Arch Virol (1991) 116: 19–31

Archives of Virology

A new typing method for the avian infectious bronchitis virus using polymerase chain reaction and restriction enzyme fragment length polymorphism

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Accepted August 22, 1990

Summary. Two primers with the length of 22 bases each and 400 bases apart on the spike protein gene of avian infectious bronchitis virus (IBV) were prepared. Using these primers, the genome RNA from twelve strains of the various serotypes were reverse-transcribed to cDNA and amplified by polymerase chain reaction (PCR). With all strains, 400 base DNA was amplified, indicating that there were no apparent insertions or deletions in this region. However, the amplified DNA showed different cleavage patterns by the restriction enzymes. These 12 strains were classified into 5 groups. The strain typing based on a comparison of the cleavage patterns was consistent with the previous serological typing. This study thus provides a simple and rapid method for typing of IBV.

Introduction

The avian infectious bronchitis virus (IBV) is a pathogen with worldwide distribution, which causes a highly contagious respiratory affliction in young chickens or reduces an egg production in laying hens. Natural outbreaks of IBV are controlled through the use of a vaccine. However, infectious bronchitis (IB) occurs occasionally even among vaccinated flocks. Various strains which have been isolated from these flocks are serologically unrelated to previous strains [14, 19, 23]. Appearances of antigenic variant of IBV thus cause a major problem in poultry industry.

Serotypes of IBV have been classified on the basis of reciprocal neutralization tests measured by LD_{50} in hen eggs [17], plaque reduction in chicken kidney (CK) monolayer cell cultures [16, 21], reduction of cilia movement in chicken tracheal organ cultures [15, 18, 22], or a hemagglutination-inhibiton (HI) method [1, 24, 25]. In some cases, these different tests lead to conflicting results concerning strain relationships [26]. Molecular markers were recently introduced for virus classification. The molecular weights of proteolytically cleaved

viral polypeptides were compared by Nagy and Lomniczi [30], and the oligonucleotide finger printing analyses of viral RNAs were studied by Clewley et al. [13] and Kusters et al. [26].

IBV, which belongs to the family *Coronaviridae*, has a single-strand RNA genome of positive polarity, 27.6 kb in length [35, 36]. The genome organization and mRNA has been studied in detail [2, 4, 5, 38]. Its virion contains three major structural proteins, nucleocapsid (N) protein, membrane (M) protein and spike (S) protein [7]. The S protein is encoded by mRNA E and is further cleaved into two glycopolypeptides, S1 (Mr 90k) and S2 (Mr 84k) [2, 8, 9]. S1 glycopolypeptide is the target of neutralizing and hemagglutination-inhibiting monoclonal antibodies [29]. Nucleotide sequences of the S gene have been published for Beaudette [2], M41 [31], 6/82 [3], and KB8523 [39] strains.

To investigate serotypic variations in IBV at the molecular level, the comparison of sequences of the S gene would be useful. As an initial approach along these lines, we used polymerase chain reaction (PCR) [33,34] and restriction enzyme fragment length polymorphism (RFLP) analysis [6, 37].

In this study, we amplified a portion of the S gene of IBV using specific oligonucleotide primers and thermostable DNA polymerase, then analyzed the RFLP patterns of amplified DNA. Based on the RFLP patterns, we have classified twelve strains of IBV into five groups and discussed the serotypic relationships between them.

Materials and methods

Virus strains, growth, and RNA extraction

The IBV strains used in this study, listed in Table 1, included 7 major American strains and 5 Japanese strains of various serotypes. Three strains, Ishida, ON and C-78, are currently used for vaccination in Japan.

