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Molecular Epidemiology of Infectious Bronchitis Virus in The Netherlands

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SUMMARY

Twelve Dutch isolates and the M41 strain of infectious bronchitis virus (IBV), a coronavirus of chickens, were characterized by cross-neutralization and T1 fingerprinting to elucidate their evolutionary relationship. The T1 fingerprinting showed that the Dutch isolates formed two clusters. The first cluster contained strains H52, H120, D387, V1259, V1385 and V1397; the estimated sequence homology is 99%. Cluster two comprised strains D207, D274, D212, D1466, D3128 and D3896, which have about 95% sequence homology. The M41 virus did not belong to either cluster. The four different serotypes which arose in the late 1970s belonged to cluster two and appeared to be different from the vaccine strains (H52 and H120) used at that time. This indicates that the strains were newly introduced and could have arisen from a common virus. On the other hand, three recently isolated field strains were genetically closely related to the vaccine strains H120 and H52 (cluster one), suggesting that these live vaccine strains themselves could have given rise to these serologically altered field isolates. The data are relevant to the development of new vaccine strategies.

INTRODUCTION

Avian infectious bronchitis virus (IBV), the prototype of the Coronaviridae, is the aetiological agent of a highly contagious respiratory disease of young chickens. Another consequence of the infection is a marked and rapid decline of egg production and quality in laying hens (Darbyshire, 1981). The disease was first described in the United States (Schalk & Hawn, 1931), but by the early 1960s it occurred all over the world. Infectious bronchitis is controlled mainly by vaccination with attenuated virus strains (e.g. H52 and H120). In The Netherlands vaccination resulted in a drastic decrease in the incidence of the disease. Since 1978, however, outbreaks in properly vaccinated flocks have been reported with increasing frequency, together with the appearance of new neutralization serotypes (Davelaar *et al.*, 1984).

Serotypes of IBV (Massachusetts and Connecticut) were first described in 1956 (Jungherr *et al.*, 1956). Until now some 20 serotypes, basically defined by cross-neutralization tests, have been found. Most of them have a worldwide distribution (Dawson & Gough, 1971; Lohr, 1976; Doi *et al.*, 1982; Locher *et al.*, 1983*a*, *b*; Cook, 1984). In some cases unequivocal assignment to a serotype of an isolate is complicated by the phenomenon of one-way neutralization: a serum against strain A neutralizes strain B but the antiserum against strain B does not neutralize strain A (Dawson & Gough, 1971; Locher *et al.*, 1983*a*, *b*). Several laboratories have tried to classify new virus isolates, using neutralization tests *in ovo* or in tracheal organ cultures (Hitchner, 1973; Cowen & Hitchner, 1975; Darbyshire *et al.*, 1979; Cook, 1984), and to construct a tree of relationship between the IBV strains and serotypes.

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Initially only the live attenuated strains H52 and H120 of the Massachusetts serotype were used in The Netherlands to vaccinate chickens against IBV (Davelaar *et al.*, 1984). When four new serotypes emerged (see Tables 1 and 2), however, virus strains from these serotypes were also included in the vaccination programme (Davelaar *et al.*, 1984). The reason for undertaking this study was to obtain information on both the serological relatedness and the nucleotide sequence homology of the genomes of IBV strains occurring in The Netherlands. We were particularly interested to know whether the recently isolated Dutch IBV strains which escaped vaccine-elicited immunity were newly introduced in the poultry population or arose by mutations of circulating Dutch IBV strains. This knowledge is important for the development of new vaccines. We have chosen RNase T1 fingerprinting of the variants to determine sequence homology at the genome level. Variants of different serotypes with a common origin should have very similar RNase T1 fingerprints. On the other hand, if marked differences between the T1 fingerprints are found, the field isolates were most probably newly introduced from elsewhere.

