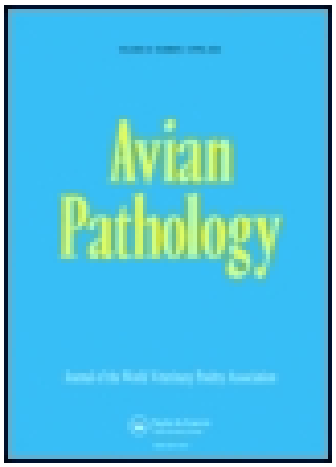

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Coronavirus IBV: Relationships among recent European isolates studied by limited proteolysis of the Virion Glycopolypeptides

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CORONAVIRUS IBV: RELATIONSHIPS AMONG RECENT EUROPEAN ISOLATES STUDIED BY LIMITED PROTEOLYSIS OF THE VIRION GLYCOPOLYPEPTIDES

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SUMMARY

After the year 1978, strains of avian infectious bronchitis virus (IBV) were isolated in the Netherlands and the UK which were assigned by neutralisation tests to four new serotypes (arbitrarily designated A, B, C and D in this communication) distinct from the long recognised US serotypes. We have labelled, with 35S-methionine, the structural polypeptides of 12 of the European isolates during growth in de-embryonated eggs. The S2, spike-anchoring, polypeptide of isolates of all four serotypes had a molecular weight of 87 000 (87K) whereas the US IBV-M41 strain had S2 of 84K. When the isolates were grouped according to the molecular weight of the other spike glycopolypeptide, S1, and the membrane (M) glycopolypeptide four groups emerged which corresponded to the serotypes based on neutralisation tests. Serotypes A (isolates D207, 1, 5 and 6) and B (isolates 7, 8 and D3896) had S1 of 91.5K while serotypes C (isolates 9 and D3128) and D (isolates D212 and D1466) had S1 of 90K. The M of serotypes A and D had a molecular weight of 30K while that of serotypes B and C was 27K. Chymotrypsin and protease V8 were used for limited proteolysis, the hydrolysis of S1 giving the greatest discrimination between serotypes. After proteolysis of S1 the UK isolates of serotype A gave identical profiles which were very similar to the Dutch serotype A isolate D207. Isolates of serotype B gave S1 profiles very similar to those of serotype A, as did D274 which is serologically related to both groups A and B. Serotypes C and D were distinguishable from each other and from serotypes A and B on the basis of the S1 peptide profiles. These results strengthen the view that the UK and Dutch isolates are closely related and that serotypes A and B are more closely related to each other than to either group C or D serotypes.

INTRODUCTION

Many serotypes of avian infectious bronchitis coronavirus (IBV) exist in the USA (Hopkins, 1974; Johnson and Marquardt, 1975; Cowen and Hitchner, 1975) but

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in Europe, in the early 1970s, it was considered that only strains of the Massachusetts M41 (Mass) serotype were important causes of disease in chickens. However since 1978 an increasing number of flocks in The Netherlands have suffered from respiratory infection and drops in egg production caused by IBV (Davelaar *et al.*, 1981; 1984). The IBV strains isolated were frequently found to be distinct, as ascertained by virus neutralisation tests, from the Mass serotype and from the five other US serotypes examined. These Dutch isolates have since been assigned to four new serotypes (Davelaar *et al.*, 1981; Lutticken and Cornelissen, 1984). Variant IBV strains have also been isolated in the UK by Cook (1983). The UK isolates were assigned to new serotypes on the basis that 20 antibody units of sera against each of 23 IBV strains did not neutralise 100 ciliostatic dose 50 of the new isolates (Cook, 1983). Although the division of IBV strains into serotypes is somewhat arbitrary (Darbyshire *et al.*, 1979), extensive cross-neutralisation tests indicated that each UK isolate belonged to one of three serotypes analogous to three of those found in the Netherlands (Cook, 1984).

IBV has two glycoproteins (Cavanagh, 1981). The surface projection or spike (S) glycoprotein comprises two glycopolypeptides S1 and S2 derived by cleavage of a precursor glycopolypeptide (Stern and Sefton, 1982a; Cavanagh, 1983b, c; Cavanagh *et al.*, 1986a). The second glycoprotein is the membrane (M) glycoprotein. This comprises a polypeptide of 25.3K molecular weight (Boursnell *et al.*, 1984) which is heterogeneously glycosylated, the major species of M having a molecular weight of 30K or 27K, depending on serotype (Collins and Alexander, 1980; Stern *et al.*, 1982; Cavanagh, 1983a). We have used the technique of limited proteolysis to examine relationships among the glycopolypeptides of the four serotypes of IBV which have been identified in Europe since 1978. Specifically we were interested to know how similar were the UK and Dutch isolates of a given serotype and whether serotypes differed slightly or extensively from each other.

