Genetic and antigenic analysis of the G attachment protein of bovine respiratory syncytial virus strains

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Antigenic and genetic studies of bovine respiratory syncytial virus (BRSV) were made on isolates obtained from three continents over 27 years. Antigenic variation between eight isolates was initially determined using protein G-specific monoclonal antibodies. Four distinct reaction patterns were observed, two of which corresponded to the previously established subgroups A and AB. A third pattern was produced by five Scandinavian strains and a fourth was observed from a single Dutch isolate. The genetic diversity of 27 strains of BRSV was investigated by comparative nucleotide sequence analysis of a 731 nucleotide fragment in the G protein gene. Nine of the BRSV strains were analysed by direct sequencing of RT–PCR amplicons whereas sequences of 18 BRSV and three human respiratory syncytial virus (HRSV) strains were obtained from GenBank. The analysis revealed similarities of 88–100% among BRSV strains and 38–41% between BRSV and HRSV. A phylogenetic tree created for BRSV revealed two main branches, one of which divided into five further lineages, each representing a geographic cluster. A correlation was evident between the positions of some strains in the phylogenetic tree and their antigenic pattern. For HRSV strains, a genetic similarity of only 62% allowed the distinction of two antigenic subgroups, A and B, a pattern which was not seen for BRSV. This study showed that genetic analysis was an accurate method for discriminating BRSV strains and that these viruses should be regarded as a single genetic and antigenic group, within which variants can be distinguished.

Introduction

Bovine respiratory syncytial virus (BRSV), a pneumovirus of the Paramyxoviridae family, is one of the major respiratory pathogens of cattle. BRSV infections affect all age groups, most frequently calves (Stott et al., 1980; Van der Poel et al., 1993) but also dairy cows (Inaba et al., 1972; Elvander, 1996). BRSV is closely related antigenically to human respiratory syncytial virus (HRSV), an important cause of severe respiratory disease in infants and young children (McIntosh & Chanock, 1990). In outbreaks of BRSV, the severity of symptoms can show considerable variation both between and within herds (Frey, 1983) and it is not clear what factors influence the outcome of disease. Naive cattle exposed to the virus for the first time can be severely affected (Ames, 1993), but the disease may also have a virtually asymptomatic course (Van der Poel et al., 1993). Antigenic variations in the major surface glycoprotein of the G attachment protein may have important implications in the epizootology and pathogenesis of BRSV infections (Prozzi et al., 1997).

Antigenic studies of HRSV have shown the existence of two major subgroups, A and B. Reaction patterns with specific monoclonal antibodies (MAbs) revealed that the main differences between these two subgroups were located in the G protein (Mufson et al., 1985). Additional differences were also detected in the F, N, M and M2 proteins (Norrby et al., 1986). Variability in the amino acid sequences of the G protein has been demonstrated between isolates of both subgroup A (Cane et al., 1991) and subgroup B (Sullender et al., 1991) of HRSV. The separation of two subgroups has also been confirmed by RT–PCR (Gottschalk et al., 1996).
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Molecular studies, based on the use of HRSV- and BRSV-specific MAbs as well as studies of the molecular mass of the F protein and its cleavage products, have independently suggested the existence of different antigenic subgroups of BRSV (Baker et al., 1992; Pastey & Samal, 1993; Furze et al., 1994; Prozzi et al., 1997). In a study by Furze et al. (1994), the reaction patterns of MAbs specific for the G attachment protein of BRSV placed the majority of investigated strains into either of two antigenic subgroups, A and B. Schrijver et al. (1996) used the same MAbs and introduced a third subgroup, AB, also recognized by Prozzi et al. (1997). Both subgroups A and AB are considered to be currently circulating, while no isolates of subgroup B have been demonstrated since 1976 (Furze et al., 1994). American strains isolated during the last 10 years were investigated by Stine et al. (1997) and were found to be different from the above-mentioned subgroups A, B and AB.