Stock viruses were inoculated into allantoic cavities to 11 day-old embryonated chicken eggs, which were incubated at 37 °C for 24 h. After being kept at 4 °C overnight, 10 to 20 ml of allantoic fluid was obtained from the inoculated eggs and centrifuged at 3,000 rpm for 10 min. The supernatant was centrifuged at 32,000 rpm for 120 min (Kontron, TFT70 rotor). The pellet was resuspended in a lysis buffer containing 10 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 1% (w/v) SDS and 50 mM NaCl. Viral RNA was then extracted with phenol/chloroform/isoamyl-alcohol (50:48:2) several times, ethanol precipitated, and redissolved in 20 µl of distilled water, which corresponded to 1/1000 volume of the original allantoic fluid (RNA solution).

Chemicals and enzymes

Restriction enzymes used in this study were purchased from Boehringer Mannheim Biochem. Co. (Federal Republic of Germany) and Takara-Syuzo Co. (Japan). Taq DNA polymerase was purchased from Parkinson Elmer-Cetus (U.S.A.). DNA synthesizing reagents were all purchased from Beckman Japan Co. (Japan). Two primers, IBP1 (TGGATAAGGTCCAAATTAATTG) and IBRP2 (AGCAAACCATTATATTCACGAG) were synthesized using an automatic DNA synthesizer (Beckman, System 1 plus).

Table 1. Descriptions of IBV strains used in this investigation

Serotype[a]	Strain	References
Massachusetts	M 41	[32]
	Be 42	[32]
Conneticut	A-5968	[20, 32]
Delaware	Holte	[40]
	Gray	[40]
Iowa 97	I-97	[20]
Iowa 609	I-609	[20]
Japanese isolates	KH	
	K-79	
	Ishida[b]	
	ON[b]	
	C-78[b]	

[a] Serotype designation proposed by S. R. Hopkins [21]
[b] Vaccine strain

cDNA synthesis

Twenty µl (1.5 µg) of the RNA solution was mixed with 5 µl of 10 × reverse transcriptase buffer (500 mM Tris-HCl (pH 8.0), 300 mM KCl, 100 mM dithiothreitol (DTT), 80 mM $MgCl_2$, 30 ng of each primer and 5 µl of 2.5 mM each of deoxyribonucleoside triphosphates (dATP, dGTP, dCTP and TTP). A reaction volume of 50 µl was obtained by the addition of distilled water. After heating at 90 °C for 5 min, 30 units of avian myeloblastosis virus reverse transcriptase (Seikagaku Kogyo Co. Japan) were added to the reaction mixture. After incubation at 37 °C for 1 h, a half volume (25 µl) of alkaline solution (150 mM NaOH, 40 mM EDTA) was added and incubated at 65 °C for 1 h. After neutralization with 25 µl of 1 N HCl, 25 µl of 1 M Tris-HCl (pH 8.0) was added to the reaction mixture. Then, cDNA was extracted with phenol/chloroform/isoamyl-alcohol (50:48:2), ethanol-precipitated, and dissolved in 10 µl of distilled water (cDNA solution).

Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) was performed in 50 µl of a mixture containing 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 30 ng of each primer, 0.5 mM each of deoxyribonucleoside triphosphates (dATP, dGTP, dCTP and TTP), 3 µl of cDNA solution, and 2.5 units of Taq polymerase. The reaction was repeated for 15 to 20 cycles under the following conditions: 1 min at 92 °C for denaturation of the template, 2 min at 52 °C for primer annealing and 3 min at 72 °C for chain extension.

Restriction enzyme analysis of PCR products

The amplified products were precipitated by adding a half volume of 4 M ammonium acetate and 2.5 volumes of iso-propanol, and redissolved in a few µl of distilled water (amplified DNA solution). Amplified DNA was digested by several restriction enzymes under the conditions recommended by the enzyme suppliers.

Determination of digestion sites on amplified DNA

To map the restriction enzyme cleavage sites, both primers were labeled with [γ-^{32}P]ATP and polynucleotide kinase. Then, PCR was performed using combinations of non-labeled and labeled primers. The amplified DNA was digested with a restriction enzyme, and analyzed by electrophoresis on 6% polyacrylamide gel and autoradiography.

