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Molecular and Epidemiological Characteristics of Infectious Bronchitis Virus Isolated in Korea

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ABSTRACT: Phylogenetic tree constructed from the nucleotide sequences of the S1 gene showed that the 15 Korean strains of infectious bronchitis virus(IBV) examined were classified into 2 genetically distinct groups, except one respiratory strain, RB86, which was clustered with Massachusetts group. All the 5 respiratory strains belonged to Korean group I and the rest 9 nephropathogenic strains belonged to Korean group II according to the analysis , based on S1 gene sequences. Like previous classifications corresponded with the geographic origin, Korean stains were discriminated from geographically distinct reference strains of IBV. The nephropathogenic strains within Korean group II sharing 96% homology were continuously isolated since 1990, and seemed to be genetically stable. Whereas the respiratory strains within Korean group I sharing 88% homology were sporadically isolated since 1986, and seemed to be genetically unstable. Because we found putative accumulated point mutation as well as recombination events in Korean group I, we discussed why genetic variations have often occurred in respiratory strains rather than nephropathogenic strains.

(Key words: infectious bronchitis virus, S1 gene, genetic variation, evolution)

INTRODUCTION

Infectious bronchitis (IB) virus causes an acute, highly contagious disease of chicken of all ages and types resulting in significant economic losses. The trachea is the primary target organ for virus attachment and replication, but some strains can also replicate in the kidney (Cavanagh and Naqi, 1997). Until now, a number of serotypes as well as antigenic variant strains, basically defined by viral neutralization and cross-protection studies, have been found worldwide IB outbreaks continue to occur in vaccinated flocks, because different IB virus (IBV) serotypes or antigenic variant strains did not induce complete cross-protection (Cavanagh and Naqi, 1997).

In 1986, IB was first described in Korea (Rhee et al., 1986), and Massachusetts (Mass) type live IB attenuated vaccine as well as inactivated killed oil-emulsion vaccine were applied to prevent and control the incidence of the disease. The most commonly used live IB vaccine strain is H120 and only some other Mass type strains were allowed for IB vaccination in Korea until now. In 1986, the most frequently observed clinical signs of IBV infection were respiratory distress in broilers, and egg production and egg quality declines in laying flocks, and these field IB outbreaks were easily controlled by vaccination with H120. Since 1990, however, the incidence of nephritis in broilers and decrease in egg production in laying flocks caused by variant IBV infection were increased, even in areas where Mass type IB vaccines were widely and correctly used. Further, we isolated new variant respiratory strains of IBV in Mass type IB vaccinated flocks since 1997. The nephropathogenic strains of IBV has become a major concern in Korea nowadays, however the respiratory strains of IBV still issued (Kim et al., 1992; Song et al., 1988).

IBV, a member of the *Coronaviridae* family, has a

single-stranded RNA genome, approximately 20kb in length, of positive polarity which specifies the production of three major structural proteins; the phosphorylated nucleocapsid protein, the membrane glycoprotein, and the spike (S) glycoprotein which is post-translationally cleaved into N-terminal S1 and C-terminal S2 subunits (Binns et al., 1985). The S glycoprotein extends from the viral membrane and the S1 glycoprotein is anchored to the viral membrane by the S2 glycoprotein (Cavanagh, 1983a,b,c). Neutralizing, haemagglutination-inhibiting, and serotype-specific antibodies are directed against the S1 glycoprotein (Karaca et al., 1992; Koch et al., 1990). Wang et al. (1994) previously described that point mutations as well as genetic recombination provide a mechanism for antigenic and pathogenic evolution. Although, mutations will occur at random throughout the IBV genome, the mutation of the S1 glycoprotein gene is more important than that of other structural protein genes of IBV, because S1 glycoprotein has virus neutralizing epitopes on its S1 gene (Karaca et al., 1992; Koch et al., 1990). When the vaccine-induced antibodies do not neutralize a mutant strain, it will rapidly spread through the chicken flocks. The existence of different serotypes in genetically very closely related viruses presents the evidences that the generation of genetic variants is easily created in nature. However, not all the strains of IBV isolated in certain area cause epidemic situation. Some of them seem to persist throughout the years, while others appear to re-emerge after periods of non-detection.