Genetic studies on a number of BRSV strains showed the G protein to be the most variable, in terms of both nucleotide and amino acid sequences (Lerch et al., 1989, 1990; Mallipetdi & Samal, 1993; Prozzi et al., 1997; Stine et al., 1997). The mutations contributing to amino acid changes were not equally distributed along the gene but were largely concentrated in the extracellular domain and C-terminal parts of the protein (Lerch, 1990), as seen in HRSV strains (Cane et al., 1991; Sullender et al., 1991). It has been suggested that the central hydrophobic region of the ectodomain encompassing amino acids 166–186 is a putative receptor binding site in the G protein of HRSV (Johnson et al., 1987). This region has also been shown to be immunodeterminant in both HRSV (Åkerlind-Stopner et al., 1990) and BRSV (Langedijk et al., 1996a, b). In BRSV, this region is variable between different strains despite their high genetic similarity and confirms, according to Prozzi et al. (1997), the antigenic variation seen by MAb mapping.

The present study gives antigenic and genetic analysis of BRSV isolates obtained from three continents over a 27 year period. As a pilot study, the reaction patterns of four G protein-specific MAbs were determined for eight BRSV isolates, including five novel Scandinavian isolates and representatives of currently circulating subgroups of A and AB. Furthermore, for nine BRSV isolates, including the eight mentioned above, nucleotide and deduced amino acid sequence data were obtained by direct sequencing of G protein gene amplicons in an RT–PCR assay. To determine the variability and phylogenetic relationships of as many strains as possible, the published sequences of 18 BRSV strains were obtained from GenBank. In addition, the region involving 243 amino acids was studied to determine variations between investigated strains.

Methods

- **Virus strains.** Primary cultures of bovine foetal turbinate or bovine foetal lung cells (Elvander, 1996) were used to propagate virus and aliquots were stored at −70 °C until processed. The following field isolates of BRSV were obtained from natural outbreaks in Sweden, 187SW (1992), 504SW (1993), 271SW (1995) and 4546SW (1995), and Denmark, 2020Dk (1994). The Japanese strain NMK7 (1968) was kindly provided by I. T. van Oirschot (ID-DLO, Lelystad, The Netherlands) and has been described by Inaba et al. (1970). Strain Rb94 (1975) was provided by M. Merza (NNI, Uppsala, Sweden), the field strain 37NL (1974) was kindly provided by J. Kramps (ID-DLO, Lelystad, The Netherlands) and strain 375USA, described by Lehnhkuhl et al. (1979), was obtained from the ATCC.

The sequences of the following strains were taken from GenBank: 220-60, 4042, 127, 220-69, WBH and Snoook described by Furze et al. (1994, 1997), BovX, MVR553, Lelystad, Dorset and 85-1330 described by Prozzi et al. (1997), and the American isolates 234-489, NDKS-7, 87-14594, 236-652, 88-294, 1540R and 1344R described by Stine et al. (1997).

- **Antibodies.** The four MAbs used were generously donated by J. Furze (Compton, UK). The MAbs, named 44, 46, 62 and 70, were raised against three strains of BRSV (391-2, Snoook and 127) and recognized three antigenic domains on the G protein (Furze et al., 1994).

Hyperimmune bovine BRSV serum was prepared by inoculating a young castrated bull with BRSV antigen. Virus for this antigen was propagated in testis cells from the same animal using its own serum. The hyperimmune serum produced was specific for BRSV and had a neutralizing antibody titre of 1:32000 (Utenthal et al., 1996).

- **Peroxidase labelling assay (PLA) for the MAb reaction pattern.** The PLA typing technique for virus isolates was modified after Have et al. (1992). Virus strains were grown on primary foetal calf lung cells in 96-well culture microplates. After 5–7 days, when 10% of the cells showed cytopathic effect, the plates were washed three times in ice-cold PBS and rinsed once in ice-cold 99% ethanol. Finally, 100 µl 99% ethanol was added to each well and the plates were stored at −20 °C.