Results

Location of the oligonucleotide primers for polymerase chain reaction

A high frequency of antigenic variation is common in IBV, probably due to high rates of genetic mutations. The major antigenic determinant of IBV is the viral surface glycoprotein called spike (S) protein. S protein has been known to play an important role in virus neutralization.

Therefore, we focused on the region of the S gene and first compared four S sequences. Figure 1 shows the comparison results, in which the number of nucleotides different from those present in the other three strains was scored for every 20 nucleotides. The nucleotide change at the N-terminal region of the S2 protein was relatively high in all four strains. In addition, this region is flanked by relatively unchanged conserved sequences. This region was thus

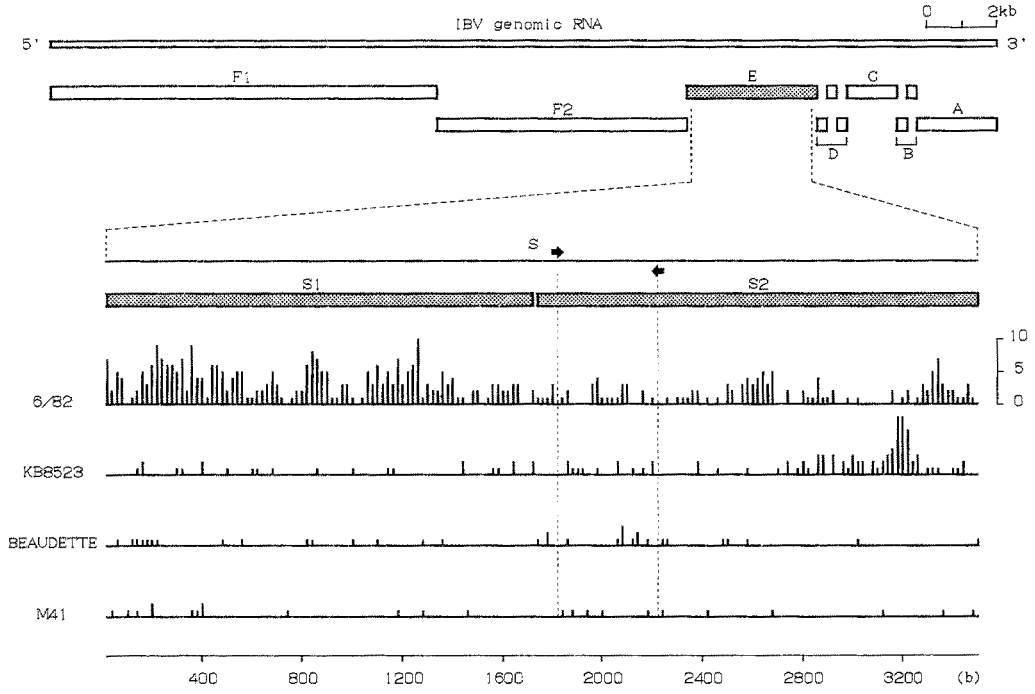

Fig. 1. Genomic organization of IBV and position of PCR primers. The genes encoding proteins are indicated by boxes. Letters indicate the name of encoding mRNA. The gene encoding S protein magnified under the genome organization shows the location of the S1 and S2 glycopolypeptides. The bars indicate the sum of nucleotides, which are different from those present in other three strains, scored for every 20 nucleotides. The positions of two oligonucleotide primers for PCR are marked by the arrows

selected as a PCR target. Two primers, IBP1 and IBRP2, which corresponded to these common flanking regions were synthesized.

DNA amplification using the IBV M41 strain

Using IBV M41 as a standard strain, viral RNA was reverse-transcribed to cDNA and amplified as described in Materials and methods. DNA with a length of 400 bp was amplified in accordance with the reaction cycles (Fig. 2). The amplified DNA was as long as that expected from the DNA sequence of the designated region and was not seen when the reverse transcriptase was omitted from the reaction mixture in the first step nor when mock infected fluid was used (data not shown). The cleavage patterns with several restriction enzymes was the same as that of the S gene sequence of IBV M41. Together, these observations indicate that the amplified DNA did not originate from a contaminated unknown DNA but was a product of the designated region of the S gene.