METHODS

Enzymes and chemicals. Ribonuclease T1 was purchased from Calbiochem. T4 polynucleotide kinase, calf intestine phosphatase (CIP), proteinase K, *Escherichia coli* tRNA and yeast RNA were from Boehringer. Yeast RNA was treated with proteinase K, deproteinized and degraded by RNase T1 as described for the viral RNAs (see below). Rabbit liver glycogen, obtained from BDH, was purified as previously described (Stern & Kennedy, 1980). [γ-^{32}P]ATP (3000 Ci/mmol) was purchased from Amersham.

Virus strains. Identification codes and other relevant data are summarized in Table 1. A summary of the prototype IBV strains prevalent in The Netherlands and their serotypes is presented in Table 2. All strains were obtained from the Poultry Health Institute, Doorn, The Netherlands. Strains were stored as infectious allantoic fluid at −70 °C.

Virus growth and isolation of viral RNA. Approximately 100 egg infectious doses (EID_{50}) of the stock virus was inoculated into the allantoic cavity of 10-day-old chicken embryos and incubated for 40 h at 37 °C. The eggs were kept at 4 °C for at least 4 h before the allantoic fluid was harvested. After low speed centrifugation, sodium chloride and polyethylene glycol 6000 (BDH) were added to final concentrations of 2·33% (w/v) and 10% (w/v), respectively. The mixture was stirred overnight at 4 °C and then centrifuged for 30 min at 10000 ***g***. The pellet as well as the lipids at the meniscus were resuspended in 1/50 of the original volume of TESV buffer (0·02 M-Tris–HCl, 1 mM-EDTA, 0·1 M-NaCl, pH 7·4). The concentrated virus suspension was clarified (10 min, 10000 ***g***) and layered onto a 10% (w/v) sucrose cushion (10 ml) on top of a 20 to 50% (w/v) sucrose gradient (16 ml, both in TESV buffer), and centrifuged (6 h, 10000 ***g***) in a SW27 rotor. The light-scattering virus band was collected and diluted threefold in TESV buffer. The virus was pelleted at 250000 ***g*** at 4 °C for 90 min and incubated subsequently for 30 min at 37 °C with 200 µl of TESV containing 0·5% SDS and 0·5 mg/ml proteinase K. Finally the RNA was extracted with phenol/chloroform and ethanol-precipitated from the ether-extracted aqueous phase.

Oligonucleotide fingerprinting. The lyophilized RNA pellet was dissolved in 13 µl T buffer (20 mM-Tris–HCl pH 8·0, 0·1 mM-EDTA) containing 5 units of RNase T1 and incubated at 37 °C for 30 min. After the addition of 2 µl T buffer containing 5 units of CIP the mixture was incubated for another 30 min at 37 °C, followed by an incubation of 30 min at 56 °C. Subsequently the mixture was deproteinized and RNA was recovered by ethanol precipitation as described above. RNase T1-resistant oligonucleotides were labelled by adding 25 µl of T4 polynucleotide kinase mixture (34 mM-Tris–HCl pH 7·6, 10 mM-$MgCl_2$, 5 mM-DTT, 0·4 µM γ-^{32}P-labelled ATP and 200 units of T4 polynucleotide kinase/ml) to the lyophilized RNA pellet. The mixture was incubated for 30 min at 37 °C followed by another 30 min at 56 °C. After the addition of 175 µl TESV, the mixture was extracted once with phenol/chloroform/isoamyl alcohol (25 : 24 : 1). Unincorporated ATP was removed by three ethanol precipitations. The lyophilized RNase T1-resistant oligonucleotides were resuspended in 5 µl of water and prepared for electrophoresis by the addition of 20 µl of sample mixture (8·5 M-urea, 37% w/v sucrose, 0·75% w/v xylene cyanol FF, 0·25% w/v bromophenol blue, 1 mg/ml RNase T1-degraded yeast RNA and 2·5 mg/ml *E. coli* tRNA). The mixture was heated to 56 °C for 15 min and immediately layered onto the first dimension gel.

