94

DOES IBV CHANGE SLOWLY DESPITE THE CAPACITY OF THE SPIKE PROTEIN TO VARY GREATLY?

D. Cavanagh,[1*] K. Mawditt,[1] A. Adzhar,[1] R. E. Gough,[2] J.-P. Picault,[3]
C. J. Naylor,[1] D. Haydon,[4] K. Shaw,[1] and P. Britton[1]

[1]Institute for Animal Health
Compton Laboratory
RG20 7NN, United Kingdom
[2]Central Veterinary Laboratory
Weybridge, KT15 3NB, United Kingdom
[3]Laboratoire Central de Reserches Avicole et Porcine
22440 Ploufragan, France
[4]Department of Zoology
University of Oxford, OX1 3PS

1. SUMMARY

We have sequenced that part of the spike protein (S) gene which encodes the amino-terminal and most variable quarter (hypervariable region, HVR) of the S1 subunit of 28 isolates of the 793/B (also known as CR88 and 4/91) serotype of infectious bronchitis virus (IBV) and the whole of S1 for nine of them. The isolates were from France and Britain between the years 1985 (first isolation) and 1996. The maximum nucleotide and amino acid differences between the first isolate and the others were 4.1% and 7.6%, respectively, for the whole of S1 and 7.1% and 14.6%, respectively, in the HVR. Analysis within clearly recognisable subgroups suggested that even in the HVR the nucleotide mutation rate was only 0.3 to 0.6% per year. However, there was no evidence that mutations had become fixed in a progressive manner; this serotype did not appear to be evolving. Strains isolated several years apart could be more similar than those isolated in a given year. It is likely that the amino acid changes are largely at positions where amino acid differences are tolerated rather than as a consequence of immune pressure. Reasons for this conclusion are discussed.

Coronaviruses and Arteriviruses, edited by Enjuanes *et al.*
Plenum Press, New York, 1998

2. INTRODUCTION

IBV is known to exist as many serotypes, defined by haemagglutination-inhibition (HI) or virus neutralization (VN) tests, and doubtless many more await discovery. The epitopes which define serotype are formed largely by amino acids within the first and third quarters of the S1 subunit (Kant et al., 1992). It is in these regions that most amino acid differences occur between serotypes and within serotypes (Cavanagh, 1995). Serotypes frequently, but not invariably, differ by 20 to 25% of S1 amino acids. The amino-terminal quarter of S1 is the most variable, this hypervariable region (HVR) differing between serotypes by 35% or more of amino acids. Such differences might suggest that IBV mutates rapidly.

In the winter of 1990/91 in Britain chickens suffered respiratory disease, and unusual pathology, caused by a serotype of IBV not previously detected in Britain (Gough et al., 1992; Parsons et al., 1992). The serotype was named 793/B (also 4/91) after one of the earliest British isolates. It differs from all known IBV serotypes at 21 to 25% of S1 amino acids (Adzhar et al., 1997). It subsequently transpired that a large number of strains isolated in France in 1988 (prefixed by CR88) were of the same serotype (Picault et al., 1995). Retrospective serological analysis revealed that one French isolate of 1985 was also of this serotype. These epidemiological studies presented us with an opportunity to examine the manner and extent to which changes were occurring in this serotype over a decade.

3. MATERIALS AND METHODS

The French isolates (number examined in parenthesis) were from years 1985 (1), 1988 (3) and 1994 (6) and the British isolates were from years 1991 (7), 1993 (6), 1995 (2) and 1996 (4).

The S1 part of the S gene of the 1991 and 1993 isolates was amplified in a reverse transcriptase polymerase chain reaction (RT-PCR) using 'universal' IBV oligonucleotides as primers (Adzhar et al., 1997). The DNA products were cloned prior to sequencing. For all other isolates sequencing was performed directly on the PCR products. The HVR was amplified using two primers specific for the 793/B genotype.

The phylogenetic relationships between the strains were investigated using the DNAML (maximum likelihood) package of Felsentein (1993); 11 'hill climbs' were perfomed. Estimates of the mean corrected (Jukes and Cantor, 1969) numbers of synonymous changes per synonymous site (d_s) and non-synonymous changes per non-synonymous site site (d_{ns}) per sequence pair were obtained using the method of Nei and Gojobori (1986).