MATERIALS AND METHODS

IBV strains

The Dutch strains D207, D3896 and D3128 were obtained from Dr B. Kouwenhoven, Poultry Health Institute, Doorn, The Netherlands and strains D274, D212 and D1466 were supplied by Dr W.G. Hesselink, Intervet International, Boxmeer, Holland. UK isolates 1, 5, 6, 7, 8 and 9 (Cook, 1984) were provided by J.K.A. Cook of this Institute. Isolates 1, 5 and 6 are of the same (A) serotype; 1 and 6 were isolated at approximately the same time but from locations about 150 miles apart. Isolate 5 was obtained from a site near to that of isolate 1 but 8 months later. The Massachusetts M41 strain has been described (Darbyshire *et al.*, 1979). For the present report each isolate was passaged once in embryonated chicken eggs to prepare a working stock.

Preparation of radiolabelled virus

Radiolabelled preparations of each strain were made in pairs of de-embryonated eggs using 250 μCi of 35S-methionine (Amersham International, UK) per egg and purified by ammonium sulphate precipitation followed by sucrose gradient sedimentation (Cavanagh, 1981). Virus in the density range 1.15 to 1.21 g/ml was pelleted by centrifugation for 3 hours at 90Kg max at 4°C in a 6 x 14 ml swing-out rotor (MSE).

SDS-polyacrylamide gel electrophoresis (PAGE) and electroelution.
Pelleted virus was dissolved in 250 μl of 5% SDS and 5% 2-mercaptoethanol in dissociation buffer (Laemmli, 1970) and heated at 100°C for 3 min prior to SDS-PAGE in 5 to 10% acrylamide gradient gels using Laemmli buffers (Laemmli, 1970). After electrophoresis the gels were immediately dried without fixation and autoradiographs prepared on Kodak XAR X-ray film. After the S1, S2 and major M glycopolypeptide bands had been located they were cut from the gel and rehydrated in water. The gel pieces were then placed in dialysis tubing (about 2.5 cm wide) containing elution buffer (25mM tris, 192mM glycine, 0.1% SDS and 5 mM dithiothreitol), most of the medium expelled without introducing air bubbles and the ends sealed with Mediclips (Spectrum Medical Industries Inc.). The tubing was placed between two platinum electrodes 18 cm apart in a plastic box and just covered with buffer. The glycopolypeptides were electroeluted at 100 volts. After 3 hours the current was reversed for 2 min. The buffer containing the glycopolypeptides was then removed from the bag, dialysed against 0.05% SDS in water and then lyophilised.

Limited proteolysis
The lyophilised polypeptides were dissolved in buffer containing 125 mM Tris-HCl pH 6.8, 10% glycerol, 0.0001% bromphenol blue to give a 10-fold concentration. Volumes of polypeptide solutions from different virus isolates containing the same amount of radiolabel were taken and the volume made to 59 μl with buffer containing 0.5% SDS (proteolysis buffer; PB). A 5 μl volume of PB containing 5 μg of bovine serum albumin was then added followed by 16 μl of either chymotrypsin (Type VII, TLCK-treated; Sigma) or *Staphylococcus aureus* V8 protease (Miles) diluted in PB; controls received 16μl of PB only. After 1 hour at 37°C, 20 μl of a solution of 5% SDS and 5% 2-mercaptoethanol was added and the samples heated at 100°C for 2 min. The peptides from the M glycopolypeptide were analysed in a 15% acrylamide gel (Laemmli, 1970) while those from S1 and S2 were analysed on gels containing a 10 to 18% acrylamide gradient containing 7 M urea based on the procedure described by Hashimoto *et al.* (1983). The 10% acrylamide solution contained 0.45 M Tris-HCl pH 8.8, 0.1% SDS, 7 M urea, 0.024% TEMED, and 0.025% ammonium persulphate. The 18% acrylamide solution was similar but contained 0.0064% ammonium persulphate and 10% glycerol. The solutions were not deaerated before use; under these conditions no premature polymerisation of the solutions occurred. The 15% gels were run for 5 hours at 200 volts, while the gradient gels were run at 90 volts for 16 hours followed by 2 hours at 180 volts. After staining the gels with Coomassie Brilliant Blue and destaining, the gels were soaked in 1 M sodium salicylate (Chamberlain, 1979) containing 4% glycerol and then dried without heat under a weak vacuum. Fluorographs were prepared using Kodak XAR X-ray film.