In previous study, we classified the 40 strains of IBV isolated between 1986 and 1997, into 5 genotype by reverse transcriptase-polymerase chain reaction and restriction fragment length polymorphism (RFLP) analysis (Song et al., 1998). More recently, we identified new variant respiratory strains of IBV, which belong to new genotype VI, in Mass type IB vaccinated flocks. Results from the RFLP analysis showed that the IBV strains belong to genotype III were constantly found since 1990 and mainly cause nephritis, whereas the IBV strains belong to the other 5 genotypes were sporadically found and mainly cause respiratory disease.

In this study, we evaluated evolutionary relationships between the respiratory and nephropathogenic strains of IBV at the molecular level.

MATERIALS AND METHODS

1. Viruses and Cells

Six respiratory strains, RB86, B4, EJ95, EY95, K620/97 and K348/99, of IBV and 9 nephropathogenic strains, KC90, KM91, K604/97, K151/98, K152/98, K083/98, K242/99, K451/99 and K576/99, of IBV were used to analyze the S1 gene sequence. Each strain was isolated from natural outbreaks of IB in different types of commercial chicken flocks between 1986 and 1999. The 6th-embryo-passage of each strain was propagated in 10-day-old specific-pathogen-free embryonated chicken eggs (Hyvac) at 37℃ for 48 hours. The allantoic fluid from eggs infected with each strain was harvested and clarified by low speed centrifugation.

2. Viral RNA Extraction and Amplification of the S1 Gene of IBV

Viral RNA extraction and polymerase chain reaction (PCR) were performed according to the methods as described previously (Song et al., 1998). Briefly, two primers, which included of 20 bp sequence complimentary to region at the 5′ end (BES1F1) and 3′ end (BES1R1) of the S1 gene of reference IBV Beaudette strain, were used to amplify the complete S1 gene of IBV. The sequence of primer BES1F1 was 5′-CGCGGATCCTTTGAAAACTGAACAAAAGA-3′ and that of primer BES1R1 was 5′-CGCGGATCCCATAA CTAACATAAGGGCAA-3′. *Bam*HI sites (underlined) were added to the 5′ ends of each primer to facilitate cloning. These primers were flanked a 1722-base sequence containing the whole S1 gene. Reverse transcription (RT) of mRNA purified from allantoic fluid was done with Moloney murine leukemia virus reverse transcriptase (GibcoBRL) and primer BES1R1. For PCR reaction, Ampli*Taq* DNA polymerase (Perkin-Elmer Cetus) and primer BES1F1 were added to RT reaction. The PCR was performed by 40 cycles of denaturation (94℃, 10 sec), annealing (45℃, 90 sec), and polymerization (72℃, 90 sec). The pre-denaturation step was at 94℃ for 20 sec and post-polymerization step was at 72℃ for 300 sec. The PCR products were electrophoresed on a 1.0 % agarose gel and confirmed the S1 band with a predicted of 1722 bp (Song et al., 1998).

3. Cloning of the S1 Gene of IBV

The PCR product from agarose gel was purified using the Geneclean kit (BIO 101) and the purified DNA was ligated into plasmid pCRII using a TA Cloning kit (Invitrogen) according to the manufacturer's recommendations. The plasmid including the complete S1 gene of IBV was transformed into *Escherichia coli* INVαF′ cells and ampicillin-resistant colonies were screened by restriction enzyme analysis. For sequencing, large amounts of plasmid DNA was produced using the QIAGEN Plasmid Midi Kit (QIAGEN).

