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Genetic grouping for the isolates of avian infectious bronchitis virus in Taiwan

C.-H. Wang and **C.-T. Tsai**

Department of Veterinary Medicine, National Taiwan University, Taipei, Taiwan

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Summary. In order to differentiate recent isolates of avian infectious bronchitis virus (IBV) in Taiwan, polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP), and direct sequencing methods were used to type 25 IBV Taiwan isolates. Two conserved sequences that flank the hypervariable region I (HVR I) in the N-terminus of S1 protein gene were chosen as primers. Sequences of 228–231 base pairs (bp) were amplified by PCR from 25 Taiwan isolates and 4 reference strains (H120, Conn, JMK, Holte). PCR products were digested with 5 restriction endonucleases, *Bso*FI, *Dde*I, *Mbo*II, *Alu*I, *Rsa*I, and different IBV isolates were grouped according to their RFLP patterns. The RFLP patterns of the 4 reference strains in this study matched the published sequences in GenBank. Except 1 vaccine strain, the other 24 Taiwan isolates were different from these 4 and 18 other IBV strains whose sequences were published. The data from PCR-RFLP and sequencing of IBV genomes showed that the 24 Taiwan isolates can be divided into 2 distinct groups, I and II. Seven RFLP patterns are identified in group I and only 1 in group II.

Introduction

Avian infectious bronchitis virus (IBV) causes a highly contagious respiratory disease in chickens which is economically devastating to farmers who raise birds under intensive conditions [10]. The isolation and identification of IBV isolates throughout Taiwan are important, because vaccines are selected based on the serotypes of IBV in specific geographic areas. Traditionally, IBV serotypes are identified by the virus-neutralization (VN) test which is labor-intensive and time-consuming. However, ambiguous results are sometimes obtained using a serological approach [8, 17]. Recently, restriction fragment length polymorphism (RFLP) and sequencing have offered more sensitive and specific methods for identifying IBV strains [2–6, 16]. All known IBV viruses contain three major virus-encoded structural proteins which include the surface projection glycoproteins, the membrane protein, and the nucleocapsid protein [9, 10]. The VN

antibodies that normally form the basis for comparison of IBV isolates are induced largely by the N-terminus of the S1 protein [7, 9, 11]. In the present study, we used reverse transcription polymerase chain reaction (RT-PCR) coupled with restriction fragment length polymorphism (RFLP) of the RT-PCR products to classify our isolates. Using these techniques, we present a convenient method for identifying the RFLP patterns of IBV isolates in Taiwan which are different from those of known IBV strains.

Materials and methods

Viruses

Twenty-five Taiwan isolates of IBV were used in this study. They were collected between 1991 and 1995 (A1121, A1131, A1171, A1210, A1211, A1246, A1449, A1793, A1916, A1927, A1928, A1950, A1952, A1953, A1955, A1960, A1963, A1967, A1972, A1980, A2003, A2012, A2054, A2056, A2137). Seventeen of the twenty-five isolates came from flocks that had been vaccinated with a eye drop method using IBV vaccines (mostly H120) on day 1 or day 4. Four reference strains were also used which included H120 (a vaccine strain from Intervert, Holland), Conn, JMK, and Holte. These reference strains were provided by Taiwan Provincial Research Institute for Animal Health (PRIAH, Tamsui, Taiwan), which were orginated from USA. The viral isolates and reference strains were propagated in 10-day-old specific-pathogen-free (SPE) embryonated chicken eggs (PRIAH) at 37 °C.

Grouping of published avian infectious bronchitis virus

The S1 gene nucleotide sequences of 21 IBV strains in GenBank + EMBL and IBV Iowa 609 from Dr. Collison (Texas A&M University, College Station, Texas, USA) were analyzed and grouped using the Clustal method in the Megalign program (Lasergene, Madison, USA). The 21 IBV strains used for the computer analysis included D207, 6/82, UK/142/86, D274, D3896, UK/123/82, UK/167/84, UK/918/67, Gray, JMK, PP14, Ark99, SE17, Holte, H120, KB8523, Beaudette, M41, Conn, D1466, and V1397.