RESULTS

Two sets of radiolabelled virus preparations were examined. Set 1 contained isolates 1, 5, 6, 7, 8, 9, D207, D3128, D3896, and M41 while set 2 comprised isolates 1, 7, 9, D207, D274, D212, D1466 and M41. The M41 strain was included as a reference strain. The protease preparations used with set 1 had lower specific activities than those used with set 2 viruses. The nucleocapsid polypeptides of the viruses were also examined. However variable but extensive aggregation of this

polypeptide precluded unequivocal conclusions.

Molecular weights of the structural glycopolypeptides

Studies with IBV-M41 (Cavanagh, 1983a) and IBV-Beaudette (Stern *et al.*, 1982) have shown that the small amount of unglycosylated M polypeptide in IBV preparations has an apparent mol.wt. by SDS-PAGE of 23K and that the glycosylation of M is heterogeneous. The major species of M in these two strains has a mol.wt. of about 30K, with minor species in the 26K to 36K range. The mol.wt. of the major species of M in this study was 30K in six isolates and 27K in five other isolates (Fig. 1; Table 1). The mol.wt. of M from D212 was 29K.

Table 1. Molecular weight of the S1, S2 and major M glycopolypeptides of IBV isolates

IBV strain	Serotype	Mol. wt. (x 10^{-3}) of S1	S2	M
D207	A	91.5	87	30
1	A	91.5	87	30
5	A	91.5	NM[a]	30
6	A	91.5	NM	30
D274	A,B	91.5	87	30
D3896	B	91.5	NM	27
7	B	91.5	87	27
8	B	91.5	NM	27
D3128	C	90	87	27
9	C	90	87	27
D212	D	90	87	29
D1466	D	90	87	30
M41[b]	–	90	84	30

[a] NM: not measured because S2 not resolved.

[b] IBV-M41 belongs to the Massachusetts serotype.

With the exception of M41, in which S2 had a mol.wt. of 84K, S2 of the other strains, where measurable, had a mol.wt. of 87K (Fig. 1; Table 1). S2 was not readily distinguishable in every case, in part because it was obscured by a host cell polypeptide which gave a sharp band of about 87K and in part because some S2 had aggregated, as confirmed by peptide mapping (not shown). S1 had a mol.wt. of 90K and 91.5K in four and eight isolates respectively (Fig. 1; Table 1). When the isolates were grouped according to the mol.wt. of S1 and M, four groups emerged (Table 1) and these groups corresponded to the serotypes to which these isolates have been assigned (Davelaar *et al.*, 1984; Cook, 1984). Thus these isolates could be distinguished by physical criteria in addition to virus neutralisation.

Limited proteolysis of M

The differences observed in the pattern of the proteolytic products of the M glycopolypeptide could largely be attributed to differences in the molecular weight of the starting M glycopolypeptides (data not shown).