DNA amplification from various IBV strains

As our amplification system was confirmed to be correct, 12 IBV strains, including 7 major American strains and 5 strains isolated in Japan, were used

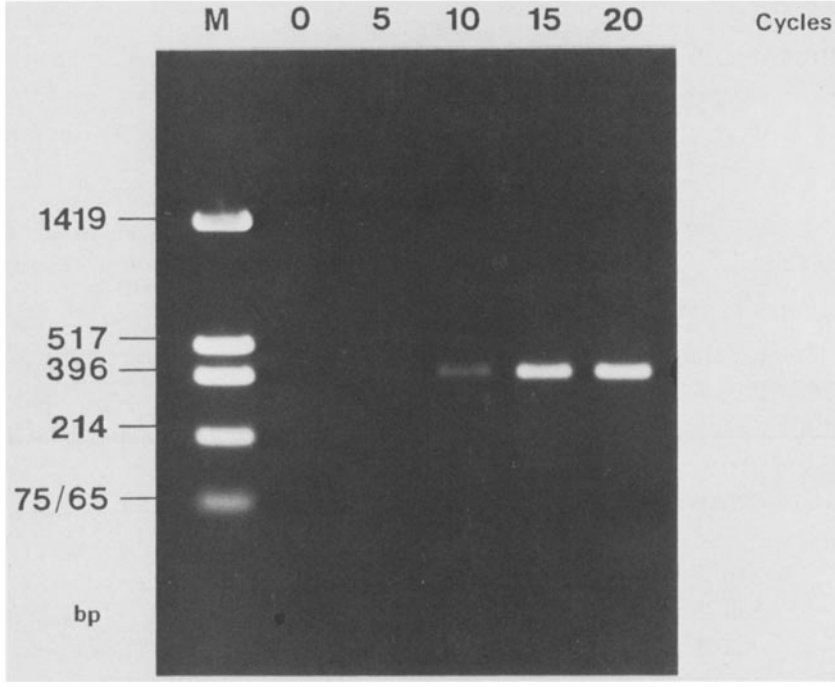

Fig. 2. Agarose gel analysis of cDNA amplification in IBV M41. Portions of the reaction mixture are collected every 5 cycles and analyzed by electrophoresis on 1.5% (w/v) agarose gel in Tris-borate buffer containing 0.5 μg/ml of EtBr. The numbers marked above the lanes indicate the repeated PCR cycle. HinfI digested pUC19 DNA is used as the molecular size marker (*M*)

