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Amino acids within hypervariable region 1 of avian coronavirus IBV (Massachusetts serotype) spike glycoprotein are associated with neutralization epitopes

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Summary

The spike glycoprotein (S) gene of IBV codes for a precursor protein which is cleaved into the N-terminal S1 and C-terminal S2 glycopolypeptides. The S1 glycopolypeptide, which induces neutralizing antibody, comprises approximately 520 amino acid residues. We have determined the nucleotide sequence of S1 of seven strains of the Massachusetts (Mass) serotype and the first 337 bases of two additional Mass strains. Despite the fact that the strains had been isolated over three decades in Europe and the U.S.A. there was only 4% base and 6% amino acid variation within the group. Nearly one third of the 32 amino acid differences in S1 were in two hypervariable regions (HVRs 1 and 2) comprising residues 38–51 and 99–115, identified by Niesters et al. (1986), showing that HVRs 1 and 2 are a feature of the Mass serotype. Amino acid variation within HVRs 1 and 2 was 29% and 40% respectively. Five vaccine strains could be distinguished from each other by sequencing of the first 337 nucleotides. Variants of M41 which resisted neutralization by two monoclonal antibodies (A13 and A38) had the same, single base change at position 134, resulting in substitution of proline residue 45 by histidine. This indicates that residues within HVR 1 are associated with epitopes which induce neutralizing antibody.

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Introduction

Extensive antigenic variation is a feature of IBV and new serotypes, based on neutralization tests, have been detected frequently (Cook and Huggins, 1986). Only recently has the molecular basis for this variation been sought. Most attention has been focused on the spike glycoprotein (S), the major inducer of neutralizing antibody (Cavanagh et al., 1984, 1986a), which is a highly glycosylated protein of approximately 1160 amino acids which is cleaved into two glycopolypeptides, S1 and S2, of about 520 and 625 residues, respectively (Binns et al., 1985; Cavanagh et al., 1986b). S1 and S2 are derived from the amino-(N-) and carboxy-(C-) halves of the precursor polypeptide, respectively. Cloning and sequencing of the S gene of a number of serotypes have revealed that amino acid differences between them occur twice as frequently in S1 than in S2 (Binns et al., 1985, 1986; Niesters et al., 1986, Niesters, 1987). Experimental proof that S2 anchors S in the membrane has led to the suggestion that the distal part of S is formed largely by S1. These observations, considered with the findings that two neutralizing monoclonal antibodies (MAbs) reacted with S1 (Mockett et al., 1984) and that urea-released S1 can induce neutralizing antibody (Cavanagh et al., 1986a) show that the multimeric S1 subunit of S (Cavanagh, 1983) is a major inducer of neutralizing antibody.

Sequence comparisons of two strains of the Massachusetts (Mass) serotype (M41 and Beaudette) indicated that S1 had two hypervariable regions (HVRs) (Niesters et al., 1986). Circumstantial evidence that HVRs 1 and 2 have significance with respect to serotype-specific antibody has been produced by Niesters (1987) who showed that some IBV serotypes differed by as few as 7 amino acids and that 5 of these differences were within HVRs 1 and 2. To examine further these HVRs and to establish the extent of variation within a single serotype we have sequenced part of the S gene of nine additional Mass strains. The most widely used vaccines have been developed from strains of this serotype. We have also sequenced variants which escaped neutralization by two MAbs and show that the mutation lies within HVR 1.

Materials and Methods

Virus strains

The following strains of the Mass serotype were used (year and place of isolation in parenthesis), those marked ‘*’ being vaccine strains: M41 (1941, USA); H52*, H120* (1955, The Netherlands); Ibvax* (in or before 1960, USA); HVI-140 (1968, England); Bronchimmune* (details unavailable); VF69/149 (1969, Northern Ireland); HV2 (1974, England); MM* (1977, USA). Serological studies with strains HVI-140, VF69/149 and HV2 have been reported (Darbyshire et al., 1979). Chicken embryo tracheal organ cultures were used for neutralization tests (Cook et al., 1976).

Western and dot-blotting

Western immunoblotting of M41 polypeptides was done as previously described (Cavanagh et al., 1986a) and reaction of MAbs with virus (treated with 1% Nonidet P40 or SDS) dotted onto nitrocellulose was essentially by the same procedure.
RNA sequencing

This was performed by the dideoxynucleotide method using oligonucleotides to prime the reverse transcription of the S gene of IBV-M41 virion RNA (Cavanagh and Davis, 1988).