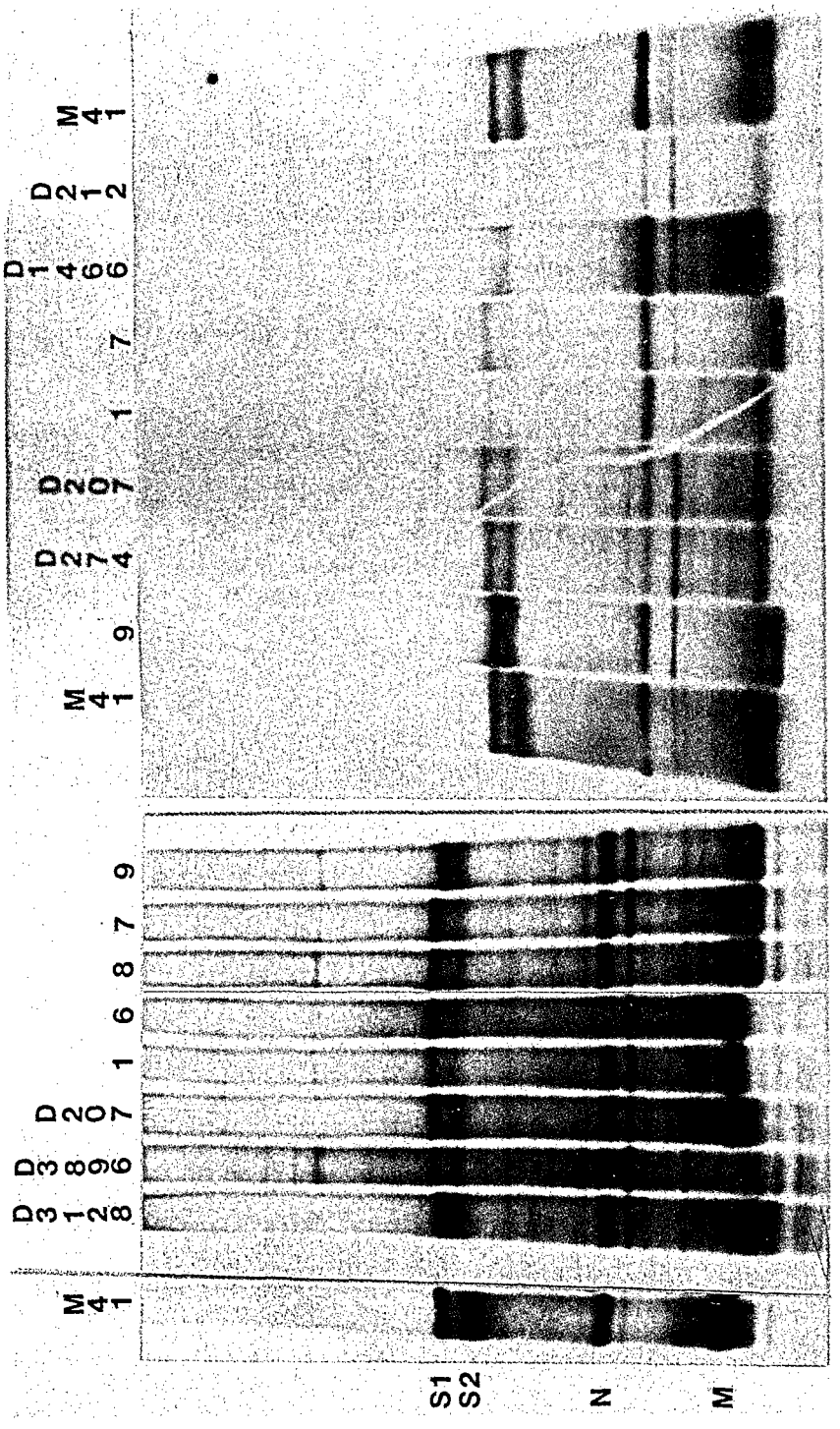


Fig. 1. SDS-polyacrylamide gel electrophoresis in 5 to 10% acrylamide gels of 35S-methionine-labelled IBV.