PCR oligonucleotide primers

Four sets of primers successfully used by other [1, 12, 13, 18] and one new set of primers were used in this study. The published primers included S1 [12], S2 [13], M-N [1], and N primers [18]. For the new primers, the S1 sequence of the 22 published IBV were analyzed using the Clustal method to find conserved sequences. The new primers (C2U-C3L) were located in the N-terminus of the S1 gene which contains hypervariable region I (HRV I, nucleotides, nt 142–320); this region is reponsible for induction of antibodies to the IBV that have been the basis for defining of different IBV serotypes [9, 11]. The sequences and locations of the new primers used for the PCR were C2U: TGGTT GGCAT TTACA CGGGG (114–133) and C3L: CAATG GGTAA CAAAC AC (341–325, as in Beaudette strain).

RNA extraction

Viral RNA was extracted as described [12] with some modification. Briefly, guanidine thiocyanate (Sigma), and NaOAC were added to allantoic fluid, and extracted with phenol-chloroform-isoamylalcohol. The RNA was further precipitated in isopropanol (Merck) and resuspended in alcohol and stored at −20 °C until it was used in RT-PCR.

RT-PCR

All glassware and double distilled water (DDW) were treated with 0.1% diethyl- pyrocarbonate (Sigma) for removing RNase contamination. The RT-PCR was accomplished in one step. The mixture included 10 μl of pfu polymerase 10 × buffer (Stratagene, USA), 8 μl of 2.5 mM dNTP (Stragene), 0.4 μl of 40 U/μl RNasin (Promega), 0.3 μl RTase (AMV, Promega), 1 μl primers (20 μM), 0.8 μl of 2.5 U/μl pfu polymerase (Stragene), 60 μl DDW and 20 μl viral RNA. The RT-PCR was conducted in a FTS-960 thermal cycler (Corbett Research, Australia) where the RT was performed at 42 °C for 1 h. PCR was then performed during 35 cycles of denaturation at 94 °C for 30 sec, annealing at 45 °C for 1 min, and polymerization at 72 °C for 1 min. The final polymerization step was conducted at 72 °C for 7 min. The PCR products were analyzed on a 2% gel containing ethidium bromide (0.5 μg/ml), and the amplified DNA was detected using an ultaviolet transilluminator.

Specificity of the PCR amplification

Rapid RNA extraction was conducted on allantoic fluid from uninoculated eggs and from eggs inoculated with Newcastle disease virus (A970, National Taiwan University), Egg drop syndrome virus (PRIAH), fowl adenovirus type I (PRIAH), infectious bursal disease virus (PRIAH) and infectious laryngotracheitis virus (Vaccine strain, Intervet, Holland). Extraction of RNA and RT-PCR were performed using the same methods described above.

Restriction endonuclease selecting and restriction fragment length polymorphism analysis

Five restriction endonucleases, *Bso*FI, *Dde*I, *Mbo*II, *Alu*I, and *Rsa*I, were chosen based on the 22 published IBV sequences using the Mepdraw program (Lasergene). The predicted PCR products were equally divided into five tubes, and digested with each enzyme (BioLabs, Beverly, MA, USA) under the conditions recommended by the enzyme supplier. The restriction fragment patterns were observed following electrophoresis on a 2.5% metaphor (FMC Bioproducts, USA) + 1% pure agarose gel (BBL) at 100 V constant voltage. The 25 Taiwan isolates were grouped according to their RFLP patterns.

Sequencing of PCR products

The amplified DNA was amplified again using the same primers (C2U-C3L) to obtain pure PCR products (secondary PCR). The secondary PCR products were sequenced by the dideoxynucleotide method using Taq DyeDeoxy Terminator Cycle Sequencing kit (ABI 3370, Applied Biosystems, USA). Four kinds of fluorescent dye-labeled ddNTPs were added with a single-strand primer. The sequences was analyzed by an automatic DNA sequencer (Applied Biosystems). Both strands C2U-C3L PCR products were sequenced for each isolates to ensure the accuracy of results. The Megalign program (Lasergene) was used to align the nucleotide sequences and to generate a similarity plot. If the identity was less than 80%, isolates were classified into different groups (I and II). A phylogenetic tree was drawn using the Megalign program (Lasergene).