Two-dimensional gel electrophoresis was carried out by using a modification of the system described previously (Spaan *et al.*, 1982). The first dimension gel had the following composition: 10% (w/v) acrylamide, 0·33% (w/v) *N,N'*-methylene bisacrylamide, 6 M-deionized urea and 25 mM-citric acid pH 3·5. Electrophoresis was carried out at 4 °C and 250 V until the bromophenol blue dye marker had migrated 19 cm. A 1·5 cm wide gel strip, corresponding to 6 to 30 cm from the origin was cut out for second dimension electrophoresis. For this, the composition of the gel was 22% (w/v) acrylamide, 0·7% (w/v) *N,N'*-methylene bisacrylamide in TBE (100 mM-Tris–borate pH 8·3, 2·5 mM-EDTA). Electrophoresis was carried out at 4 °C and 600 V until the bromophenol blue marker had migrated 24 cm. Autoradiography was performed with Fuji RX films at 4 °C, using a DuPont Lightning Plus intensifier screen.

Table 1. *IBV strains used in this study**

Code	Approx. no. of egg passages	Year of isolation	Reference
M41†	–‡	1941	Van Roekel *et al.* (1950)
H52†	52	1956	Bijlenga (1956); Hoekstra & Rispens (1960)
H120†	120	1956	Bijlenga (1956); Hoekstra & Rispens (1960)
D387	–	1970	Davelaar *et al.* (1984)
D207	56	1979	Davelaar *et al.* (1984)
D274†	–	1979	Davelaar *et al.* (1984)
D212	56	1979	Davelaar *et al.* (1984)
D1466†	–	1979	Davelaar *et al.* (1984)
D3128	67	1979	Davelaar *et al.* (1984)
D3896	54	1978	Davelaar *et al.* (1984)
V1259	–	1984	Field isolate
V1385	–	1984	Field isolate
V1397	–	1984	Field isolate

* With the exception of M41, all strains are Dutch isolates.
† Vaccine strain.
‡ Not known.

Table 2. *Summary of data on Dutch IBV serotypes*

Serogroup	Reference strain(s)	Additional strain
A (Massachusetts)	M41, H52, H120	D387
B	D207	D274*
C	D212	D1466
D	D3128	
E	D3896	D274*

* Strain reactive with reference sera from two different serogroups (Davelaar *et al.*, 1984).

Antisera and virus neutralization assays. The monospecific antisera to the various virus strains were prepared as previously described (Davelaar *et al.*, 1984). The anti-D274 chicken hyperimmune serum was supplied by Duphar (Weesp, The Netherlands). The sera were heated at 56 °C for 30 min and centrifuged at 10000 *g* and 20 °C for 15 min. Aliquots were stored at −20 °C. Tenfold dilutions of the serum were mixed with an equal volume (50 µl) 3% tryptose phosphate broth containing 100 EID_{50} of virus. The mixture was incubated for 1 h at 37 °C and inoculated into the allantoic cavity of 10-day-old embryonated eggs. The assay was read after 7 days. Titres are expressed as the reciprocal of the antibody dilution causing 50% survival of embryos. A similar assay has been used by others (Darbyshire *et al.*, 1979; Locher *et al.*, 1983*a*, *b*) to classify IBV strains. Two strains under comparison are considered to belong to different serotypes when neutralization titres between the homologous and heterologous reaction differ by a factor of 20 or more (Kapikian *et al.*, 1967).

RESULTS

IBV strains prevalent in The Netherlands

To protect flocks against the newly arisen serotypes, strains D274 and D1466 (serotype B/E and C, respectively) were recently included in the vaccination programme. Furthermore, strain M41 of the Massachusetts serotype was added to the vaccine.

Some recent field isolates (V1259, V1385 and V1397) could not be assigned to any of the established serotypes using sera against vaccine strains M41 (serotype A), H52 (serotype A), D274 (serotype B/E) and D1466 (serotype C) or against D3128 and D3896, the prototype strains of the two recent Dutch serotypes D and E, respectively (Table 3). The cross-neutralization experiments showed that the variant strains V1259 and V1385 were not recognized by antisera against any of the vaccine strains; therefore they most probably belong to one or two new serotypes. On the other hand, strain V1397 is recognized by antisera against both strains M41 and D1466, implying partial homology in the immunological response elicited by these two strains. Vaccine strain D274 was already known to belong to both serotypes B and E, since anti-D274 serum also neutralizes D3896 (serotype E) (Davelaar *et al.*, 1984).