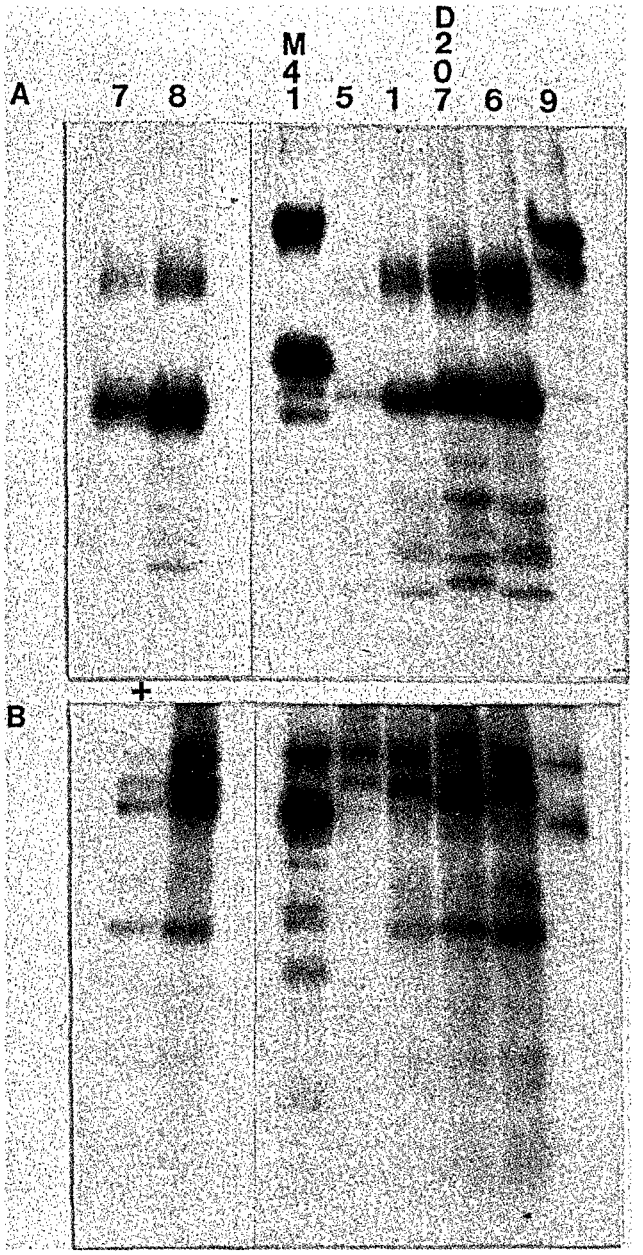
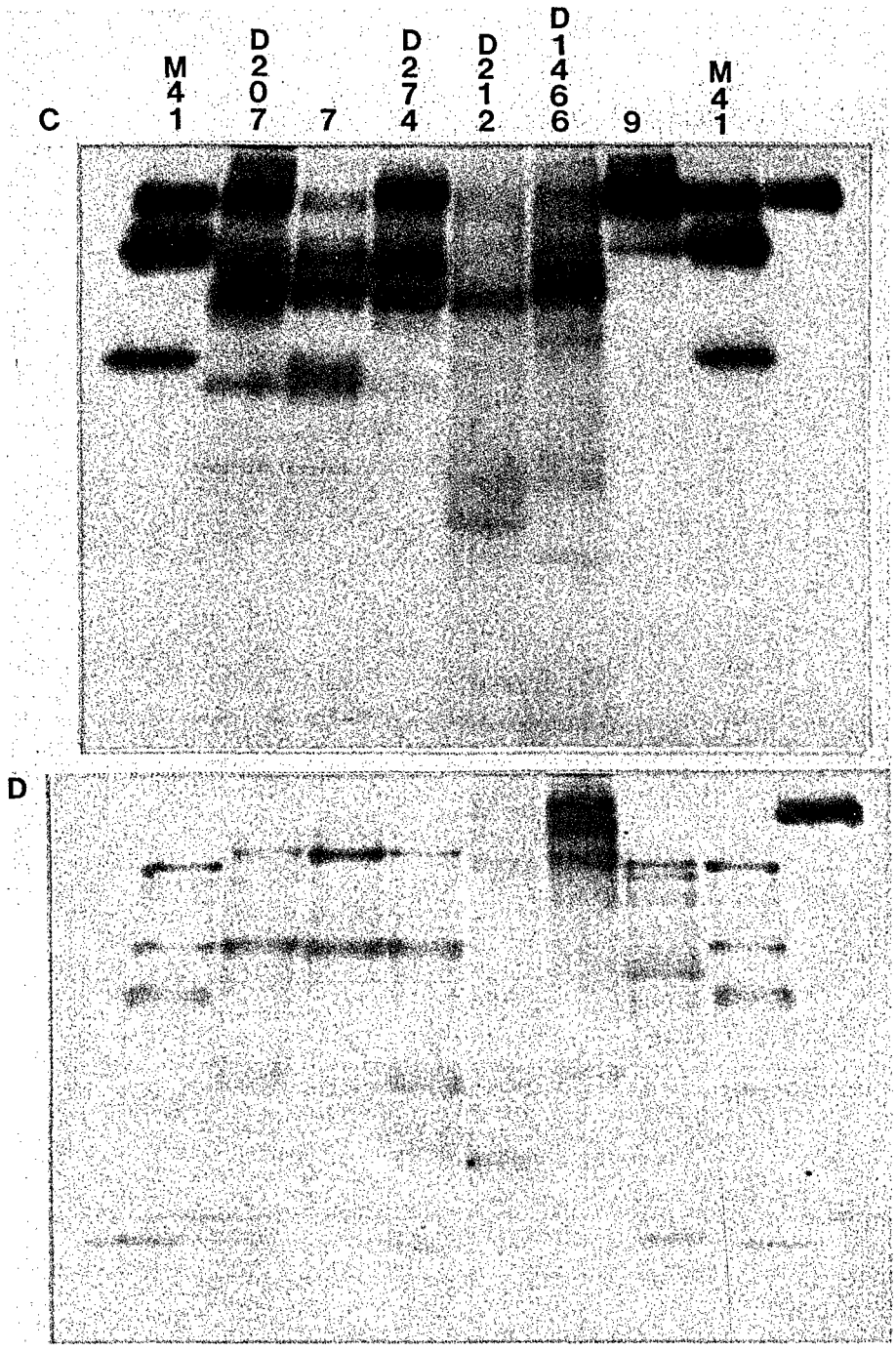


Fig. 2. Limited proteolysis of the 35S-methionine-labelled IBV S1 glycopolypeptide by (A) protease V8 (100 μg/ml), (B) chymotrypsin (25 μg/ml), (C) protease V8 (100 μg/ml) and (D) chymotrypsin (100 μg/ml). The peptides were analysed in 10 to 18% acrylamide gels. The last track in C and D contained non-hydrolysed S1 from IBV-M41. Panel B contains the same strains as panel A except that the track marked with a cross (+) contains D3896, of the same serotype as strain 7.