4. RESULTS

4.1. Nucleotide Sequence Comparisons

It is not suggested that the British strains had developed directly from the French strains in this study or, indeed, from any other French strains. It is highly likely that this serotype was prevalent in Continental Europe, but not in Britain, by 1990 (Cook et al., 1996). Notwithstanding, we have used CR85131, the earliest isolate of the 793/B serotype, as a reference point.

The sequence of that part of the S gene encoding the whole of the S1 subunit was determined for nine of the isolates. Comparison of each nucleotide sequence with that of

Table 1. Percentage differences between the nucleotide and amino acid sequence of the whole of S1 of the earliest 793/B isolate (CR85131) with those of eight other strains of the same serotype

Isolate	Year	Country	Difference (%) from whole S1 sequence of CR85131	
			Nucleotides	Amino acids
CR88061	1988	France	3.0	5.6
2/91	1991	Britain	3.3	6.5
3/91	1991	Britain	3.1	5.9
5/91	1991	Britain	3.2	5.8
7/91	1991	Britain	3.3	6.5
7/93	1993	Britain	3.9	7.6
CR94047	1994	France	4.1	6.7
1233-95	1995	Britain	3.7	6.5

the earliest isolate (CR85131) of the 793/B serotype showed that the strains differed at 3.0 to 4.1% of nucleotides (Table 1).

In order to get a more thorough picture of the sequence differences exhibited by this genotype we sequenced the HVR (approximately 450 nucleotides) of an additional 19 isolates. Relationships among the 28 isolates in this region are illustrated by a maximum likelihood phylogeny unrooted tree in Figure 1. A number of subgroups can be discerned.

The best estimate of the rate at which these viruses are mutating can be deduced from the subgroup at the top of Figure 1. This subgroup comprises 13 strains isolated in Britain between 1991 and 1996. The greatest difference between two of the isolates, isolated in 1991 and 1996, respectively, was only 1.5% of nucleotides. This is very low when one considers that these figures refer to the HVR. It is likely, therefore, that all these isolates have a common origin. If that is the case then there has been a change of only 1.5 % of nucleotides in five years, or 0.3%/year. The rate for the whole of the S1 part of the S gene would be approximately half this figure.

The other British subgroup comprises five isolates. The greatest difference in the HVR, between a 1991 and a 1995 isolate, was 2.6%. If the 1995 isolate had developed from the 1991 isolate then this indicates a rate of change of 0.6%/year.

The five 1994 French isolates which are on the same branch as the earliest (1985) isolate (CR85131) have differences of 2.6 to 3.9% from this isolate. If these strains developed directly from CR85131 then this difference over nine years suggests a mutation rate of 0.3 to 0.4% in the HVR. This is similar to the rate estimated for the two British subgroups and supports the phylogenetic analysis that these French 1994 isolates have developed from CR85131.

The three 1988 French isolates differed from CR85131 by 4.7 to 5.8%. Given the mutation rates estimated above this would suggest that these three isolates were introduced into France independently of CR85131.

4.2. Amino Acid Sequence Analysis

The amino acid sequence of S1 of CR85131 differed from that of the other eight strains for which the whole of S1 was sequenced by 5.6 to 7.6% (Table 1). The ratio (% amino acid difference:% nucleotide differences) was approximately 1.8.