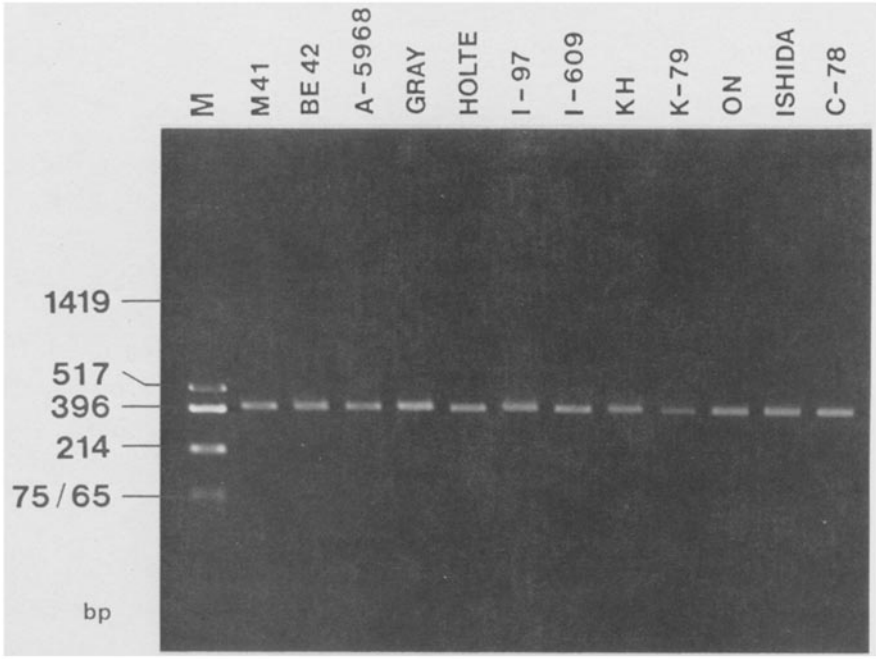

Fig. 3. Amplification of cDNA from several strains. The twelve strains listed above the lanes are amplified by PCR in 20 cycles. HinfI digested pUC19 DNA is used for the molecular size marker (*M*). The numbers at the left indicate the length of fragments at bp

to amplify the DNA. All amplified DNA showed almost identical mobilites in 1.5% agarose gel (Fig. 3). There were no apparent deletions or insertions within this genome region of the strains. These IBV strains cannot be distinguished on the basis of the length of these amplified DNAs.

Restriction enzyme digestion of the amplified DNA

To see whether there were sequence divergences within the amplified DNA, the DNA was digested by several restriction enzymes. Nine enzymes, DdeI, HaeIII, HincII, HinfI, HpaII, MaeIII, PstI, ScaI and XhoII, were chosen by referring to the published S gene sequences of IBV. Representative cleavage profiles by HpaII and MaeIII digestion are shown in Fig. 4. In the case of HpaII digestion, the cleavage profiles can be classified into two types. The first type is a group which has no cleavage site, (C-78, K79, I-609, Holte, Gray, ON, I-97, and A-5968 strains). The second type is a group which has one cleavage site (the remaining 4 strains) (Fig. 4 A).

In the case of MaeIII, the test strains can be classified into three types. The first group with no cleavage site includes the I-609, Be42, Ishida, KH, I-97, and A-5968 strains. The second one with one cleavage site includes the M41, Holte, Gray, and ON strains. The remaining 2 strains (C-78 and K-79) can be classified into the third group with two cleavage sites (Fig. 4 B).

Using nine restriction enzymes, 17 cleavage sites were recognized among these strains, and the cleavage pattern for each strain is summarized in Table