Results

Grouping of avian infectious bronchitis virus strains

By using the Clustal method in the Megalign program (Lasergene), the 22 published IBV strains in GenBank and EMBL were divided into 4 distinct genetic groups, i.e. Mass, American, European and Dutch when analyzing the

Table 1. Amplification of IBV by RT-PCR data using different primer sets

Reference IBV strains	Primer sets				
	S1	S2	M-N	N	C2U-C3L
H120	+	+	+	+	+
JMK	+	+	−	+	+
Holte	−	+	+	−	+
Conn	+	−	−	+	+
Positive rates IBV Taiwan isolates	3/4	3/4	2/4	3/4	4/4
A1121	−	+	−	−	+
A1131	−	−	−	−	+
A1171	−	−	−	−	+
A1210	−	+	−	+	+
A1211	−	+	−	+	+
A1246	−	+	−	+	+
A1449	−	−	−	+	+
A1793	ND	+	−	+	+
A1916	+	ND	ND	ND	+
A1927	ND	+	−	+	+
A1928	ND	−	−	+	+
A1950	ND	−	−	+	+
A1952	ND	−	−	−	+
A1960	ND	−	−	+	+
A1967	ND	−	−	−	+
Positive rate	1/8	6/14	0/14	9/14	15/15

ND Not done. + PCR product positive. − no PCR product. S1 primers, PCR product including the whole S1 gene [12]. S2 primers, PCR product locating the N-terminus of S2 gene [13]. M-N primers, PCR product including the junction part of M and N genes [1]. N primers, PCR product including N gene [18]. C2U-C3L primers, a new set of primers at the N-terminus of S1 gene

whole S1 gene as well as the C2U-C3L (nt 114–341) region in the N-terminus of the S1 gene. The data obtained from analysis of the C2U-C3L region resulted in the same classification as those obtained from the whole S1 gene.

Amplification of IBV genes using different primers

Only 2 or 3 of the 4 reference strains could be amplified using the published primers (Table 1). Positive responses with the Taiwan isolates were even lower using these published primers. By contrast, the new primers (C2U-C3L) which

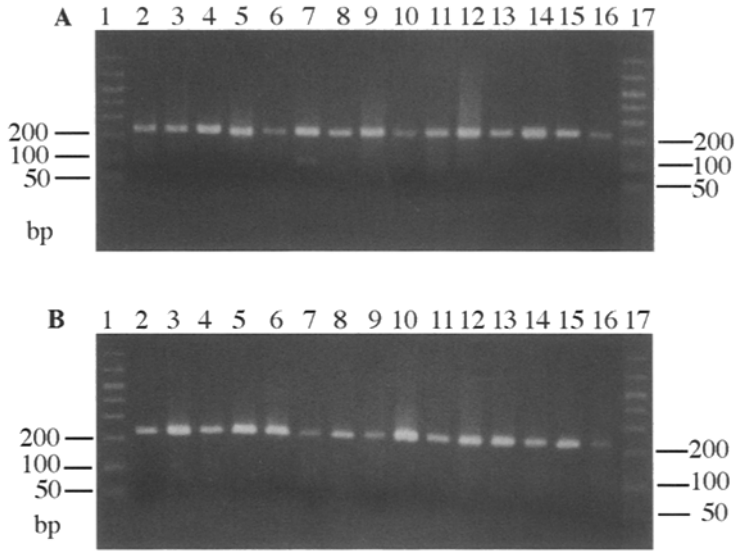

Fig. 1. Agarose gel electrophoresis of polymerase chain reaction (PCR) products produced by amplifying the reference strains and Taiwan isolates of infectious bronchitis virus (IBV) using C2U-C3L primers. **A** *1*, *17* GelMarker (50–1000 bp) (Promega), *2* H120, *3* JMK, *4* Holte, *5* Conn, *6* A1121, *7* A1131, *8* A1171, *9* A1210, *10* A1211, *11* A1246, *12* A1449, *13* A1793, *14* A1916, *15* A1927, *16* A1928. **B** *1*, *17* GelMarker (50–1000 bp), *2* A1950, *3* A1952, *4* A1953, *5* A1955, *6* A1960, *7* A1963, *8* A1967, *9* A1972, *10* A1980, *11* A2003, *12* A2012, *13* A2054, *14* A2056, *15* A2137, *16* H120