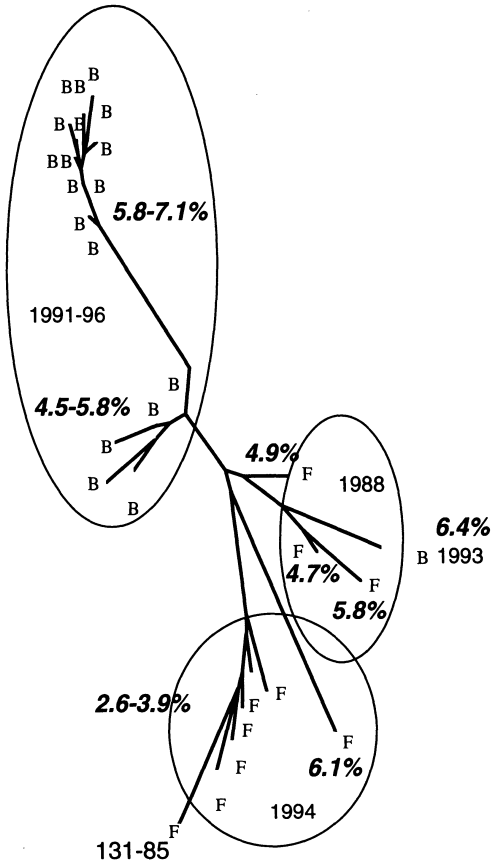

Figure 1. Relationships among 28 isolates of the 793/B serotype, using data corresponding to the hypervariable region, illustrated by a maximum likelihood phylogeny unrooted tree. The French and British isolates are indicated by 'F' and 'B', respectively. The ellipses encircle strains isolated in the years shown. The differences (%) between the nucleotide sequence of the HVR of the oldest isolate, CR85131, and the others is shown.

In the HVR the range of amino acid differences from CR85131 was 7.6 to 14.6%. The ratio (% amino acid difference:% nucleotide difference) was in the range 1.8 to 2.3 for the majority of strains, indicating a generally higher proportion of non-synonymous mutations in the HVR than in the S1 gene as a whole.. The exceptions were four of the isolates in the same branch as CR85131 in Figure 1, where the ratio was 2.6 to 2.8, indicative of a higher proportion of non-synonymous mutations than in the other isolates.

We have analysed the ratio of synonymous to non-synonymous mutations in the HVR region of 12 British 793/B strains isolated in 1991–93 and compared it to the same ratio obtained from an analysis of a 360 nucleotide partial sequence from the nucleocapsid protein (N) gene of 21 strains of IBV of many serotypes (Zwaagstra et al., 1992). The N protein is much less variable than the S1 protein. The expected value of d_s/d_{ns} (see Materials and Methods) for the N gene was 6.48 (standard error 1.36) and for the HVR of S1 gene was 1.35 (standard error 0.46). These ratios are significantly different from each other ($p<0.0002$), demonstrating that non-synonymous substitutions were relatively more common with respect to synonymous substitutions in the S1 gene than the N gene. Indeed the value of 1.35 demonstrates that a high proportion of mutations in the HVR of S1 led to changes in amino acid composition.

5. DISCUSSION

A d_s/d_{ns} ratio of 1.0 is often cited as evidence for the presence of positive Darwinian selection for amino acid changes (Hughes and Hughes, 1995; Seibert et al., 1995). A ratio of slightly greater than 1.0 could result either from a selection pressure arising from immune surveillance, or as a consequence of reduced structural constraints on the protein structure thereby permitting a wider diversity of tolerated amino acid substitutions. While both of these factors probably interact to determine the observed d_s/d_{ns} ratio, we favour the latter—low structural constraints—as being the major factor.

In Britain alone there are approximately 700 000 000 meat-type chickens in a year. Most of these live for only 6 to 8 weeks and are quickly replaced with day-old chicks. Throughout the country the chicks receive a similar vaccination regime, typically one, sometimes two, vaccinations with live IBV vaccine of the Massachusetts serotype. Chicks vaccinated with Massachusetts vaccine develop clinical signs when challenged with IBV of the 793/B type (Parsons et al., 1992). If a 793/B-type strain can successfully infect a flock of meat-type chickens, and our epidemiological studies show that this is common, there is little immune pressure on the S1 protein to change as the replacement flock will have virtually the same immune status. Therefore it is likely that many of the amino acid differences, which arise from random mutations, are simply tolerated because of low structural constraints in this part of the protein. It is the equivalent part of the S protein of porcine transmissible gastroenteritis virus (TGEV), canine coronavirus, feline coronavirus and human coronavirus 229E which shows the greatest variation among those species. Indeed, in the respiratory variant of TGEV known as porcine respiratory coronavirus, the corresponding part of the S protein is missing (Cavanagh, 1995).

Egg-type chickens number approximately 60 000 000 in Britain. They live for a year or more, sufficient time for more than one infection by IBV. Immunity developed to infection by a 793/B strain during the first few weeks of life might be expected to have some protective effect against subsequent infections by strains of the same serotype. Changes in the S protein during the first infection might give some selective advantage when the virus infects a chicken that has previously been infected with the 793/B serotype. Further changes in the S protein may occur during this second infection and be selected.