Selection of MAb-resistant variants

Variants of M41 were selected by MAb A38 (Mockett et al., 1984) by the following procedure. Five hundred microlitres of mouse ascitic fluid containing MAb A38 was mixed at room temperature for 30 min with 50 μl of M41-containing allantoic fluid which had a titre of 8.1 log_{10} median ciliostatic doses/ml. The mixture was inoculated into eleven-day-old chicken embryos (100 μl/embryo) and incubated at 37°C for 2 days. The allantoic fluid from individual embryos was harvested. After dilution (1/1000) the allantoic fluid (100 μl) was mixed with 100 μl of the ascitic fluid. Serial 10-fold dilutions were then inoculated into tubes containing tracheal organ cultures, 5 tubes per dilution. After 3 days the fluid in those tubes showing ciliostasis at the limiting dilution was harvested. One hundred microlitre volumes were mixed with 100 μl of MAb diluted 1/5 and the virus was titrated. After a further 3 days the limiting dilution tubes were harvested and the titer of the virus amplified by growing in embryonated eggs. Five clones of variant virus (vA38) selected in this way were chosen and shown to be resistant to neutralization by MAb A38 but not by polyclonal antiserum to IBV-M41. Two attempts to select variants in this way using MAb A13 were unsuccessful; all virus was neutralized. A successful attempt was made to isolate variants to this MAb by mutagenising virus with nitrous acid, essentially as described by Scheid and Choppin (1976), followed by a selection procedure similar to that described for MAb A38.

Results

Sequence differences within the Mass serotype

We have sequenced the S1 part of the S gene, starting at the codon which codes for the amino terminus of the mature S1 i.e. excluding the signal sequence. The M41 sequence generated by reverse transcription of virion RNA was identical to that obtained from cloned M41 RNA (Binns et al., 1986) except at base 323 where we identified U (as did Niesters et al., 1986) instead of G. The nucleotide differences between M41 and the other Mass strains are shown in Table 1, including differences from the Beaudette strain (Houghton variant) (Binns et al., 1985). This strain, isolated in the USA in 1936, which has been passaged in embryonated eggs considerably more times than even vaccine strains, has also been adapted to growth in chick kidney cells and has been passaged in and handed on by several laboratories. Niesters et al. (1986) have shown that the Houghton and Salk strains of IBV-Beaudette differed at 20 nucleotide and 14 amino acid positions within the S gene. Thus these differences have arisen during laboratory manipulation and are likely to have little relevance to IBV evolution in the field. Preliminary sequencing of isolate HVI-140 indicated that two strains were present. Consequently it was
cloned by passage at limiting dilution in tracheal organ cultures. Sequencing of six clones, shown by neutralization tests to be of the Mass serotype, revealed that they had identical S1 sequences.

Strain VF69/149 had the same S1 nucleotide sequence as HV2. Excluding those 16 bases where only Beaudette (Houghton strain) differed from any of the other

|     | 10 | 20 | 30 | 40 | 50 | 60 | 70 |
|-----|----|----|----|----|----|----|----|
| M41 | ALYDSSYYVYYQSAFRPPNGWHLHGGAYAVVINISSENNAGSSPGCIVGTIGHGCRVVNASSIAMTPSS |
| Beau | V   | S   | Q   | F   | S   | T   | I   |
| HV1-140 | V   | VS  | S   | F   | S   | T   | D   |
| HV2 | T   | S   | T   | I   |
| H52 | T   | S   | T   | I   |
| H120 | V   | S   | T   | I   |
| MM | V   | S   | T   | D   |
| Ibvax | V   | S   | T   | I   |
| Bronch | V   | S   | T   |
| vA38 |   |   |   |   |   |   |   |

|     | 80 | 90 | 100 | 110 * | 120 | 130 | 140 |
|-----|----|----|------|-------|----|----|-----|
| M41 | GMMAWSSQFCTAHCNFSSTDFTVFTHCYDGCPITGMQRKNFLRVSMKNGQLFYNYLTVSVAKYPTFKSF |
| Beau |   |   | HG  | L    | Q   |     | I   |
| HV1-140 | TN |   | HG  | L    | Q   | SI  | E   |
| HV2 | Y   | H   | Q   |
| H52 | Y   | HG  | Q   |
| H120 | Y   | HV  | Q   |
| MM |     | H   | Q   |
| Ibvax | Y   | H   | QHS  |
| Bronch |     | HG  | QH  |

