains

Anti-serum	A1246	A1960	A1211	A1928	A1449	A1927	A1171	A2012	A2296	Mass	Conn
A1246	256[a]	133	64	32	8	36	≤ 4	< 4	11	10	4
A1960	501	454	398	103	87	126	128	256	< 4	22	22
A1211	186	29	644	89	16	32	7	4	11	9	< 4
A1928	22	28	64	146	4	6	< 4	< 4	< 4	6	5
A1449	6	9	12	49	87	32	< 4	4	< 4	16	13
A1927	64	82	32	50	64	534	256	< 4	< 4	32	16
A1171	89	6	8	11	16	16	126	5	8	8	32
A2012	7	< 4	< 4	6	< 4	< 4	< 4	158	16	13	11
A2296	5	32	10	16	< 4	7	< 4	32	106	34	4
Mass	24	13	< 4	7	< 4	11	< 4	4	< 4	256	–[b]
Conn	7	7	7	< 4	< 4	< 4	< 4	6	4	–	200

[a]Homologous titers with underlines
[b]Not done

Serotyping of IBV by one direction VN test

Two reference IBV strains and all nine Taiwan isolates were examined against anti-Mass or anti-Conn antisera. Viruses were considered to be members of the same serotype if the antiserum protected 50% or more of embryos [7]. None of the nine Taiwan isolates were protected by anti-Mass or anti-Conn antisera with reducing embryo mortality by 50% or more. So the nine isolates were neither Mass nor Conn serotypes.

Serotyping of IBV by cross VN test

Table 1 shows cross VN titers. Serum dilutions less than 1:4 were not determined. In general, homologous titers were higher than heterologous titers. Comparison of the homologous and heterologous titers showed that the viruses could be divided into two serotypes. The genotype TW I strains, IBV isolates A1246, A1960, A1211, A1928, A1449, A1927, and A1171 had higher titers than the isolates in Genotype Taiwan II, A2012, A2296. The r-values calculated from r1 and r2 are shown in Table 2. Almost all the isolates had $\leq 5\%$ values with Mass or Conn serotypes. The serotypes of Taiwan isolates differed from the two standard strains. Among the Taiwan isolates, the former seven isolates were of one serotype, and the latter two isolates belonged to another serotype. The serotype distinction was similar to that of the genetic grouping (Table 3).

Pathogenicity analysis

All infected chickens, except those in the control group, showed signs of head shaking and depression at 24 to 48 h after virus inoculation. Sick chickens showed varying degrees of coughing, sneezing, tracheal rales, open mouth breathing, and watery faeces. The clinical sign scores differed in different groups (Table 4).

Table 2. The r-values of IBV Taiwan isolates, Mass, and Conn strains

	A1246	A1960	A1211	A1928	A1449	A1927	A1171	A2012	A2296	Mass	Conn
A1246	100										
A1960	75.7	100									
A1211	26.9	19.9	100								
A1928	13.7	20.9	24.6	100							
A1449	4.6	14.1	5.9	12.4	100						
A1927	13.0	20.6	5.5	6.2	21.0	100					
A1171	<10.5	11.6	2.6	<4.9	<7.6	24.7	100				
A2012	<2.6	<11.9	<1.3	<3.2	<3.4	<1.4	<3.2	100			
A2296	4.5	<5.2	4.0	<6.4	<4.2	<2.2	<4.9	17.5	100		
Mass	6.1	4.9	1.5	3.4	<5.4	5.1	<3.1	3.6	<7.1	100	
Conn	2.3	4.1	<1.5	<2.6	<5.5	<2.4	<7.1	4.6	2.7	–	100

Table 3. Comparison of serotype and genotype for IBV Taiwan isolates

Serotype	Genotype	RFLP pattern	IBV
1	I	1–2B	A1246
	I	1–3B	A1960
1	I	1–3A	A1211
	I	1–1	A1928
1	I	1–2A	A1449
	I	1–3C	A1927
1	I	1–4	A1171
2	II	2	A2012
	II		A2296

Genotype and RFLP pattern see [20]

Mortality ranged from 0% to 100%. The main lesions were swollen and urate deposited in both kidneys, and mucin increased in trachea. Lesion scores varied from 4 to 25 in different groups. According to their pathogenicity indexes, IBV isolates could be divided into high, intermediate, and low pathogenicity. No relationship between pathotype and genotype was observed.