Our analysis of the S1 gene sequences of the 28 strains in this study suggest that the mutation rate is not especially high. While many of the mutations are non-synonymous there is no obvious fixation of amino acid changes. Rather, different amino acids can be tolerated at some locations, especially within the first and third quarters of S1. The maximum amino acid difference between two 793/B isolates over the whole of S1 was 7.6%, approximately one-third of the difference between many IBV serotypes. It may take a considerable time for an IBV to accumulate a change of 20% of its S1 amino acids. A live 793/B vaccine was introduced recently. If this becomes widely used two scenarios may be envisaged. Firstly, the virus may accumulate amino acid changes in S1, and possibly other proteins, more quickly, in response to immune pressure. Secondly, the vaccine may reduce the amount of 793/B in the environment to such an extent that a 'vacuum' is created, paving the way for another serotype to become dominant. The dominant serotype/genotype (D274) in Continental Europe during the early/mid 1980s decreased greatly in the later part of the decade, during which time D274-specific vaccines were introduced. Concomitant with the decline of D274 was the rise of 793/B.

ACKNOWLEDGMENTS

We acknowledge the financial support of the British Chicken Association and the Ministry of Agriculture, Fisheries and Food, Great Britain.

REFERENCES

A. Adzhar, R. E. Gough, D. Haydon, K. Shaw, P. Britton & D. Cavanagh., 1997, Molecular analysis of the 793/B serotype of infectious bronchitis virus in Great Britain. *Avian Pathol.* 27. In press.

Cavanagh, D., 1995, The coronavirus surface glycoprotein, in: S.G. Siddell (Ed) *The Coronaviridae*, 73–114, New York, Plenum Press.

Cook, J.K.A., Orbell, S.J., Woods, M.A. & Huggins, M.B., 1996, A survey of the presence of a new infectious bronchitis virus designated 4/91 (793B). *Vet. Rec.* **138**: 178–180.

Felsentein, J., 1993, PHYLIP (phylogeny inference package), version 3.5c. Department of Genetics, University of Washington, Seattle.

Gough, R.E., Randall, C.J., Dagless, M., Alexander, D.J., Cox, W.J. & Pearson, D., 1992, A 'new' strain of infectious bronchitis virus infecting domestic fowl in Great Britain. *Vet. Rec.* **130**:493.

Hughes, M.K. & Hughes, A.L., 1995, Natural selection on plasmodium surface proteins. *Mol. Biochem. Parasitol.* **71**: 99–113.

Jukes, T.H. & Cantor, C.R., 1969, Evolution of protein molecules, in: Munro, H.N. (Ed) *Mammalian Protein Metabolism*, 21–132.

Kant, A., Koch, G., Van Roozelaar, D.J., Kusters, J.G., Poelwijk, F.A.J. & Van Der Zeijst, B.A.M., 1992, Location of antigenic sites defined by neutralizing monoclonal antibodies on the S1 avian infectious bronchitis virus glycopolypeptide. *J. Gen. Virol.* **73**: 591–596.

Nei, M. & Gojobori, T., 1986, Simple methods for estimating the numbers of synonymous and non-synonymous nucleotide substitutions. *Mol. Biol. Evol.* **3**: 418–426.

Parsons, D., Ellis, M.M., Cavanagh,D. & Cook, J.K.A., 1992, Characterisation of an infectious bronchitis virus isolated from vaccinated broiler breeder flocks. *The Vet. Rec.*: **131**: 408–411.

Picault, J.P., Drouin, P., Lamande, J., Allee, C., Toux, J.Y., Le Coq, H., Guittet, M. & Bennejean, G., 1995, L'epizootie recente de bronchite infectieuse aviaire en France: importance, evolution et etiologie. 1ères Journee de la Recherche Avicole, Centre de Congres, d'Angers.

Seibert, S.A., Howell, C.Y., Hughes, K.A., & Hughes, K.L., 1995, Natural selection on the gag, pol and env genes of human immunodeficiency virus 1 (HIV-1). *Mol. Biol. Evol.*, **12**: 803–813.