|     | 150 | 160 | 170 | 180 | 190 | 200 | 210 |
|-----|-----|-----|-----|-----|-----|-----|-----|
| M41 | QCYNNLTSTVLYNLGDLVYTSNETTDVTSSAGVFYKAGGPIYKVMREVKALAYFVNGTATQDVILCDGSPGL |
| Beau |   |   |     | R   |
| HV1-140 |   |   |     |
| HV2 |   |   |     |
| H52 |   |   |     |
| H120 |   |   |     |
| MM |   |   |     |

|     | 220 | 230 | 240 | 250 | 260 | 270 | 280 |
|-----|-----|-----|-----|-----|-----|-----|-----|
| M41 | IACQYNTCNFSGDGYPFINSSLVQKIFIVYRENSVNTTTFLHNFTFHNETGMPNPGCVQNIQTYQTFTA |
| Beau | T   |     | C   | I   |     | K   |
| HV1-140 | T   |     |     |     |     | P   |
| HV2 | T   |     |     |
| H52 | T   |     |     |
| H120 | T   |     |     |
| MM | Y   | S   |     |

Fig. 1. Amino acid differences between the S1 moiety of the S protein of M41, a MAb escape variant of M41 (vA38) and other Mass strains. The sequence begins with the N-terminus of S1, excluding the signal sequence. The N-terminus of S2 is at residue 520. Isolate VF69/149 had the same sequence as HV2 and is not shown. The entire S gene of vA38 was sequenced but only the first 70 amino acids are indicated as there was only one difference between vA38 and wild type M41. Beau and Bronch are the Beaudette (Houghton strain) and Bronchimmune strains respectively. Only the first 112 amino acids of Ibvax and Bronchimmune were determined. The dotted lines (...) show regions where sequence data was not obtained. An asterisk (*) shows where the Salk strain of Beaudette was identical to M41 (Niesters et al., 1986).
strains there were only 62 bases (4.0%) in S1 where one or more strains differed (Table 1). At the amino acid level, and again excluding those 10 positions at which only Beaudette varied, there were differences at 32 (6.2%) of residues (Fig. 1).

Niester et al. (1986) observed that when the S sequence of the M41 and Beaudette strains were compared there were two regions in S1 where amino acid differences were concentrated. These were able regions (HVRs 1 and 2) are at amino acid positions 38–51 and 99–115 in Fig. 1. Our results for S1 of six additional strains, plus partial sequence for two others, shows that at most residues within HVRs 1 and 2 strains resembled either Beaudette or M41.

**MAb-resistant variants have a mutation in HVR 1**

Two MAbs (A38 and A13), each of which can neutralize virus infectivity and prevent haemagglutination, were available for the selection of variants which escaped neutralization. We have previously shown that these MAbs reacted with S1 and competed with each other in an enzyme-linked immunosorbent assay (Mockett

| 290 | 300 | 310 | 320 | 330 | 340 | 350 |
|-----|-----|-----|-----|-----|-----|-----|
| M41 | QSGYNYFMNFSFLSSFVYKESNFMGYHPSCNFRLETINNGLFNLSVSISIAYGFLQGCKQSVSFGRAT |
| Beau | K |
| HVI-140 | L |
| HV2 | |
| H52 | |
| H120 | |
| MM | |

| 360 | 370 | 380 | 390 | 400 | 410 | 420 |
|-----|-----|-----|-----|-----|-----|-----|
| M41 | CCYAASYGGSPLCKGVYSGELDLNFECGLLLVVTYKSGSRIQTATEPPVITRHNYNNTLNTCVDYNTY |
| Beau | H |
| HVI-140 | Q |
| HV2 | L |
| H52 | L |
| H120 | L |
| MM | H |

| 430 | 440 | 450 | 460 | 470 | 480 | 490 |
|-----|-----|-----|-----|-----|-----|-----|
| M41 | RIGGQFIINTIDSAVSYNLADAGLALIDTSGSIDFVVQGEYGLTYYKVNFCEDVQFVSVGGKLVGI |
| Beau | N |
| HVI-140 | S N |
| HV2 | S N |
| H52 | S N |
| H120 | S N |
| MM | |

| 500 | 510 | 520 |
|-----|-----|-----|
| M41 | LTRSRNFGSQLLENQFYKITNGRRPRSRITE |
| Beau | |
| HVI-140 | C H |
| HV2 | G |
| H52 | |
| H120 | |
| MM | |