Table 3. *Cross-neutralization titres of sera against vaccine and reference strains of IBV**

Virus	Antiserum to (serotype)					
	M41 (A)	H52 (A)	D274 (B/E)	D1466 (C)	D3128 (D)	D3896 (E)
M41	**3·1**	1·5	–†	–	2·3	–
H52	1·8	**3·5**	–	–	–	–
D274	–	–	**2·1**	–	1·6	2·3
D1466	–	–	–	**2·8**	–	2·0
D3128	–	–	1·0	–	**4·3**	–
D3896	–	–	1·7	–	2·3	**2·8**
H120	–	2·3	–	–	2·3	–
D207	–	–	1·4	–	1·5	1·5
D212	1·6	–	–	1·3	1·1	–
D387	–	3·3	–	–	1·6	–
V1259	1·6	2·0	–	–	–	–
V1385	–	1·8	–	–	2·3	–
V1397	2·1	1·0	–	2·7	1·3	1·3

* Titres are expressed as $\log_{10}$. The serotype of each virus strain (Table 2) is indicated. Homologous neutralization titres are in bold type. Significant cross-neutralization titres are underlined.

† Neutralization titre less than 0·8.

It is clear from Table 3 that the classification of the Dutch IBV strains into five serotypes (Table 2) is not clean-cut. Not only are new serotypes arising but, more confusingly, there is an apparent overlap between 'serotypes', as has been found by others (Darbyshire *et al.*, 1979).

Oligonucleotide fingerprinting

In order to evaluate the sequence homologies between the genomes of different IBV strains, RNase T1 fingerprints were made. Fingerprint analyses were performed on all 13 strains listed in Table 1 and the resulting oligonucleotide maps were grouped according to their degree of homology. In addition to the normal spots some relatively faint but reproducible spots were observed in all maps, except for the M41 fingerprint. These spots were not only reproducible within different preparations of the same virus, but were also present at the same relative intensity in closely related strains. Recloning of strains H52, H120, D274 and D1466 did not eliminate or change the pattern of these faint spots; thus they do not reflect heterogeneity of the viral RNA population. The low intensity spots might have been due to non-uniform incorporation of the ^{32}P label into the RNase T1-resistant oligonucleotides. This has been observed before for IBV (Stern & Kennedy, 1980) as well as for other viruses (Clements *et al.*, 1980; Lee & Fowlks, 1982; Monath *et al.*, 1983; Nerome *et al.*, 1984) and is presumably due to some secondary structure within the RNA. We included these faint spots in our comparison, but omission would have had no effect on the conclusions drawn.

Fig. 1 shows one group of IBV strains with similar T1 fingerprints. Within this cluster there are few differences in the pattern of the large (>15 residues) characteristic oligonucleotides. Strains H52 and H120, both of the Massachusetts serotype (A), gave almost identical RNase T1 fingerprints. This was to be expected since these two strains represent different passage numbers of the same parental strain (Hoekstra & Rispens, 1960). Closely related to these two strains were strains V1259, V1385 and V1397. These strains had more than 98% of all spots in common with strains H52 and H120; this corresponds to a sequence homology of 99·5% (Aaronson *et al.*, 1982). Strain D387 diverged in about 5% of its characteristic T1 spots from the other strains in this cluster and hence its genome differs from the other strains by about 1%.

A second fingerprint cluster is represented by strains D207, D212, D274, D1466, D3128 and D3896 (Fig. 2). The overall relatedness in this cluster is less than in the first one. Nevertheless, the strains have more than 50% of their large oligonucleotides in common (95% homology at the level of the genomic RNA).