After washing, the monolayers were incubated with 50 µl MAbs diluted 1:100 in PBS supplemented with 0.1% Tween 20 (PBST) and 5% normal swine serum. The plates were incubated for 1 h at room temperature, then washed three times with PBST and a conjugated antiserum was added for 1 h at room temperature. The antiserum used were either a peroxidase-conjugated rabbit anti-mouse antibody (Dakopatts), diluted 1:250, for murine MAbs, or a peroxidase-conjugated goat antimouse IgG (Kirkegaard & Perry), diluted 1:1000, for bovine antibodies. Finally, substrate (3-aminophenyllethylcarbazole) was added and the mixture was left for 30 min at room temperature. Specific staining of affected cells was regarded as positive.

- **Isolation of RNA.** Isolation of RNA was performed as described previously (Vilcsek et al., 1994). In brief, 300 µl PBS, 25 µl 20% SDS and 5 µl proteinase K (10 mg/ml) (Boehringer-Mannheim) were added to 200 µl BRSV-infected cell culture supernatant. The mixture was incubated at 56 °C for 60 min. RNA was extracted with an equal volume of phenol (saturated to pH 4.3) and chloroform (1:1, v/v) (Sigma). The water phase was precipitated overnight at −20 °C with 2 vol. cold 95% ethanol and 0.1 vol. 3 M sodium acetate, pH 4.0. The RNA was pelleted by centrifugation, re-suspended in 10 µl sterile double-distilled water and stored at −20 °C.

- **Synthesis of cDNA.** The method was performed as previously described (Vilcsek et al., 1994) in a final volume of 25 µl. The mixture for synthesis of cDNA, consisting of 4 µl extracted RNA, 1 µl (0.02 U) random hexamers (Pharmacia) and 5 µl water, was denatured at 65 °C for 5 min and immediately chilled on ice for 5 min. RNAguard (1 µl (24 U); Pharmacia), 5 µl first-strand reaction buffer (0.25 M Tris–HCl, pH 8.3, 0.375 M KCl, 15 mM MgCl₂), 2.5 µl 2 mM each dNTP (Pharmacia) and
1 µl (200 U) Moloney murine leukaemia virus reverse transcriptase (Gibco) were added. The reaction mixture was incubated at 37 °C for 90 min and enzyme was then inactivated at 98 °C for 5 min. Synthesized cDNA was immediately used for PCR or stored at −20 °C.

### PCR
A 493 bp DNA fragment from the F protein gene of BRSV was amplified using primers B1 and B4A as described previously (Vilcék et al., 1994). A 731 nucleotide fragment from the G protein gene of BRSV was amplified using primers G1 and G2 (Stine et al., 1997). The end of the gene was amplified by combining primer B7A (5′ CATCAATCCAAA-GCACACACGTGTC 3′) from the G gene and primer F1 (5′ CACGG-ATCCTGCTTTGCGCATCC 3′), located in the 5′ end of the F gene. The reaction mixture contained 5 µl 10 × reaction buffer (100 mM Tris–HCl, pH 9.0, 500 mM KCl), 3 or 5 µl 25 mM MgCl₂, 1 µl 10 mM each dNTP (Pharmacia), 15 pmol each primer, 0.2 µl (1 U) Taq DNA polymerase (Perkin Elmer) and 2–4 µl cDNA. The final volume was adjusted to 50 µl with sterile water and the reaction mixture was overlaid with 2 drops mineral oil (Sigma). In vitro amplification of DNA was carried out in Perkin Elmer thermocyclers using denaturation at 94 °C for 1 min, annealing at 50 °C for B1 × B4A or 60 °C for G1 × G2 and B7A × F1 primer pairs, respectively, for 1 min, and extension at 72 °C for 90 s. After 35 cycles, the last step of the extension was prolonged for 7 min. PCR products (amplicons) were separated in 2% agarose gels, stained with ethidium bromide and visualized under UV light. The PCR assays were performed by applying the routine precautions and safety procedures of our laboratory (Belák & Ballagi-Pordány, 1993).