Fig. 1 (continued).
et al., 1984). We have previously shown that MAb A13 can bind to S1 after its removal from virions by urea, in which circumstances the S1 exists as a monomer and not as an oligomer (Cavanagh et al., 1986a). However, neither MAb bound to S1 that had been transferred to nitrocellulose or to SDS-treated virus which had been immobilised on nitrocellulose, indicating that some element of conformation was a feature of the epitopes.

We sequenced the entire S gene of two clones of variant (vA38) selected using MAb A3g in addition to that of the wild-type M41. Only one nucleotide difference between the variants and the wild-type was detected. This was a change from C to A at base position 134 (Fig. 2), resulting in the substitution of amino acid residue 45 (proline) by histidine in HVR 1 (Fig. 1). Three other clones of vA38 were sequenced in this region; the same base difference was detected. After nitrous acid treatment a variant resistant to MAb A13 was obtained. Sequencing of all of the S1 part of the gene of two clones of variant showed only one nucleotide difference, the same as

| Base No. | M41 Beaudette | HV1 | HV2 | M22 | M120 | MM | Ibvax | Bronchirome | Amino acid change | Base No. | M41 Beaudette | HV1 | HV2 | M22 | M120 | MM | Ibvax | Bronchirome | Amino acid change |
|----------|----------------|-----|-----|-----|------|----|-------|--------------|-------------------|----------|----------------|-----|-----|-----|------|----|-------|--------------|-------------------|-------------------|
| 2        | C              | U   | U   | U   | U    | +  | 488   | C            | U                | +       |                |     |     |     |      |    |       |             |                   |                   |
| 37       | H              | C   | C   | C   | C    | G  | 560   | A            | G                | +       |                |     |     |     |      |    |       |             |                   |                   |
| 36       | C              | U   | U   | U   | U    | +  | 615   | A            | G                | +       |                |     |     |     |      |    |       |             |                   |                   |
| 48       | U              | C   | C   | C   | C    | C  | 683   | U            | C                | C       | +               |     |     |     |      |    |       |             |                   |                   |
| 59       | A              | G   | U   | U   | +    | 714 | U     | A            | G                | +       |                |     |     |     |      |    |       |             |                   |                   |
| 75       | C              | A   | U   | U   | U   | U   | U     | +            | 746   | U              | G     | +               |     |     |     |      |    |       |             |                   |                   |
| 81       | G              | U   | +  | 747 | U    | C  | +       |                |                   |         |                   |     |     |     |      |    |       |             |                   |                   |
| 82       | G              | U   | +  | 757 | A    | U   | +       |                |                   |         |                   |     |     |     |      |    |       |             |                   |                   |
| 83       | G              | U   | +  | 764 | U    | G   | +       |                |                   |         |                   |     |     |     |      |    |       |             |                   |                   |
| 84       | U              | G   | G   | G   |      | 792 | U     | A        | A                | A       | A              | +     |     |     |      |    |       |             |                   |                   |
| 88       | U              | C   | 807 | C   | C    |     |        |              |                   |         |                   |     |     |     |      |    |       |             |                   |                   |
| 99       | G              | U   | +  | 818 | U    | C   | +       |                |                   |         |                   |     |     |     |      |    |       |             |                   |                   |
| 100      | C              | U   | +  | 832 | C    | A   | +       |                |                   |         |                   |     |     |     |      |    |       |             |                   |                   |
| 102      | U              | A   | +  | 886 | G    | C   | +       |                |                   |         |                   |     |     |     |      |    |       |             |                   |                   |
| 108      | C              | U   | U   | U   | U    | +  | 936   | U            | A                | +       |                |     |     |     |      |    |       |             |                   |                   |
| 113      | C              | U   | +  | 963 | U    | U   | +       |                |                   |         |                   |     |     |     |      |    |       |             |                   |                   |
| 114      | C              | U   | +  | 1037| G    | A   | +       |                |                   |         |                   |     |     |     |      |    |       |             |                   |                   |
| 123      | C              | U   | +  | 1039| G    | A   | +       |                |                   |         |                   |     |     |     |      |    |       |             |                   |                   |