flanked the HVRI detected all 4 reference strains and all new Taiwan isolates. The PCR products of the 4 strains and all isolates tested in this study appeared to be approximately 231 base pairs (bp) in length (Fig. 1).

Restriction fragment length polymorphism patterns of reference strains

The RFLP patterns of the 4 reference strains generated by digestion with *Bso*FI, *Dde*I, *Mbo*II, *Alu*I, and *Rsa*I are shown in Fig. 2. The fragments predicted from the published sequences in GenBank is shown in Table 2. There was only one apparent exception noted in lane 5 of Fig. 2A where *Mbo*II was supposed to generate 3 fragments, 54, 64, and 110 bp but only 2 bands were seen (110, 60 bp). Apparently the two smaller fragments migrated together. Figure 2 and Table 2 summarize the RFLP patterns of the 4 reference strains by showing the presence or absence of cleavage sites in each strain.

Restriction fragment length polymorphism patterns of Taiwan isolates

The RFLP pattern of one Taiwan isolate (A1916) was similar to H120. This isolate was isolated from chickens diagnosed with Newcastle disease virus and not IBV infection. This isolate was confirmed to be a vaccine virus. The RFLP

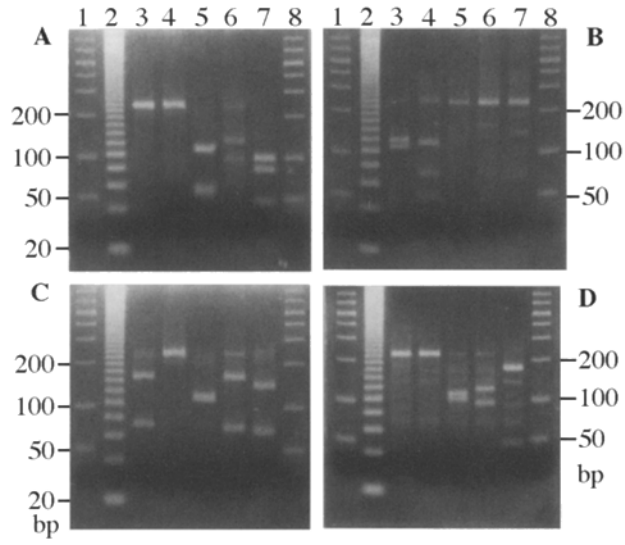

Fig. 2. RFLP patterns of reference IBV strains. *1*, *8* GelMarker (500–1000 bp), *2* 20 bp ladder marker. The restriction endonucleases used are *3* *Bso*FI; *4* *Dde*I; *5* *Mbo*II; *6* *Alu*I; *7* *Rsa*I. Numbers of fragment; **A** H120: 1, 1, 2, 2, 3; **B** JMK: 2, 3, 1, 1, 1; **C** Holte: 2, 1, 1, 2, 2; **D** Conn: 1, 1, 2, 2, 2 (these data are given in Table 2)

Table 2. The sizes (base pairs) of fragments of 4 reference IBV strains generated by different restriction endonucleases

IBV strains	*Bso*FI	*Dde*I	*Mbo*II	*Alu*I	*Rsa*I
H120	228	228	54	93	43
			64	135	86
			110		99
JMK	118	45	228	228	228
	110	65			
		118			
Holte	72	228	114	71	24
	156		114	157	67
					137
Conn	216	216	102	97	47
			114	119	169

patterns of the other 24 Taiwan isolates in this study differed from those of the C2U-C3L S1 gene of the 4 published strains described above plus 18 other published strains whose sequences were analyzed by the Megalign program (Lasergene). The RFLP of these strains were compared and divided into 8 RFLP patterns (Table 3 and Fig. 3). The representative RFLP patterns are shown in Fig. 3. One of the examples was isolate A1967. The numbers of fragments of this isolate digested with these 5 restriction endonucleases were 1, 1, 3, 2, 2 (Fig. 3A).