### Sequencing of PCR products
PCR amplicons were purified using microcon columns and directly sequenced in both directions with the same primers used to generate the PCR products. Sequencing was done in an ABI PRISM sequencing device based on the incorporation of fluorescein-labelled dideoxynucleotide terminators.

### Computer analysis
Nucleotide sequence analysis of the selected regions of the F and G genes was performed by comparing the corresponding regions of the nine BRSV isolates of the present study, as well as sequences of 18 BRSV and three HRSV strains obtained from GenBank. The nucleotide sequences were aligned using the multiple alignment programs from the DNASTAR software package. The phylogenetic tree was created using the PHYLIP computer program package (Felsenstein, 1993). The neighbour-joining method (Saitou & Nei, 1987) was applied with the Kimura two-parameter method and a transition/transversion ratio of two. The DRAWGRAM program was used to plot the tree graphics.

### Results

#### MAb typing of BRSV strains
The reaction patterns of five Scandinavian, one Dutch, one Belgian and one Japanese strains were tested by MAbs 44, 46, 62 and 70. The four Swedish viruses and the Danish isolate reacted identically with MAbs 70 and 44 only. The Japanese strain was the only one not to react with MAb 70, but did react with all other MAbs. The Belgian strain reacted with all MAbs, whereas the Dutch strain reacted with all MAbs except MAb 46 (Table 1).

### Sequence analysis of G attachment protein gene
The alignment of a deduced 243 amino acid sequence fragment of 27 strains of BRSV is given in Fig. 1. As shown, amino acid substitutions were distributed along the entire length of the selected part of the G gene, but occurred at a higher frequency in the extracellular region. The Scandinavian strains showed a uniform pattern of mutations, which were located at different sites compared to isolates from other countries, as shown in Fig. 1. In these strains, the amino acids were shifted at eight unique positions (Fig. 1; marked with black squares) of which changes at positions 12, 73, 133, 206 and 219 created a shift into a different amino acid functionality (polar to non-polar or vice versa). Four cysteine residues, at positions 173, 176, 182 and 186 (Fig. 1; marked with black diamonds), were highly conserved in all BRSV strains tested.

The phylogenetic tree constructed from amino acid data showed two major branches of BRSV termed I and II (Fig. 2). Branch I could be subdivided into Ia and Ib, and then further divided into five lineages, each representing a geographic cluster. As shown in Fig. 2, branch Ia held strains of antigenic subgroup A, whereas branch Ib consisted of European strains of subgroup AB. The Scandinavian strains did not segregate into any of these subgroups. Branch II represented strains of antigenic subgroup B only.

The addition of HRSV strains A2 and Long (antigenic subgroup A) and 18537 (antigenic subgroup B) to the BRSV dendrogram showed that they branched separately, with the creation of two additional groups. The comparatively high genetic distance between BRSV and HRSV caused compression of the phylogenetic tree within the BRSV groups (data not shown).

The overall similarity between the 27 BRSV strains was 87–7–100% at the nucleotide level and 82–7–100% at the amino acid level. A comparison between strains of branch I and II showed similarities of 87–7–92-7% at the nucleotide level and 82–7–87-7% at the amino acid level (Table 2). The

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**Table 1. Binding of G protein-specific MAbs 70, 62, 46 and 44 to a panel of eight BRSV isolates in a peroxidase labelling assay**

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<td>NMK7 J</td>
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The origin of strains is denoted as follows: SW, Sweden; DK, Denmark; NL, The Netherlands; B, Belgium; J, Japan. +, Positive (binding of MAbs); −, negative (no binding of MAbs).
Fig. 1. The alignment of deduced amino acid sequences of BRSV and HRSV strains in the region corresponding to amino acids 8–250 of the G attachment protein. The chosen sequences are truncated to allow comparison with sequences obtained from GenBank. Black squares indicate unique positions with a shift of amino acid in the Scandinavian strains. Black diamonds mark the position of the conserved cysteines. C, T and E are cytoplasmic, transmembrane and extracellular domains, respectively.