| 134      | C              | +  | 1085| U    | C   | C   | C       |                |                   |         |                   |     |     |     |      |    |       |             |                   |                   |
| 135      | A              | +  | 1086| U    | C   | C   | C       |                |                   |         |                   |     |     |     |      |    |       |             |                   |                   |
| 143      | U              | C   | C   | C   | C    | C   | +       | 1073   | G                | A       | +               |     |     |     |      |    |       |             |                   |                   |
| 152      | C              | U   | U   | U   | U    | +  | 1082   | C            | U                | U       | +               |     |     |     |      |    |       |             |                   |                   |
| 164      | C              | A   | +  | 1086| G    | U   |         |                |                   |         |                   |     |     |     |      |    |       |             |                   |                   |
| 208      | C              | C   | +  | 1116| U    | C   | +       |                |                   |         |                   |     |     |     |      |    |       |             |                   |                   |
| 227      | C              | C   | +  | 1118| U    | A   | A       | +               |                   |         |                   |     |     |     |      |    |       |             |                   |                   |
| 230      | G              | A   | +  | 1122| U    | C   | +       |                |                   |         |                   |     |     |     |      |    |       |             |                   |                   |
| 246      | C              | U   | U   | U   | U    | +  | 1205   | G            | A                | A       | A              | +     |     |     |      |    |       |             |                   |                   |
| 255      | C              | U   | +  | 1207| C    | A   | +       |                |                   |         |                   |     |     |     |      |    |       |             |                   |                   |
| 291      | U              | C   | C   | C   | C    | C   | +       | 1290   | G                | A       | +               |     |     |     |      |    |       |             |                   |                   |
| 295      | U              | C   | C   | C   | C    | C   | +       | 1335   | G                | A       | +               |     |     |     |      |    |       |             |                   |                   |
| 304      | G              | U   | G   | U   | G    |      |      | 1398   | G                | A       | A              | +     |     |     |      |    |       |             |                   |                   |
| 310      | A              | U   | +  | 1397| C    | A    | A       | A        | A                | +       |                |     |     |     |      |    |       |             |                   |                   |
| 328      | A              | C   | C   | C   | C    | C   | +       | 1413   | C                | U       | +               |     |     |     |      |    |       |             |                   |                   |
| 331      | G              | U   | C   | C   | C   | C   | +       | 1479   | U                | C        | C             |     |     |     |      |    |       |             |                   |                   |
| 334      | U              | C   | +  | 1512| C    | U    | U       | +       | 1521   | C                | U       | +               |     |     |     |      |    |       |             |                   |                   |
| 335      | U              | C   | +  | 1524| U    | C    | C       | C        | C                | C       | +               |     |     |     |      |    |       |             |                   |                   |
| 337      | U              | A   | A   | A   | A    | +  | 1543   | C            | G                | +       |                |     |     |     |      |    |       |             |                   |                   |
| 372      | U              | U   | U   | U   | U    | -   | -       | A          | A                | A       | -               |     |     |     |      |    |       |             |                   |                   |
| 397      | A              | G   | -   | -   | -    | +  |         |              |                   |         |                   |     |     |     |      |    |       |             |                   |                   |

* A short line (-) means base not determined
Fig. 2. RNA sequencing of wild-type (W) M41 and two clones of a variant (V) of M41 selected using MAb A38. Virion RNA was sequenced by the dideoxy method and the reaction products (U, G, C, A) were analyzed on a 6% sequencing gel. The autoradiograph shows nucleotide positions 120–141 and reveals that the variant has an A in place of a C at base position 134.

that obtained with MAb A38. It may, therefore, have been merely fortuitous that a variant to MAb A13 was obtained after but not before nitrous acid treatment. As anticipated vA38 was resistant to neutralization by MAb A13. Of the 9 Mass strains sequenced 6 (M41, H52, H120, MM, VF69/149 and HVI-140) have been treated with MAbs A38 and A13; only M41 was neutralized (Mockett et al., 1984).

Thus these results indicate that residues within HVR 1 are associated with epitopes which induce neutralizing antibody.