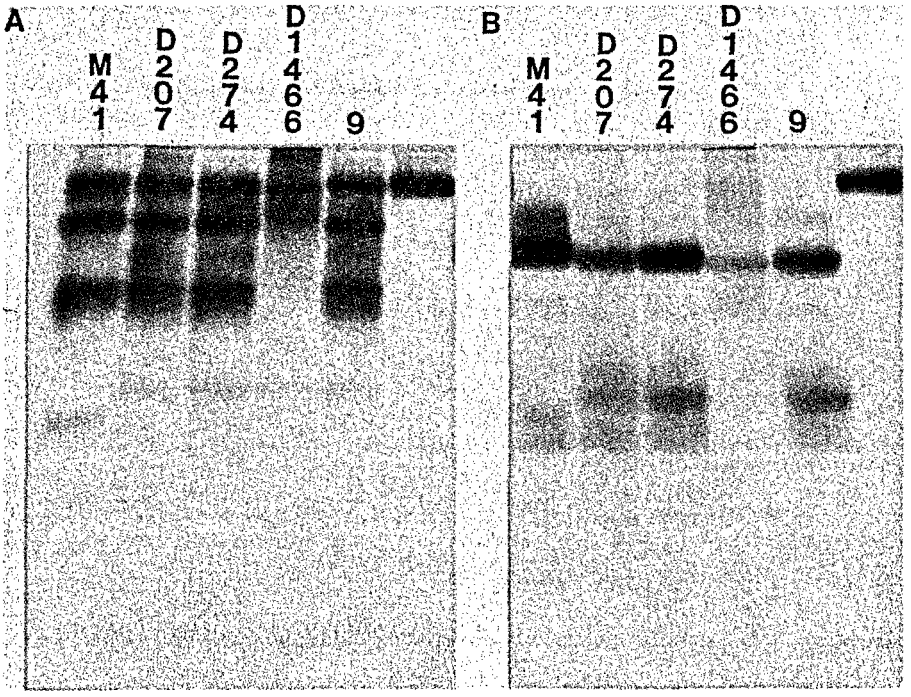


Fig. 3. Limited proteolysis of the 35S-methionine-labelled IBV S2 glycopolypeptide by (A) chymotrypsin (25 μg/ml) and (B) protease V8 (400 μg/ml). The peptides were analysed in 10 to 18% acrylamide gels. The last track in A and B contained non-hydrolysed S2 from IBV-M41.

Limited proteolysis of S1 and S2

Analysis of the first set of isolates (Fig. 2A, B) by both chymotrypsin and protease V8 showed that isolates 1, 5 and 6 gave identical S1 peptide profiles, and might therefore be isolates of the same strain, while that of D207, of the same (A) serotype was distinguishable but very similar. Serotype B isolates 7, 8 and D3896 gave identical profiles which were very similar to those of isolates 1, 5, 6 and D207. In contrast isolate 9 (serotype C) was readily distinguishable from the others. Further analysis, using the second set of virus preparations (Fig. 2C, D) showed that isolates D207 and 7 gave similar profiles while D274 was identical to D207. Isolate 9 (serotype C) was again distinguishable from the others. Isolates D212 and D1466 (serotype D) did not give identical profiles but 6 were distinguishable from the other isolates. The finding that the UK and Dutch isolates within a serotype had the same mol.wt. for S1, S2 and M and gave very similar peptide profiles for S1 is further evidence that the isolates from these two countries are closely related.

The peptide profiles of S2 from D207 (serotype A), D274 (related to serotype A and B) and 9 (serotype C) were virtually identical while that of D1466 was distinguishable (Fig. 3).

DISCUSSION

UK and Dutch strains assigned to the same serotype by cross-neutralisation tests had very similar molecular weights for the virion glycopolypeptides, and S1 and S2 gave very similar peptide maps. This indicates that not only do they have the major neutralising-antibody epitopes in common but also have extensive similarity throughout S1 and S2. Thus the spike proteins of the UK and Dutch strains within a given serotype appear to be closely related.