4. DNA Sequencing and Computer Analysis

DNA sequence was determined using Dye Terminator Cycle Sequencing method and analyzed with a ABI 377 autosequencer. The sequences were aligned using the MegAlign program in the Lasergene package (DNASTAR). Phylogenetic relationship were established with the computer program TREECON for Windows. For determining the phylogenic relationship, the nucleotide sequence of the entire S1 gene of the 15 Korean strains of IBV were compared with 20 published sequences of S1 genes of IBV isolated in the USA (M41-GenBank accession numbers X04722; Beaudette-A12747; Connecticut-L18990; Arkansas99-M99482; PP14 -M99483; Gray-L14069; SE17-M99484; Holte-L18988), Europe (H120-GenBank accession numbers M21970; UK/6/82-X04723; UK/793B-Z83979; D274-X15832; D207-M219-69; D3896-X52084; D1466-M21971), Japan (KB8523-GenBank accession numbers M21515) and Australia (Vic S-GenBank accession numbers U29519; N2/75-U29523; N9/74-U29452; Q3/88-U29451). Among the 20 IBV reference strains, 8 strains (Arkansas99, Connecticut, D274, D1466, H120, M41, UK/973B, Vic S) were commercially available vaccine strains worldwide.

RESULTS AND DISCUSSION

1. Phylogenetic Tree Analysis

1) Relationships between Korean and Rreference Strains of IBV

Phylogenetic tree constructed from the nucleotide sequences of the S1 gene showed that the 15 Korean strains of IBV were divided into 2 genetically distinct groups, except one respiratory strian, RB86, which was clustered with Masss group (Fig. 1). All the 5 respiratory strains examined belonged to Korean group I, whereas the other all the 9 nephropathogenic strains examined belonged to Korean group II. As wang et al. (1994) previously described that the phylogenetic classification corresponded with the geographic origin of the strains within a group, Korean strains of IBV were also discriminated from geographically distinct reference strains of IBV.

2. Sequence Analysis

1) Comparison of the S1 Nucleotide Sequences

The S1 nucleotide sequence of the oldest known Korean strains of each group were compared with other strains (Table 1). The nephropathogenic strains within Korean group II had approximatrely 95% or greater identity. Interestingly, nephropathogenic strains seemed to be genetically stable,

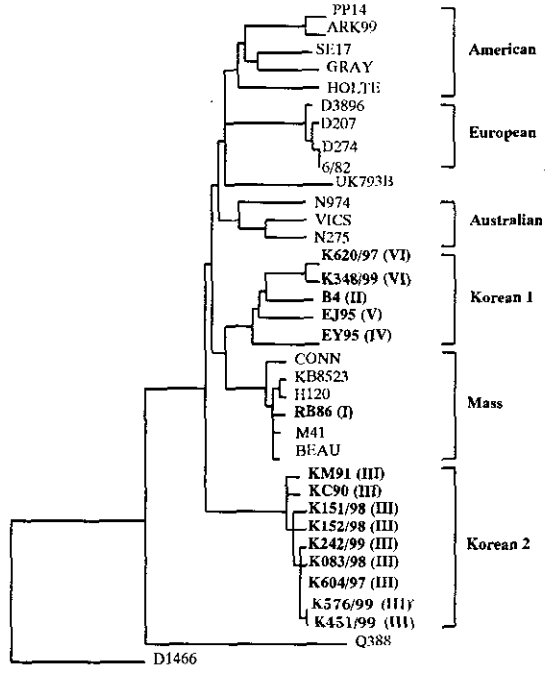

Fig. 1. Phylogenetic tree and genomic relatedness based on the nucleotide sequences of the S1 gene for 15 Korean strains of IBV, compared with published sequences for 20 other strains of IBV. Fifteen Korean strains of IBV are marked in bold.