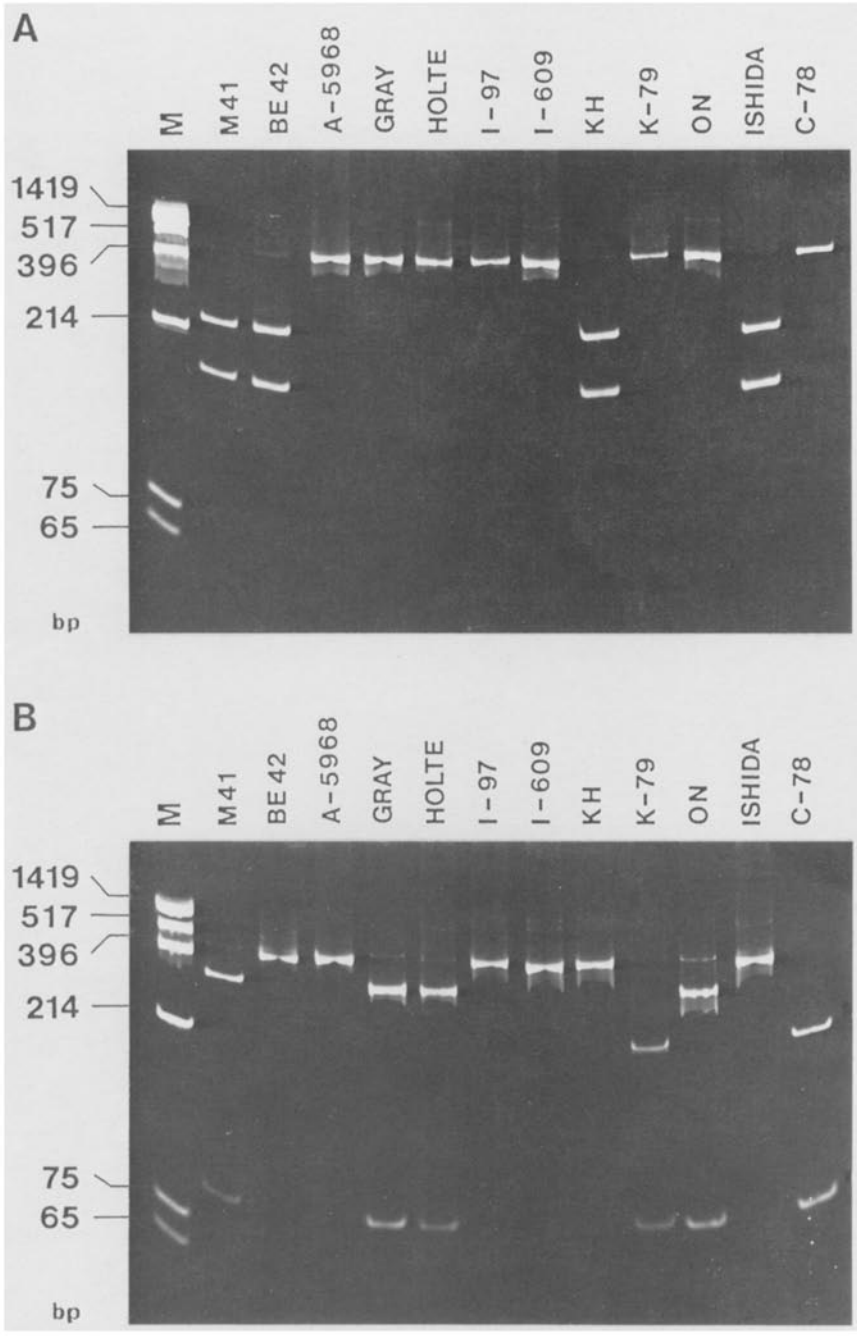

Fig. 4 A, B. Polyacrylamide gel electrophoretic patterns of PCR products of each strain digested by restriction enzyme. The twelve strains listed above the lanes are digested by the restriction enzyme HpaII (**A**) or MaeIII (**B**). The PCR products of C-78 and K-79 strains are cleaved at 2 sites by MaeIII. Two of three bands overlap in slow migrate. HinfI digested pUC19 DNA is used as the molecular size marker (*M*). Digested fragments were then electrophoresed on 6% polyacrylamide gel and analyzed by EtBr staining

Table 2. Restriction enzyme digestion profiles of twelve strains

Strain	Restriction enzyme sites[a]																
	Pst I	Hae III	Hae III	Mae III	Hpa II	Dde I	Dde I	Sca I	Hinc II	Sca I	Xho II	Hinf I	Mae III	Hinf I	Pst I	Hae III	Xho II
C-78	–[c]	–	–	O[b]	–	O	–	O	–	–	O	O	O	–	O	O	O
K-79	–	–	–	O	–	O	–	O	–	–	O	O	O	–	O	O	O
I-609	O	–	O	–	–	O	–	O	O	–	O	O	–	O	O	–	O
Be 42	O	–	O	–	O	O	–	–	–	–	–	O	–	–	O	–	–
KH	O	–	O	–	O	O	–	–	O	–	O	O	–	–	O	–	–
Ishida	O	–	O	–	O	O	–	–	O	–	O	O	–	–	O	–	–
M 41	O	–	O	O	O	O	–	–	O	–	–	O	–	–	O	–	–
Holte	O	–	O	O	–	O	–	–	O	–	–	O	–	–	O	–	–
Gray	O	O	O	O	–	–	–	–	O	–	O	O	–	–	O	–	–
ON	O	O	O	O	–	–	O	–	O	–	O	O	–	–	O	–	–
I-97	–	–	O	–	–	–	–	–	O	–	–	O	–	–	O	–	–
A-5968	–	–	O	–	–	–	O	–	O	O	O	O	–	–	O	–	–