Our data also permit some comment on the differences between the A, B, C and D serotypes. On the basis of the indistinguishable molecular weights of S1 and the similar S1 peptide maps, serotypes A and B appear to be more closely related to each other than to either serotype C and D. The suggestion that S1 of serotypes A and B are closely related is supported by the finding that sera against strains of both these serotypes have high neutralising antibody titres against isolate D274 (Davelaar *et al.*, 1984) and that a vaccine produced from D274 has been reported to induce good protection against both A and B serotypes (Davelaar *et al.*, 1983). The spike protein is the major inducer of neutralising antibody (Cavanagh *et al.*, 1984) and has been implicated in the induction of protective immunity. Thus whereas the intramuscular inoculation of inactivated IBV induced some resistance to challenge in the trachea of chickens there was no such resistance in chickens that had been inoculated with virus from which S1, but not any other protein, had been removed (Cavanagh *et al.*, 1986b). Also, inoculation of S1 alone did result in the production of virus neutralising antibody. Thus the superficial similarity of S1 of serotypes A and B, observed by peptide mapping, is in agreement with neutralisation tests and protection experiments. The most striking difference between the serotype A and B isolates observed in our study was the molecular weight of the major species of M, this being 30K and 27K respectively. However it is most unlikely that this would affect the serotype designation of an isolate since serotyping is based on neutralisation tests and M does not induce neutralising

antibody. Nucleotide sequencing has shown that the M glycopolypeptide (mol.wt. 30K) of IBV-Beaudette can be associated with a maximum of two glycans, situated at residues 2 and 5 from the amino terminus (Boursnell *et al.*, 1984). The mol.wt. of the unglycosylated M polypeptide, derived by sequencing, is 25.3K; This is compatible with the proposition that both glycosylation sites are occupied since high mannose glycans, the type which predominate on M of IBV (Stern and Sefton, 1982b; Cavanagh, 1983a) have a mol.wt. of about 2K (Klenk and Rott, 1980). The IBV-M41 minor species of M with mol.wts. less than 30K are associated with less carbohydrate than the 30K glycopolypeptide (Cavanagh, 1983a). It is probable therefore that the 27K glycopolypeptide of the B and C serotype isolates have only one glycan.

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RESUME

Coronavirus de la bronchite infectieuse: Etude des relations entre les souches récemment isolées en Europe par protéolyse partielle des glycopolypeptides du virion.

Postérieurement à l'année 1978, des souches de virus de la bronchite infectieuse aviaire (IBV) ont été isolées en Hollande et en Grande-Bretagne et ont été classées par des tests de neutralisation en quatre nouveaux sérotypes (arbitrairement désignée A, B, C et D dans cette communication), distincts des sérotypes américains déjà connus. Il a été procédé pendant leur multiplication sur oeufs embryonnés, au marquage avec la méthionine-35S, des polypeptides structuraux de 12 des souches isolées en Europe. Le polypeptide S2 de spicule des isolats de quatre sérotypes a un poids moléculaire de 87000 (87 K) alors que la souche IBV-M41 américaine a un S2 de 84 K. Quand les isolats sont groupés en fonction du poids moléculaire de l'autre glycopolypeptide de spicule, S1, et du glycopolypeptide de membrane (M), quatre groupes apparaissent qui correspondent aux sérotypes définis par les tests de neutralisation. Le sérotype A (isolats D207, 1, 5 et 6) et B (isolats 7, 8 et D3896) ont un S1 de 91,5 K alors que la sérotype C (isolats 9 et D3128) et D (isolats D212 et D1466) ont un S1 de 90 K. Le M des sérotypes A et D a un poids moléculaire de 30 K alors que pour les sérotypes B et C il est de 27 K. La chymotrypsine et la protéase V8 ont été utilisés pour une protéolyse partielle, l'hydrolyse de S1 donnant la plus grande discrimination entre les sérotypes. Après protéolyse de S1, les isolats de Grande-Bretagne de sérotype A ont donné un profil identique, très semblable à l'isolat D207 du sérotype A hollandais. Les isolats de sérotype B ont donné des profils de S1 très semblables à ceux du

sérotype A, comme c'est le cas du D274 qui, sérologiquement, appartient à la fois aux groupes A et B. Les sérotypes C et D se distinguent l'un de l'autre et des sérotypes A et B sur la base des profils des peptides S1. Ces résultats suggèrent fortement que les isolats hollandais et anglais sont très voisins et que les sérotypes A et B sont plus proches l'un de l'autre que ne le sont les sérotypes des groupes C et D.

ZUSAMMENFASSUNG

IBV Coronavirus: Die Verwandschaft innerhalb neuer europäischer Isolate, festgestellt auf Grund der Untersuchungsergebnisse mit einer begrenzten Proteolysis der viralen Glykopolypeptide.