The following sequences were taken from published data: 220-60, 4642, 127, 220-69, WBH and Snook (Furze et al., 1994, 1997); BovX, MVR553, Lelystad, Dorset and 85-1330 (Prozzi et al., 1997); 234-489, NDKS-7, 87-14594, 236-652, 88-294, 1540R and 1344R (Stine et al., 1997).
nucleotide similarities within branch I were 91.4–99.9% for branch Ia and 96.2–100% for Ib; at the amino acid level, these values were 87.2–99.6 and 93.8–100%, respectively. The similarity within branch II was 95.8–99.7% at the nucleotide level and 93.8–100% at the amino acid level. For HRSV strains, the similarities between strains of antigenic subgroups A and B were 62 and 51% at the nucleotide and amino acid levels, respectively. A comparison between HRSV and BRSV revealed similarities of 38–41% at the nucleotide level and 27–32% at the amino acid level (Table 2).

### Table 2. Prototype strains showing nucleotide and amino acid similarities for the 21–752 nucleotide region of the G gene of BRSV and HRSV isolates

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**Genetic variability of the F gene region**

In a minor study including the nine BRSV isolates, a very low genetic variability was observed in a 417 nucleotide fragment of the F gene. The European, American and Japanese BRSV strains showed high similarity (95–100%). However, human and bovine strains were clearly different, with similarities of approximately 67–71%. Due to the low nucleotide variability, the phylogenetic tree of the F gene region showed less resolution (data not shown).
Discussion

MAb typing of eight selected BRSV strains, four Swedish isolates and one Danish isolate showed an identical reaction pattern with the four MAbs used and this was different from those of the Japanese, Dutch and Belgian strains. The nomenclature of antigenic grouping of BRSV has been defined with a panel of G protein-specific MAbs, first published by Furze et al. (1994), and later by Prozzi et al. (1997). According to their definitions, strains of subgroup A react with all but MAb 70, members of antigenic subgroup B react exclusively with MAb 70, and antigenic subgroup AB reacts with all MAbs of this panel. Strains not fitting into any of the patterns were termed ‘intermediate’. Using the same MAbs, the Japanese strain NMK7 was determined to be a member of subgroup A and the Belgian strain, Rb94, belonged to the newly established subgroup AB. In comparison with this data, the Scandinavian strains did not belong to any known subgroup. The Dutch strain, which did not fit any pattern, could be regarded likewise. In addition, the American strains investigated by Stine et al. (1997) formed an additional antigenic subgroup. In comparison with antigenic subgroups A and B of HRSV, it does not seem likely that at least six different antigenic subgroups of BRSV should exist. The assumption of antigenic subgroups of BRSV is further contradicted by the high genetic similarity (88–100%) between BRSV isolates; for HRSV subgroups A and B, similarity as low as 62% was found.

The results of the genetic studies indicate, in accordance with published data (Walravens et al., 1990; Lerch et al., 1991; Sullender et al., 1991), that the G gene region of BRSV is more variable than the F gene region. The observed divergences were located in a region corresponding to the extracellular domain and C-terminal parts of RSV strains (Cane et al., 1991). The Scandinavian strains showed a uniform pattern of residue substitutions which, at five positions, involved non-conservative amino acid changes. The implications of this shift are not yet established, but may indicate an evolutionary lineage that has to be further investigated, as suggested by Prozzi et al. (1997).