Table 1. Comparision of the S1 nucleotide sequences of the oldest known isolates with other isolates of Korean group Ⅰ and Ⅱ

IBV strains (Genotype by RFLP)	% Identity	
	B4 (group Ⅰ)	KC90(group Ⅱ)
Korean group Ⅰ		
B4(Ⅱ)	100	8.4
EJ95(Ⅴ)	88.2	78.7
EY95(Ⅳ)	77.8	79.6
K620/97(Ⅵ)	88.4	77.9
K348/99(Ⅵ)	88.2	78.4
Korean group Ⅱ		
KC90(Ⅲ)	73.1	100
KM91(Ⅲ)	72.2	96.9
K604/97(Ⅲ)	71.9	95.9
K151/98(Ⅲ)	72.0	96.6
K152/98(Ⅲ)	73.3	96.9
K083/98(Ⅲ)	72.6	97.1
K242/99(Ⅲ)	71.7	95.9
K451/99(Ⅲ)	71.8	95.3
K576/99(Ⅲ)	71.5	96.1

because they only showed 2.9% to 5.7% nucleotide differences since the first nephropathogenic strain, KC90, was isolated in 1990. Whereas the respiratory strains within Korean group I shared approximately 88% homology, except EY95. To consider that B4 strain was isolated from first outbreak case in Korea at 1986, EJ95, K620/97 and K348/99 strains seemed to be originated from B4 strain. These genetic variations seemed to be as a result of accumulated point mutations (data not shown).

2) Comparison of the Amino Acid Sequences

Localized sequence variations or dramatic shifts in homology were difficult to find out by examining the phylogenetic tree and overall sequence comparisons. In the present study, we compared the individual amino acid sequence for the possible recombination regions of the S1 genes. One potential crossover region (amino acid 486~526) and two different amino acid characteristic regions similar to B4 and KM91 were found on the strain EJ95 (Table 2 and Fig. 2). The overall identity of the amino acid sequence at the 5' end of the S1 from EJ95 was 84.8% homology with B4 in contrast to 75.5% with KM91, whereas the identity of the remaining sequences of EJ95 had 100% homology with KM91 and only 70.6% with B4. Evidence for the homology shifts was also found in the S1 gene of the EY95. The EY95 had two

Table 2. Comparison of the deduced amino acid sequences and homology shifts within the S1 genes of IBV stains

Korean IBV Strains	Region[a]	Percentage identity					
		H120	RB86	B4	KM91	EY95	EJ95
RB86	Entire	96.1[b]	–	73.3	81.8	84.0	75.6
	51-150	93.0	–	52.3	65.7	85.9	60.7
B4	Entire	73.5	73.3	–	75.2	79.3	84.4
	51-150	50.0	52.3	–	64.1	51.6	75.0
KM91	Entire	82.0	81.8	75.2	–	77.6	76.3
	51-150	63.6	65.7	64.1	–	62.2	62.1
EY95	Entire	86.4	84.0	79.3	77.6	–	82.0
	51-150	90.9	85.9	51.6	62.2	–	56.2
	1-253	94.6	89.6	68.9	73.9	–	71.5
	254-520	79.0	79.0	88.8	81.6	–	93.3
	521-538	72.7	72.7	100	63.6	–	63.6
EJ95	Entire	76.1	75.6	84.4	76.3	82.0	–
	51-150	61.3	60.7	75.0	62.1	56.2	–
	1-526	75.9	75.3	84.8	75.5	82.4	–
	527-544	82.4	82.4	70.6	100	70.6	–

[a]Positions of amino acid residues.

[b]Greater than 90% homology is shown in bold.