Nach 1978 wurden in den Niederlanden und in UK Stämme des Infektiösen Bronchitisvirus (IBV) isoliert, die gemäß den Neutralisationstesten vier neue Serotypen (in dieser Mitteilung willkürlich als A, B, C und D bezeichnet) zugeordnet wurden. Wir haben die Strukturpolypeptide von 12 der europäischen Isolate während des Wachstumes in exembryonierten Eiern 35S-Methionin markiert. Die S2, an den Spikes verankerte Polypeptide, der Isolate aller vier Serotypen hatten ein Molekulargewicht von 87000 (87 K), während die US IBV-M41-stämme eine S2 von 84 K besaßen. Wurden die Isolate gemäß dem Molekulargewicht der anderen Spikeglykopolypeptide S1 und der Membran (M) glykopolypeptide eingeteilt, so entstanden vier Gruppen, die den auf Grund der Neutralisationsteste aufgestellten Serotypen entsprachen. Die Serotypen A (Isolate D207, 1, 5 und 6) und B (Isolate 7, 8 und D3896) besaßen S1 von 91,5 K, während die Serotypen C (Isolate 9 und D3128) und D (Isolate D212 und D1466) ein S1 von 90 K hatten. Die M der Serotypen A und D besaßen ein Molekulargewicht von 30 K, während das von den Serotypen B und C 27 K war. Mit Chymotrypsin und Protease V8 wurde eine begrenzte Proteolyse durchgeführt. Die Hydrolyse von S1 ergab die größten Unterschiede zwischen den Serotypen. Nach der Proteolyse von S1 ergaben die UK Isolate von Serotyp A identische Profile, die denjenigen des holländischen Serotyp A Isolates D207 weitgehend glichen. Isolate des Serotyps B ergaben S1 Profile, die denjenigen von Serotyp A sehr ähnlich waren. Das tat gleichfalls D274, welcher serologisch sowohl mit Gruppe A als auch mit B verwandt war. Die Serotypen C und D unterschieden sich von einander und von den Serotypen A und B im Bezug auf die S1 Peptidprofile. Diese Ergebnisse unterstreichen die Ansicht, daß die UK und die holländischen Isolate eng verwandt sind und daß die Serotypen A und B enger mit einander verwandt sind als mit jedem der C und D Serotypen.

RESUMEN

Coronavirus de la bronquitis infecciosa: relación entre los aislamientos europeos recientes estudiados por proteolisis limitada de los glipolipéptidos del virion

Después del año de 1978 se han aislado cepas del virus de la bronquitis infecciosa aviar (VBI) en los Países Bajos y en al Reino Unido, las cuales han sido clasificados por medio de pruebas de neutralización en cuatro nuevos serotipos (designados arbitrariamente en A, B, C y D, en esta comunicación), y que son distintos a los serotipos largamente reconocidos de los E.U.A. Hemos etiquetado con metionina 35 S, a los polipeptidos estructurales de 12 aislamientos europeos durante su crecimiento en huevos desembrionados.

Los polipeptidos del receptor de anclaje S2 de los aislamientos de los cuatro serotipos tenían un peso molecular de 87000 (87K) mientras que la cepa norteamericana IBV. M41 tenía un S2 de 84K. Cuando dichos aislamientos fueron agrupados de acuerdo al peso molecular del glicopolipéptido del otro receptor S1 y del glico-

polipéptido emergieron cuatro grupos los cuales corresponden a los serotipos basados en pruebas de neutralización. Los serotipos A (aislamiento D 207 1,5 y 6) y B (aislamientos 7, 8 y D 3896) tenían un S1 de 91,5K mientras quel el serotipo C (aislamiento 9 y D 3128) y D (aislamiento D 212 y D 1466) tenían un S1 de 90K. La M de los serotipos A y D presentaron un peso molecular de 30 K. mientras que el peso molecular de los serotipos B y C fue de 27K. Con el objeto de haber una proteolisis limitada se empleo quimotripsina y proteasa V8. La hidrolisis de la S1 mostró una descriminación mayor entre los serotipos. Después de la proteolisis de la S1 los aislamientos del serotipo A hechos en el Reino Unido presentaron perfiles identicos los cuales fueron muy similares al serotipo holandés A aislamiento D 207. Los aislamientos del serotipo B dieron perfiles S1 muy similares a aquellos del serotipo A. Como lo hizo el D 274 el cual esta serologicamente relacionado a ambos grupos es decir al A y al B. Los serotipos C y D fueron distinguibles el uno del otro y de los serotipos A y B en base de los perfiles peptidicos del S1. Estos resultados refuerzan el punto de vista de que los aislamientos holandeses y británicos estan estrachamente relacionados y que los serotipos A y B estan más cercanamente emparentados con los serotipos del grupo C y D.