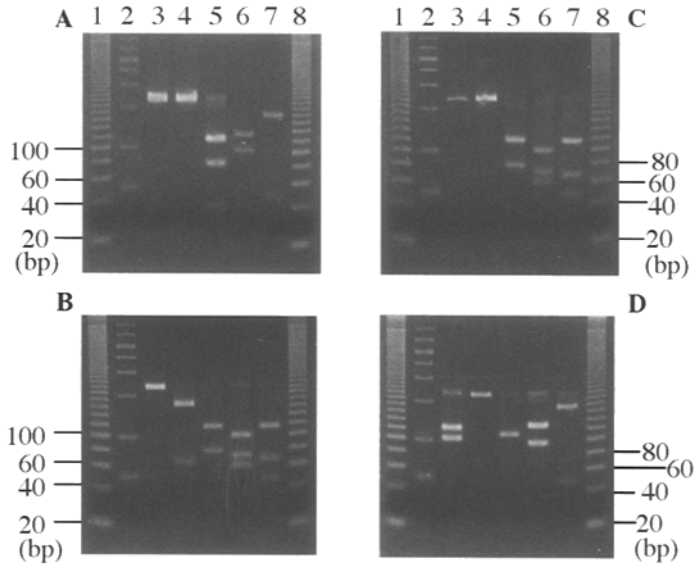

Fig. 3. RFLP patterns of IBV Taiwan isolates. *1*, *8* Ladder marker. *2* GelMarker (50–1000 bp). The restriction endonucleases used are *3 Bso*FI; *4 Dde*I; *5 Mbo*II; *6 Alu*I; *7 Rsa*I. *A* WT1-1, IBV isolate no. A1967; fragment numbers: 1,1,3,2,2. *B* WT1-2A, IBV isolate no. A1955; fragment numbers: 1,2,3,3,3. *C* WT1-2B, IBV isolate no. A2054; fragment numbers: 1,1,3,3,3. *D* WT2, IBV isolate no. A1121; fragment numbers: 2,1,1,2,2. The PCR product is cut by *Mbo*II into 2 identical fragments. Only one band is found in *5* except the original uncut PCR product (these data are given in Table 4)

Table 3. Genetic grouping of Taiwan IBV isolates

Genetic groups	RFLP patterns	IBV isolates					
M	Mass	A1916[a]					
I	WT1-1	A1928	A1967[a]				
	WT1-2A	A1449	A1955[a]	A1963	A2003[a]		
	WT1-2B	A1246	A1793	A1950	A1972	A2054[a]	A2056
	WT1-3A	A1131	A1211[a]				
	WT1-3B	A1960[a]					
	WT1-3C	A1927	A1953[a]				
	WT1-4	A1171[a]					
II	WT2	A1121[a]	A1210	A1952	A1980[a]	A2012[a]	A2137

[a]Isolates that are sequenced

The RFLP pattern of isolate A1928 was the same as that of A1967 and therefore the two isolates were considered to be the same RFLP pattern (WT1-1 Table 3). The RFLP patterns of isolates A1121, A1210, A1952, A1980, A2012 and A2137 were the same, and were quite different from the isolates in WT1 and grouped into WT2 (Fig. 3D).

Sequence of PCR products

Following the grouping of IBV isolates by PCR-RFLP, 12 representative isolates were selected from 8 RFLP patterns for direct sequencing (with "a" in Table 3). The length of the PCR products of A1211, A1960, A1953, A1955, A2003, A2054, A1967 was 231 bp and that of A1171, A1916, A1980, A2012, and A1121 was 228 bp. The sequences of the 12 isolates are shown in Fig. 4. The sizes of the fragments generated by restriction endonucleases are shown in Table 4. Since *Mbo*II cut the PCR product into 2 identical fragments (114 bp), only one band was found in the PCR product from WT2. (Fig. 3D).