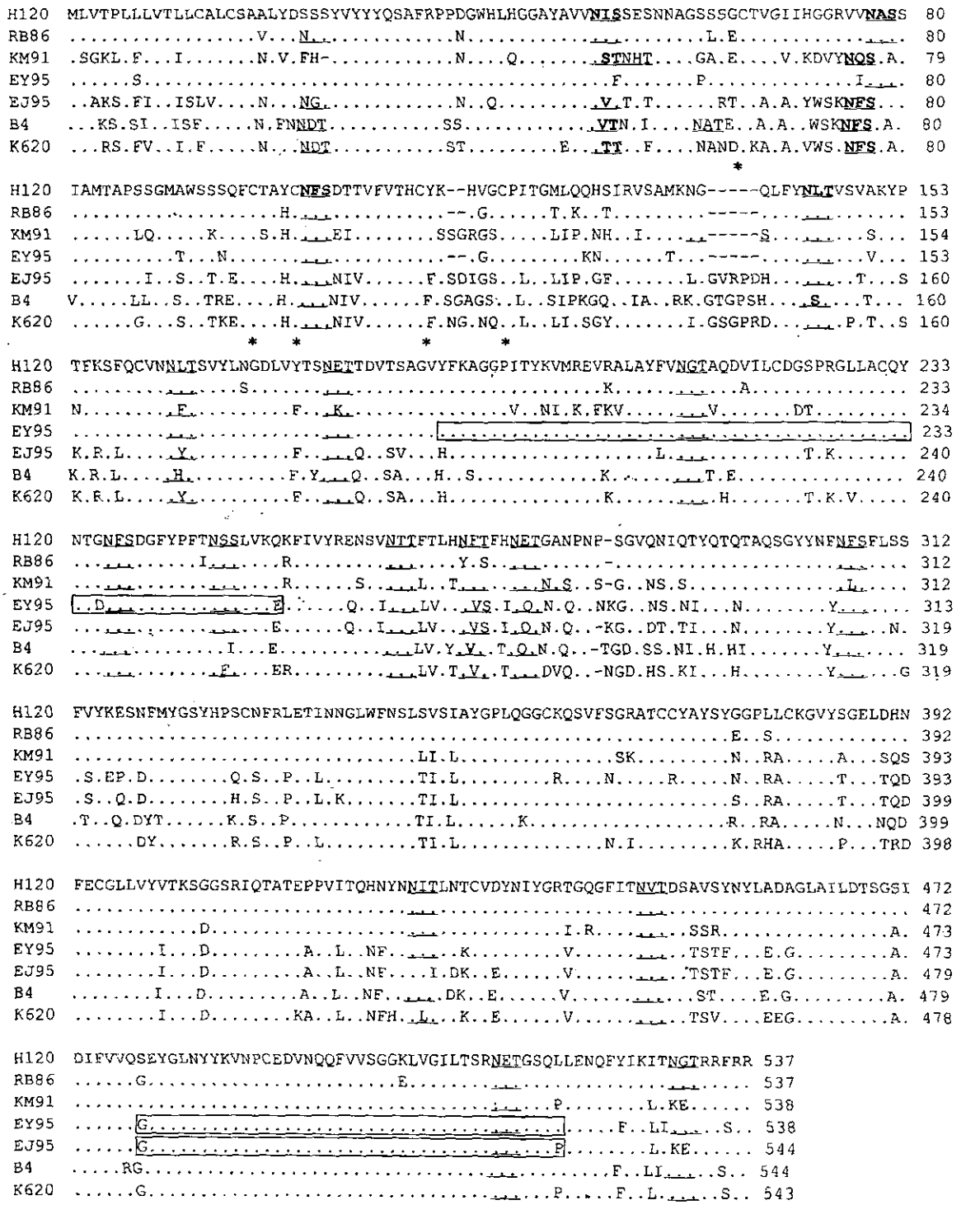

Fig. 2. Amino acid alignment of the S1 glycoprotein genes of H120 and 6 Korean strains of IBV. The complete amino acid sequence of the S1 gene of H120 is deduced and shown at the top of the panel. Gaps (dashes) were introduced in order to align the sequences, and the dots indicate the residues identical to those of H120. Potential glycosylation sites (NXS or NXT, except where X=P) are underlined and potential crossover regions are boxed. Conserved cystein residues within the HVR (amino acid 50 to 150) are marked by asterisk below the respective amino acid sequences, and the potential glycosylation sites within the HVR are marked in bold and underline, respectively.