The nucleotide sequence and deduced amino acid sequence data, enabled us to construct a phylogenetic tree of BRSV strains collected from different parts of the world. Various computer programs, i.e. the MegAlign program (DNASTAR software package) (Fig. 2), the neighbour-joining method, FITCH and maximum-parsimony (PHYLYP package), created similar trees (data not shown). In spite of only small genetic differences between the 27 BRSV strains, two major branches, I and II, were observed (Fig. 2). Branch I, consisting of strains considered to be currently circulating, was subdivided into Ia and Ib and further divided into five lineages, each representing a geographic cluster. Branch Ia held three lineages: one American, one European and one Japanese. Branch Ib consisted of two lineages, one holding the European strains and the other holding the Scandinavian strains. A correlation was found between the position of some strains in the phylogenetic tree and their suggested antigenic subgroup (Fig. 2). Strains of subgroup A were found among American, European and Japanese strains in branch Ia whereas in branch Ib, a European cluster with representatives of subgroup AB was found. Branch Ib also held a cluster of non-subgrouped Scandinavian strains. In branch II, strains of antigenic subgroup B isolated prior to 1976 were found and it has been suggested that this subgroup does no longer exist.

Regarding the high genetic similarity between all BRSV strains tested and the increasing variants of potentially new antigenic subgroups (Table 1) (Prozzi et al., 1997; Stine et al., 1997), the proposed subgrouping of BRSV by MAb typing does not seem to be appropriate. Sequencing allows both grouping and strain discrimination. Different strains of BRSV are likely to be variants within one subgroup, as suggested by other workers (Schrijver et al., 1996; Prozzi et al., 1997). The geographic distribution of strains, seen in the phylogenetic tree of BRSV, has not been observed for HRSV (Garcia et al., 1994; Cané & Pringle, 1995) and is presumably due to a much higher migration rate for man than for domestic animals.

Comparative analysis of nucleotide sequence data of the branches showed that strains within branch I were more closely related to each other than the viruses of branch I and II (Table 2) which is in agreement with the findings of Prozzi et al. (1997). The overall similarity of BRSV strains was 88–100% at the nucleotide level and 83–100% at the amino acid level. Nucleotide sequences of three HRSV strains obtained from GenBank had 38–41% similarity with BRSV at the nucleotide level and 27–32% at the amino acid level (Table 2). The lower percentage similarity at the amino acid level shows that the exchange of nucleotides frequently resulted in a change of the encoded amino acid. This can explain why certain strains that exhibit small genetic differences show a higher variability in their amino acid sequences. A similar phenomenon was observed in the G gene of HRSV (Cane et al., 1991; Sullender et al., 1991). This indicates that both HRSV and BRSV represent RNA viruses with a high sequence variability in the G gene (Garcia et al., 1994).

Lerch et al. (1990) reported that the similarity of amino acids of the G protein between BRSV and HRSV strains depends on the chosen protein domain. A similarity of 29–30% for the whole G gene was observed by comparing one BRSV (391-2) and two HRSV (A2 and 18537) strains. The cytoplasmic domain showed a similarity of 43%, whereas the similarity was 59% in the transmembrane domain and only 21–22% in the extracellular domain of the same strains. Our results agree with the above data on the extracellular domain since the amino acid similarity between the HRSV and BRSV strains tested was 20–27% in the sequenced region of this domain. The similarity between HRSV strains of subgroup A (Long, A2) and B (18537) in this region was 62%, whereas among the BRSV strains it was 88–100% (Table 2).

In conclusion, two major genetic branches of BRSV have
been differentiated in the present study. The branches were further divided into lineages which correlated with a geographic distribution that has not been previously published. The recently isolated Scandinavian strains gave an identical reaction pattern with a panel of MAbs and grouped separately from the established subgroups A, B and AB of BRSV. Additionally, they formed a separate genetic and geographic cluster. The 88–100% similarity among the different BRSV strains tested, indicates that these strains are variants of a single antigenic group and suggests that differentiating between strains should be based on analysis of genetic relationships.

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