The similarity in Taiwan isolates ranged from 65.4% to 99.6% (Table 5). The sequence relationships of the HVR I were shown in a phylogenetic tree (Fig. 5). The sequence of isolate A1916 was the same as the Mass serotype. Another 11

Table 4. The sizes (base pairs) of fragments of Taiwan isolates generated by different restriction endonucleases

RLFP Patterns	*Bso*FI	*Dde*I	*Mbo*II	*Alu*I	*Rsa*I
WT1-1	231	231	36	100	47
			78	131	184
			117		
WT1-2A	231	63	36	60	47
		168	78	71	67
			117	100	117
WT1-2B	[a]	231	[a]	[a]	[a]
WT1-3A	231	63	36	100	47
		168	78	131	91
			117		93
WT1-3B	[a]	[a]	[a]	[a]	43, 47, 141
WT1-3C	[a]	[a]	[a]	[a]	47, 184
WT1-4	66	63	111	100	47
	162	165	117	128	181
WT2	103	228	114	97	47
	125		114	131	181

[a] Same as above

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A1211   TGGTTGGCAT TTACACGGGG GCGCTTATGC AGTAGTAAAT GTTTCTTCAG AAACTAACAA
A1960   .......... .......... .......... .......... .......... ..........
A1171   .......... .......... .......... .........- A.....T.. ..........
A1953   .......... .......... .......... .......... .......... ..........
A1955   .......... .......... .G........ .......... .........C ..........
A2003   .......... .......... .......... .......... .........C ..........
A2054   .......... .......... .......... .......... .......... ..........
A1967   .......... .......... .......... .......... .......... ..........
A1980   .......... .......... .T........ ......T... ...A..A.TC G.TACGC...
A2012   .......... .......... .T........ ......T... ...A..A.TC G.TACGC...
A1121   .......... .......... .T........ ......T... ...A..AATC ..TA.GC...
A1916   .......... .......... .T..G..... G..T..T... A.....AGT. ..T....T..

A1211   TGCAGGTTCT GCTTCAGAAT GCACTGTTGG TACTATTAGA GGTGATAGAG TTGTCAATGC
A1960   .......... .......C.. .......... ..T......T ....G....A ....T.....
A1171   .......G.. .......... .....AA... ..T......T ....G...T. G.T.......
A1953   .....A.A.. .....G.... .......... ..T......T .......... ....T.....
A1955   .......A.A ...CA..G.. .......... ..T......T .......... ....T.....
A2003   .......A.A ...CA..G.. .......... ..T......T .......... ....T.....
A2054   .......A.A ...CA..... .......... ..T......T .......... ....T.....
A1967   .......CA. .......... .......... ..TAT....G .......... ....T.....
A1980   ......CAA. ..G..T.TC. .T.T..G... ..G....CA. ..C.GCTAT. CAA.T.....
A2012   ......CAA. ..G..T.TC. .T.T..G... ..G....CA. ..C.GCTAT. CAA.T.....
A1121   ......CA.. ..GC.T.T.. .T.T..G... ..G...CCA. A.C.GCTAT. .AT.T.....
A1916   ......C... T.A..T.GG. .T........ ..T....CAT ....G.C.T. ....T.....

A1211   CTCTTCTATA GCTATGACAG CACCTGTAGG TCGAGGTATG CAGTGGTCTA AGTTACAATT
A1960   .......... .......... T......... ..A....... .......... ...C......
A1171   .......... .......... .......... ..C....... .......... ...C......
A1953   .......... ........GA .......... ..A....... A.T....... ...C......
A1955   .......... ......T... .......... ..A....... .......... ...C......
A2003   T......... .......... .......... ..A....... .......... ...C......
A2054   T......... .......... .......... ..A....... .......... G..C......
A1967   .......... .......... .......... ..AC...... GG........ .A.C......
A1980   T......G.. ........C. .T.----GAA .AAT...... ACT....... CAAC......
A2012   T......G.. ........C. .T.----GAA .AAT...... ACT....... CAAC......
A1121   T......G.. ........C. .T.----GAA .AAT...... ACT....... CAGC......
A1916   T......... ........G. .......GTC ATC....... GCT....... GCAGT..G..