Discussion

Wang et al. [21] grouped the published IBV into 4 distinctly genetic groups, i.e. Mass, American, European (excluding Dutch) and Dutch by analyzing the whole S1 gene. Grouping based on the C2U-C3L region matches their S1 classification (Fig. 1). Taiwan isolates could not be classified into those 4 groups, using both grouping methods. Serologically, they are neither Mass nor Conn serotype. In our previous work, we reported a rapid method for typing IBV. In the present paper, we confirmed that the method correlates with serotyping results.

Table 4. The pathogenicity of IBV isolates in SPF chicks

Pathogenicity	Isoates	Clinical scores[a]	Mortality (%)	No. of chicks with scores > 1[b]		Pathogenicity index[c]
				trachea	kidney	
Control	–	0	0	0	0	0
High	A2296	2.77	100	8	7	25
Intermediate	A1211	2.22	60	6	5	17
	A1449	2.06	50	4	6	15
	A1928	2.02	50	5	5	15
	A1927	1.93	50	3	6	14
Low	A2012	1.65	40	1	3	8
	A1246	1.29	30	2	3	8
	A1171	0.79	20	2	2	6
	A1960	0.76	0	4	0	4

[a]Clinical scores range from 0 (normal) to 3 (severe)
[b]Lesion scores range from 0 (normal) to 3 (severe)
[c]Pathogenicity index = No. of chickens with lesion scores > 1+1 point for every 10% mortality
The pathogenicity indexes among high, intermediate, and low pathogenicity IBVs are highly significant different ($P<0.01$)

The spike glycoprotein gene of IBV codes for the N-terminal S1, which induces neutralization antibody comprising approximately 520 amino acids. There are 2 HVRs in S1 subunit, which comprise 56–69 and 117– 133 [5]. The residues within HVR 1 are associated with epitopes induce neutralization antibody. The HVR 1 in S1 is thought to be closely associated with major neutralization epitopes [10, 13, 15] and variation in this region correlates with serotype [5]. In addition, obtaining a short PCR product (228–231 bp) is easier than obtaining the whole S1 gene (1.6 kbp). Fortunately, there are two conserved sequences on both sides of HVR 1. Until now, all known Taiwan isolates can be amplified with this primer set. We have routinely used that primer set for genotyping our new isolates. The Taiwan isolates are divided into two groups, TW I and TW II [20]. In this paper, cross neutralization and one direction neutralization tests were performed for comparing the correlation between genotypes and serotypes. As predicted, serotyping results correlate with genotyping results, that genotyping based on HVR 1 is suitable for typing IBV, especially for nephrotropic isolates because almost all our isolates are nephrotropic [18].

Archetti and Horsfall [2] expressed the degree of relatedness between viruses by a numerical value r but did not define serotypes in terms of r-values. According to other reports, two viruses are considered to be different serotypes when their r-value is less than 5% [9, 14]. However the r-values of some isolates in a serotype were not always above 5%. For example, the r-value of A1246 and A1449 was below 5% (4.6% in Table 2). They are considered to be the same serotype because other isolates in the same serotype had r-values higher than 5% with either of those two isolates. For reoviruses, the r-value in different serotypes is less than

10%. Those in different subtypes within a serotype give a range of 11% to 32% [8]. The subtype based on the present serologic results does not correlate with the results obtained from genotyping. Antisera to the viruses with different restriction fragment length polymorphism patterns within a group have varying titers. From the serologic r-value data, A1246 is closer to A1960 than any other isolates in the same serotype, so they beong to the same subtype. A1211 and A1928, A1449 and A1927 belong to different subtypes. In addition, A1171 is in another subtype. Although several subtypes are present, those subtypes do not correlate with their restriction fragment length polymorphism patterns. In spite of lack of correlation between the two typing methods for different subtypes, a good correlation is present among different groups. In conclusion, the genotyping method based on HVR 1 developed by our laboratory is a convenient and useful method for typing a new IBV isolate. However, the pathotypes of IBV isolates does not correspond to their genotypes based on HVR1 analysis. This phenomenon is predictable because HVR1 is responsible for neutralization epitope, not pathogenicity. The genes determining pathogenicity of IBV need further investigation.

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