[a] Seventeen digestion sites were aligned from 5′ to 3′ end
[b] Digested by the indicated restriction enzyme
[c] Not digested

Table 3. Reciprocal restriction enzyme sites in twelve strains of IBV

Strain	C-78	K-79	I-609	Be42	KH	Ishida	M41	Holte	Gray	ON	I-97	A5968
C-78	–	0[a]	7	9	9	9	9	8	9	10	9	10
K-79	0	–	7	9	9	9	9	8	9	10	9	10
I-609	7	7	–	6	4	4	6	5	6	7	6	7
Be42	9	9	6	–	2	2	2	3	6	7	4	7
KH	9	9	4	2	–	0	2	3	4	5	4	4
Ishida	9	9	4	2	0	–	2	3	4	5	4	5
M41	9	9	6	2	2	2	–	1	4	5	4	7
Holte	8	8	5	3	3	3	1	–	3	4	3	6
Gray	9	9	6	6	4	4	4	3	–	1	4	5
ON	10	10	7	7	5	5	5	4	1	–	5	4
I-97	9	9	6	4	4	4	4	3	4	5	–	3
A5968	10	10	7	7	4	5	7	6	5	4	3	–

[a] The score of different sites

2. KH and Ishida and K-79 and C-78 showed the same cleavage pattern, while other strains showed different cleavage patterns, indicating that most of the virus serotypes analyzed in this experiment have strain specific sequences in this region.

Classification of IBV

As shown in Table 2, there are 17 cleavage sites by 9 restriction enzymes in the test region of 12 IBV strains. For each pair of strains, the difference in restriction sites was counted (0 to 17). Table 3 shows the results with 12 strains. A low rating (a difference of 0–3) signifies a group of strong relationships. A high rating signifies a group of weak relationships (a difference of more than 8). A medium rating (a difference of 4–7) signifies a group of moderate relationships. There are 5 low rating areas in Table 3. The largest one contains the Be42, KH, Ishida, M41 and Holte strains. The second low rating area contains the Gray and ON strains. The third contains the I-97 and A-5968 strains. The fourth contains the I-609 strain, the fifth contains the C-78 and K-79 strains.

Discussion

The classification of IBV strains has been performed serologically using cross-neutralization tests [7, 15, 16, 18, 21, 22]. In some cases, however, the serological classification led to conflicting results depending on the antisera or criteria used for measurements [26]. These conflicting results are probably due to the fact that almost all sera are obtained from flocks, vaccinated with live attenuated or killed IBV vaccine and that common but weak cross-neutralization epitopes are present in IBV [27, 28]. On the other hand, molecular biological methods were reportedly effective in the classification of IBV strains [11–13, 26, 27, 30].

Though these systems are highly reproducible and have led to an accurate classification, they are not easily applicable for large amounts of purified samples. To meet the need for a more accurate and practical method of identifying IBV strains, we applied PCR and RFLP analysis [6, 37].

In the present study, the region of the S2 gene between two primers was amplified in each of the 12 test strains by PCR (Fig. 3). This system required smaller samples than any other molecular method. Amplification of 12 tested cDNA indicated that the two primer regions were well conserved in at least these 12 strains. Though amplified cDNA were almost identical in size, many variations were found in the RFLP patterns among the strains (Fig. 4 and Table 2). This allowed us to discriminate 7 major American strains and 5 Japanese strains.