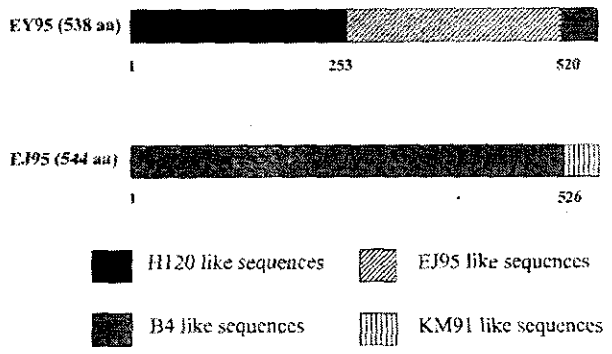

Fig. 3. A schematic presentation of the putative recombination sites in the S1 glycoproteins of EY95 and EJ95.

potential crossover regions (amino acid 189~253 and 480~520) and three different amino acid characteristic regions similar with H120, B4 and EJ95 (Table 2 and Fig. 2). The region that included 253 bases downstream of the S1 start codon of the S1 gene of EY95 had 94.6% homology with H120 and 71.5% with EJ95. However, for the next 267 bases, the EY95 had 93.3% homology with EJ95, and was only 79.0% homology with H120. Also, a region from 521 to 538 had 100% homology with B4 and only 70.6% with EJ95. A scheme of shifts in amino acid sequence homology within the S1 gene was presented in Fig. 3.

In the present study, we found that the three crossover sites were located at relatively conserved sequence nearby the hypervariable region (HVR). These results corresponded to the previously observations that putative recombination sites were often identified at position about 131 amino acid bases from the start codon of the S1 gene (Wang et al., 1994). This region usually located nearby the HVR, whereas major shifts in homology seldom identified within the HVR. A number of comparison studies using distinct IBV S1 gene identified the HVR between 50~150 amino acids from the N-terminus (Cavanagh et al., 1988; Kusters et al., 1989; Wang et al., 1994, 1997). In the present study, despite of the variations in the HVR, all five cysteins within amino acid 50~150 were completely conserved and the four putative glycosylation sites were nearly conserved within this HVR (Fig. 2). These results also corresponded to previous observation (Wang et al., 1994) and seemed to reflect on critical structural or functional features of the S1 glycoprotein of IBV. The length of the S1 genes of the 5 Korean strains varied from 537 (RB86) to 544 (EJ95) amino acids and the differences in size seemed to be resulted from insertions and deletions.

In the previous study, the 5 representative Korean strains of IBV were inoculated into day-old chicks to evaluate their pathogenicity and tissue tropism. We found that the IBV KM91 induced 50% mortality with severe renal urate deposition on the kidney and showed broad tissue tropism, but the other 4 strains only induced respiratory distress after inoculation (Song et al., 1988). In the present study, we compared the amino acid sequences of the nephropathogenic strain KM91 with those of respiratory strains RB86, B4, EY95, EJ95 and K620/97. However, we failed to identify with definite regions that could correlate with the pathogenicity of these strains. When compared the region between amino acid residues 123 and 152, which previously identified as a possible region for the nephropathogenicity of IBV Gray (Kwon and Jackwood, 1995), with that of nephropathogenic strain Holte, Vic S, N2/75 and N9/74, we could not identify any identical change (data not shown). The regions for the virulence and tissue tropism still remain to be confirmed.

Results from the present study showed that the respiratory strains of IBV seemed to be genetically unstable because point mutation, deletions, insertions, and recombination seemed to be a responsible mechanism for the evolution of the respiratory strains within Korean group I (Cavanagh et al., 1998) described the possibilities that genetic differences are driven by immune pressure or tolerated by mutability of RNA genomes. In the present study, we could assume that the genetic variation often occurred in respiratory strains than nephropathogenic strains. It seems that there are two possibilities. First, respiratory strains seemed to be more easily affected by partial immune pressure induced by Mass type IB vaccination and/or variant field IBV infection than nephropathogenic strains. Therefore respiratory strains need to mutate in order to survive. Second, nephropathogenic strains usually have more chances to infect variety of host tissues (e.g. kidney) than respiratory strains, therefore they did not need to mutate for surviving. This possibility is supported by our finding of very little change in nephropathogenic strains

within Korean group II during last ten year period.