A1211   TTGTACTGCA CACTGTAATT TTTCGGATTT TACAGTGTTT GTTACCCATT G
A1960   .......... .......... .......... .......... .......... .
A1171   .......... .......... .......C.. .......... .......... .
A1953   G......... .......... .........C .......... .......... .
A1955   .......... .....C.... ....T..... .......... .......... .
A2003   G......... .....C.... ....T..... .......... .......... .
A2054   .......... .....C.... ....T..... .......... .......... .
A1967   .......... .....C.... ....A..... .......... .......... .
A1980   .......... .....C.... .......AG. .......... ..........'.
A2012   .......... .....C.... .G.....AG. .......... .......... .
A1121   .......... .....C.... .....C.AG. .......... .......... .
A1916   .C........ T.......C. ....A...AC .......... .......... .
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Fig. 4. Sequences of PCR products of 12 representative Taiwan isolates. The first 20 nt (114–133) and the last 17 nt (325–341) are the new primers used in this study (C2U and C3L primers). The positions where nt bases missing are indicated as dashes

isolates were divided into 2 distinctly different genetic groups. The results of RFLP patterns and gene sequences of the Taiwan isolates indicated that the 24 isolates could be classified into 2 groups (I and II) and 8 RFLP patterns (WT1-1, WT1-2A, WT1-2B, WT1-3A, WT1-3B, WT1-3C, WT1-4 in group I; WT2 in group II).

Table 5. Percentage similarity and percentage divergence between different IBV strains

A

Percent Similarity

Percent Divergence

	1	2	3	4	5	6	7	8	9	10	11	12		
1		96.1	88.2	93.1	93.1	93.1	93.9	93.1	70.2	69.7	69.3	74.6	1	A1211
2	3.9		88.2	93.1	93.9	93.9	94.4	93.5	71.1	70.6	69.7	75.4	2	A1960
3	6.1	6.1		83.8	83.8	83.8	85.1	84.2	67.1	66.7	65.4	68.4	3	A1171
4	6.1	5.6	8.8		91.3	92.6	93.1	91.8	69.7	69.3	69.7	75.4	4	A1953
5	6.5	5.6	9.2	7.4		98.3	97.4	92.6	68.9	68.4	70.2	72.8	5	A1955
6	6.5	5.6	9.2	6.5	1.7		98.3	92.6	69.7	69.3	71.1	73.7	6	A2003
7	5.6	5.2	8.3	6.5	2.6	1.7		93.5	70.2	69.7	71.1	74.1	7	A2054
8	6.5	6.5	9.6	7.8	7.4	7.4	6.5		70.6	70.2	68.9	73.2	8	A1967
9	24.1	23.7	24.0	23.7	24.1	23.7	23.7	23.2		99.6	94.3	75.0	9	A1980
10	24.6	24.1	24.4	24.1	24.6	24.1	24.1	23.7	0.4		93.9	74.6	10	A2012
11	24.1	23.7	23.6	23.7	23.2	22.8	22.8	24.1	5.3	5.7		74.1	11	A1121
12	21.5	20.6	21.3	20.6	22.8	22.4	21.5	22.4	23.2	23.7	23.7		12	A1916
	1	2	3	4	5	6	7	8	9	10	11	12		