Discussion

Despite the fact the strains in this study were isolated over a thirty year period in several countries on two continents there was remarkable base and amino acid similarity. Niesters (1987) has shown that some non-Mass type IBV strains isolated in The Netherlands during the 1970s varied by as much as 50% in their base sequences of S1 whereas some pairs of strains, of different serotype, varied by less than 2%. Taken together these observations indicate that IBV evolution is complex. Thus, IBV may exhibit antigenic drift, a small number of amino acid changes resulting in a change of serotype, but without one serotype necessarily displacing others.

The finding of a small number of differences between the S1 part of S genes of the Mass serotype isolates is useful since it enables the five vaccine strains to be distinguished from each other within the first 337 bases of S1. Even the H52 and
H120 vaccines, derived by different numbers of egg passages of the same isolate, can be distinguished from each other. H52 and H120 are of particular interest not merely because they are widely used but because they differ in virulence (MacDonald and McMartin, 1976). Although there is no evidence to link this difference in pathogenicity with the S protein in preference to other gene products, it is noteworthy that we detected only one amino acid difference between H52 and H120. Moreover, this difference was within IIHR 2.

Although HVRs occur within S1 their existence alone does not show that they are immunogenic. However, our sequencing of MAbs-resistant variants shows that some residues within HVR 1 are associated with epitopes that induce neutralizing antibody, the basis of IBV serotyping. HVRs 1 and 2 are not associated with the major hydrophilic domains indicated by the hydropathicity profile of S (Binns et al., 1985). However, that HVR 1 has a surface location is indicated by our results which suggest that it induces antibody and show that it is associated with several serine residues which are usually found at protein surfaces (Schulz and Schirmer, 1979). Interestingly, HVR 1 is also associated with several glycine residues which confer flexibility to polypeptide chains (Schulz and Schirmer, 1979). While there is some dispute as to the relative importance for antigenicity of the accessibility and flexibility of an amino acid sequence there is agreement that surface exposure and flexibility are strongly correlated (Novotny et al., 1987; van Regenmortel, 1987).

The existence of HVRs 1 and 2 was first intimated following a comparison of the S genes of the M41 and Beaudette isolates (Niesters et al., 1986). However, as discussed above, Beaudette has had a chequered history in laboratories, during which time variation occurred. Our analysis of many Mass serotype strains with much simpler passage histories does, however, support the proposal that amino acid sequences 38–51 and 99–115 are hypervariable regions. Interestingly, within HVRs 1 and 2 the bases of each strain were mostly like either M41 or Beaudette. Indeed, although the Houghton and Salk strains of Beaudette differed at 8 amino acid residues in S1 (Niesters et al., 1986), at 7 of these residues either the Houghton or Salk strain was the same as M41. Although there is no evidence as to the immunogenicity of HVR 2 our current results indicate that within HVRs 1 and 2 there appears to be a limit to the variation which is compatible with retention of the Mass serotype. Circumstantial evidence that both HVRs 1 and 2 have significance with respect to serotype-specific antibody has been produced by Niesters (1987) who showed that some IBV serotypes differed by as few as 7 amino acids and that 5 of these were within HVRs 1 and 2. This suggests that in some cases a very small number of critical amino acid changes are sufficient to alter antigenicity greatly. This simple view may not, however, apply in all cases. Thus, the S1 sequence of two strains of the same serotype (D1466 and D212) differed at 50% of the amino acid residues overall and at 22 of the 30 residues within HVRs 1 and 2 (Niesters, 1987). This observation, considered with our results showing substantial variation within HVRs 1 and 2 within a single serotype, suggests that residues without these two regions are also involved in epitopes which induce neutralizing antibody. Of the 32 places within S1 where there is amino acid variation (excluding differences unique to Beaudette), 19 were within the first 113 amino-terminal residues, an incidence
five-fold greater than in the remainder of S1. Comparisons of IBV strains 6/82 and M41 (Binns et al., 1986), porcine transmissible gastroenteritis coronavirus and feline infectious peritonitis coronavirus (Jacobs et al., 1987), and the A59 and JHM strains of MHV (Luytjes et al., 1987) have shown that amino acid variation within the spike protein is greatest within the amino-terminal region.

To determine further those residues which determine IBV serotype and to analyze contemporary IBV evolution we are sequencing the S genes of a succession of field isolates, of different serotype, obtained in the UK this decade.

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