These 12 strains were classified into 5 groups (I–V) as in Table 3. Four strains, M41 and Be42 of the Massachusetts serotype, KH and Ishida, were classified into group I. The Holte strain of the Delaware serotype was found to be similar to the strains in group I. The Gray (Delaware serotype) and ON strains which had not been serologically classified belonged to group II. The I-97, I-609, and A-5968 strains had been classified into I-97 serotype, I-609 serotype, and Connecticut serotype, respectively. The present study indicated that the I-97 and A-5968 strains are relatively close to each other, and thus belong to group III. The I-609 strain, being distinct from the other two strains, was classified as group IV. The C-78 strain is prevalent in Japan (Y. Kudou, unpubl. data) and is serologically unrelated with previously isolated strains (Y. Kudou, unpubl. data). Attenuated C-78 is currently used as a vaccine strain in Japan. The RFLP data showed that both C-78 and K-79 belong to group V, which is far from other groups. These data were consistent with the serological classification (Table 1).

The most likely phylogenetic tree based on the observed variation in RFLP was inferred and calculated using the Unweighted Pair-Group Method (Fig. 5). The distances and branch lengths of this tree indicate the evolutionary relationships among the test strains. It showed the origin of new variant IBV strains causing outbreaks of infectious bronchitis.The ON strain was shown to derive from the Gray type virus. The C-78 and K-79 strains, isolated in 1978 and 1979 respectively, are neither serologically nor genetically related to any other strains, but were shown to derive from a common origin. The PCR-RFLP method thus provides a very useful system for surveying IBV strains in the field and to estimate the origin of an outbreak strain.

Due to the following findings, our designated genome region might not encode for the strain-specific neutralization epitope. Firstly, a neutralizing monoclonal antibody was found to bind to S1 glycopolypeptide [29]. Secondly, the urea-released S1 glycopolypeptide was found capable of inducing neutralizing antibodies [10]. Therefore, the epitopes of strain-specific neutralizing antibodies were thought to locate on S1 glycopolypeptide [12]. It was unclear why the RFLP results in the N terminal region of S2 glycopolypeptide were

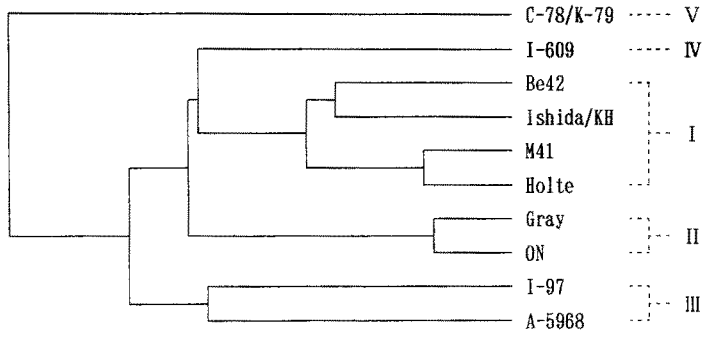

Fig. 5. Phylogenetic tree of IBV strains. The relatedness between strains was calculated on the basis of the RFLP data of the PCR amplified DNA using the Unweighted Pair-Group Method using Arithmetic Averages (UPGMA). *I–V* Group of strains

consistent with the serological classification. Probably, genetic mutation in the S2 region occurred at the same rate as that in the region involved in the serotype-specific neutralizing antibody epitopes, probably on the S1 glycopolypeptide.

The mechanisms behind the high rate of new variant strain generation in IBV are yet unknown. Although it may be explained by a misreading of viral RNA polymerase in the replication [3, 26], no other coronavirus was reported to have such a high frequency of new variant appearance as seen in IBV. Another mechanism may therefore exist in addition to the reading error of the RNA polymerase.

Acknowledgements

The authors wish to thank Mr. Kazuhiro Umeda for providing the virus stocks used in this study and Dr. Yosaburo Ohtaki (Nippon Insitute for Biological Science) for his helpful comments. We also wish to thank Dr. Akira Ishihama for his critique of this manuscript. The phylogenic tree was constructed by Drs. Y. Tateno and Akira Ishihama (National Institute of Genetics).

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Received May 30, 1990