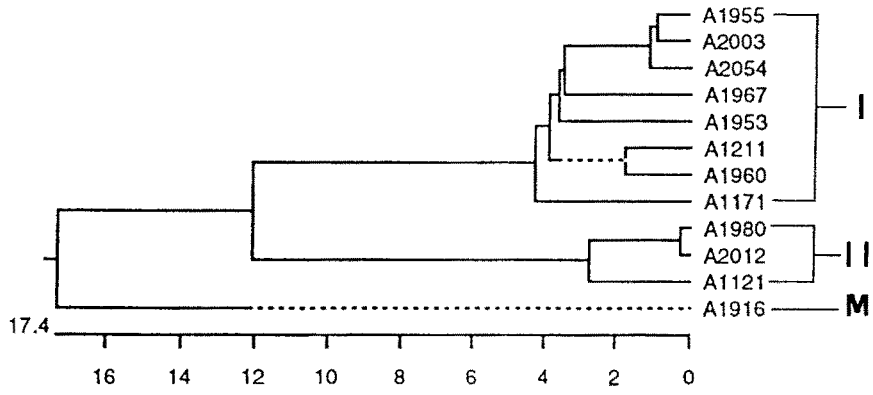

Fig. 5. Phylogenetic tree of 12 Taiwan isolates based on the sequences of PCR products. Except for one vaccine isolate (Mass, *M*), the Taiwan isolates are divided into 2 distinct genetic groups

Discussion

Due to high sequence variability between different IBV strains, it has been difficult to design PCR primers that can be reliably used to monitor all IBV isolates, especially in the S1 gene that contains hypervariable regions. In this study, we report a set of olignucleotide primers that is capable of hybridizing to the genome of all known IBV strains. The primers have now been used successfully on 4 known strains and 25 Taiwan isolates. These primers were intended to amplify a portion of the IBV genome that encodes the viral coat protein providing the antigenic determinations. This region of the S1 gene is hypervariable and apparently mutates often enough to thwart efforts to create a universal effective vaccine. The 24 isolates are related distinctly and are different from all known strains of IBV (Mass, American, European and Dutch).

The immunogenicity of Taiwan isolates needs further study by comparing with reference IBV strains.

In the past, the 22 published IBV strains have been divided into 4 groups, i.e. Mass, American, European and Dutch [15]. The results from this study are entirely consistent with that classification. The strains within the Mass, European and Dutch groups are at least 95% homologous. The C2U-C3L region (PCR oligonucleotide primers in Materials and methods) was chosen for the study reported here because phylogenetic results from the sequences in this region match those from the whole S1 gene. The 24 Taiwan isolates differ from those published foreign strains and are, therefore variant strains. Since the 24 isolates have been divided into 8 RFLP patterns it is unlikely that the IBV isolates in Taiwan have evolved from the same origin. However, the nature of their evolution is totally unknown and remains to be studied. For this, it will be necessary to type foreign IBV isolates by PCR-RFLP using the same primers (C2U-C3L) so that they can be compared to the Taiwan isolates.

The variation of IBV may also be attributed to recombination following co-infection of two distinct strains [14]. Several investigators have characterized the isolates obtained from IBV outbreaks, and showed that they had a strong relationship with the vaccine strain, suggesting that the prevalent isolates may have originated by recombination with live vaccine strains [14]. By contrast, our present observation that the 24 isolates are different from the reference strains, including current vaccine strains, suggests that they are not derived from live attenuated vaccines.

IBV causes severe poultry losses in young broiler populations despite the use of Mass serotype vaccines in Taiwan. An accurate determination of which serotypes or subtype are causing field outbreaks would be valuable to the poultry industry. Although attempts have been made to classify IBV isolates by serologic methods, it has become apparent that serology alone in not adequate [8, 17]. PCR-RFLP is a convenient method to classify new isolates, since the C2U-C3L primer set detects all Taiwan isolates. It is necessary to survey whether the prevalent IBV isolates are similar to or different from current vaccine strains. The pathogenicity and immunogenecity of different RFLP patterns of the Taiwan isolates needs further study. Since these isolates are quite different from vaccine strains used currently, it is necessary to develop a local vaccine in order to control IBV infection in Taiwan.

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Authors' address: Dr. C.-H. Wang, Department of Veterinary Medicine, National Taiwan University, 142 Chou-Shan Road, Taipei 106, Taiwan.

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