We are currently examining whether one of nephropathogenic strains could cross-protect other Korean strains of IBV. It may be a useful candidate of vaccine strain, because Korean strains of IBV showed genetical similarity in volutionary relationships as well as geographical distinction from other reference strains of IBV.

적 요

전염성 기관지염 (Infectious bronchitis : IB)은 1986년도에 국내 최초로 확인, 보고되었으며, 1990년도에는 10% 내외의 폐사를 동반하는 신장형 IB가 보고된 바 있다. 현재 국내에는 신장염, 폐사, 산란저하를 유발하는 신장형 IB 감염에 의한 피해가 1990년도 이후 전국적으로 확인되고 있으며, 호흡기 증상과 산란저하를 유발하는 호흡기형 IB 감염에 의한 피해도 산발적으로 확인되고 있다. 본 연구에서는 국내에서 유행중인 IB 한국분리주의 분자생물학적, 역학적 특성을 조사하기 위하여 1986년부터 1999년까지 국내에서 분리된 15종의 IBV S1 유전자에 대한 염기서열 분석을 실시하였고, 아울러 기존에 발표된 20종의 reference IBV S1 유전자와의 염기서열을 비교분석 하였다. IBV S1 유전자의 nucleotide 염기서열로 작성한 phylogenetic tree 분석 결과 IBV 국내 분리주들중 RB86주는 Massachusetts group에 속하였으나 그 외 14종의 국내 분리주들은 외국에서 분리된 기존의 reference IBV와는 계통발생학적으로 상이한 두 개의 유전그룹 (Korean group I, II)으로 분류되었다. 그 중 국내에서 분리된 호흡기형 IBV들은 모두 Korean group I에, 그리고 신장형 IBV들은 모두 Korean group II에 속하는 IBV임이 확인되었다. 또한 국내 분리주들간의 nucleotide 염기서열의 상동성을 비교한 결과 Korean group II에 속하는 신장형 IBV들간에는 95% 이상의 상동성을 보여 유전학적으로 안정한 IBV로 추정되었다. 반면에 Korean group I에 속하는 호흡기형 IBV들간에는 88% 정도의 낮은 상동성을 보였을 뿐만 아니라 아미노산 염기서열 분석 결과 일부 아미노산의 결손, 삽입, recombination 등이 확인되어 유전학적으로 불안정한 IBV로 추정되었다. Korean group I에 속하는 호흡기형 IBV들이 유전학적으로 불안정한 이유는 이들 호흡기형 IBV들은 주로 닭의 호흡기도에서만 증식이 되기 때문에 국내에서 보편화되어 있는 Massachusetts형 IB 생독백신의 광범위한 사용과 야외 변이형 IB 감염에 따른 partial immune pressure에 의한 영향을 신장형 IBV보다 많이 받기 때문에 바이러스의 생존을 위해 유전자의 변이가 보다 쉽게 일어난 것으로 추정하고 있다. 반면에 Korean group II에 속하는 신장형 IBV들은 호흡기형 IBV들에 비하여 tissue tropism이 넓기 때문에 유전학적으로 유전자의 변이의 가능성이 적은 것으로 추정하고 있으며, 이러한 가정은 과거 10년간 국내에서 분리된 신장형 IBV S1 유전자의 nucleotide 염기서열 변이율이 2.9~5.7% 정도로 매우 낮음에 근간을 두고 있다. Korean group II에 속하는 신장형 IBV는 유전학적으로 매우 안정할 뿐만 아니라 과거 10년간 국내에서 지속적으로 분리된 주 유행주이기 때문에 국내 유행 IB의 효과적 예방을 위한 차세대 생독백신 후보주로서의 연구가 현재 진행 중에 있다.

(색인어 : 전염성기관지염, S1 유전자, 유전적다형, 진화)

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