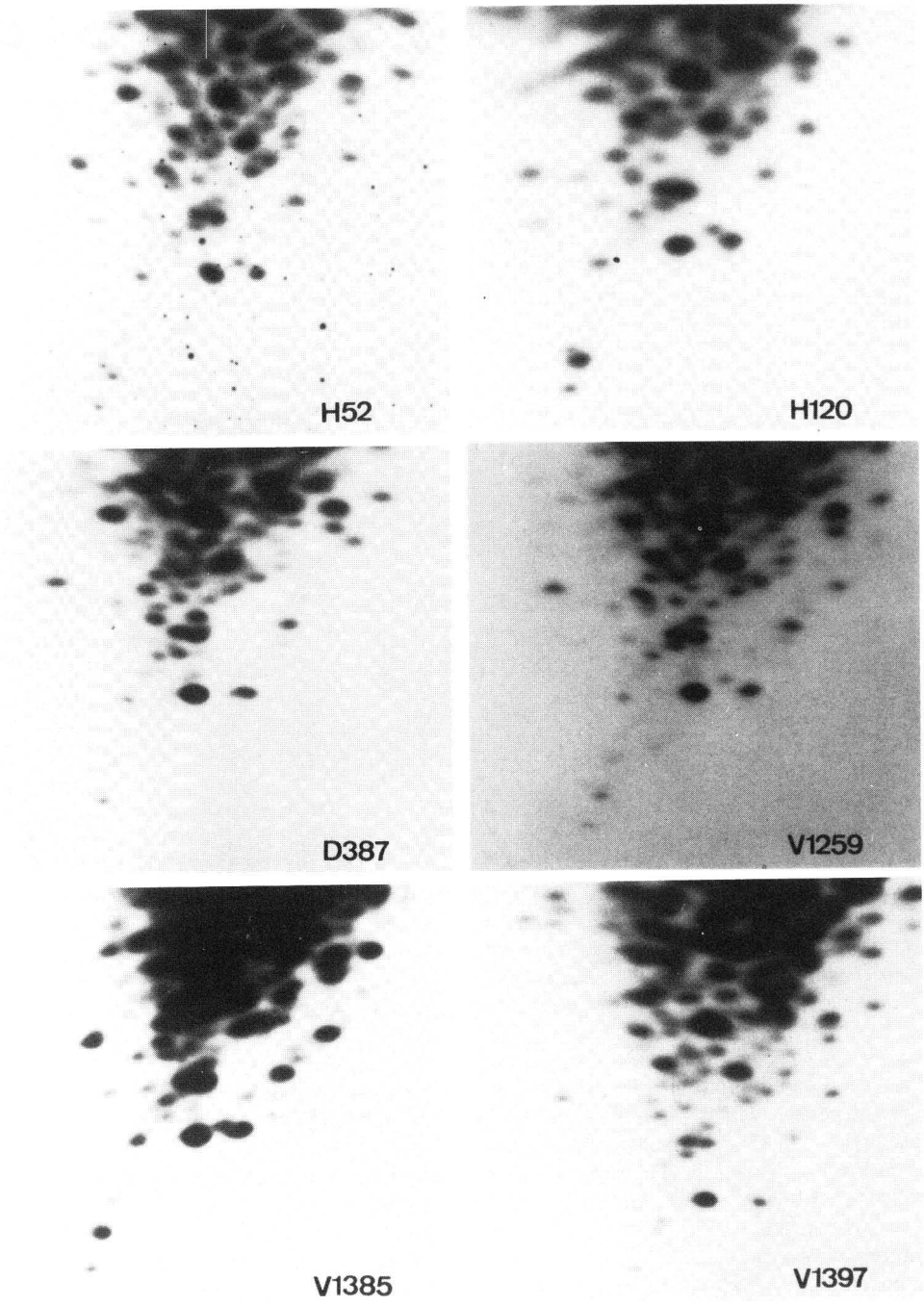


Fig. 1. Oligonucleotide fingerprints of the genomic RNA of six genetically related Dutch IBV isolates, strains H52, H120, D387, V1259, V1385 and V1397. Electrophoresis in the first dimension was from left to right, in the second dimension from bottom to top. The origin towards the bottom left-hand corner is not visible.

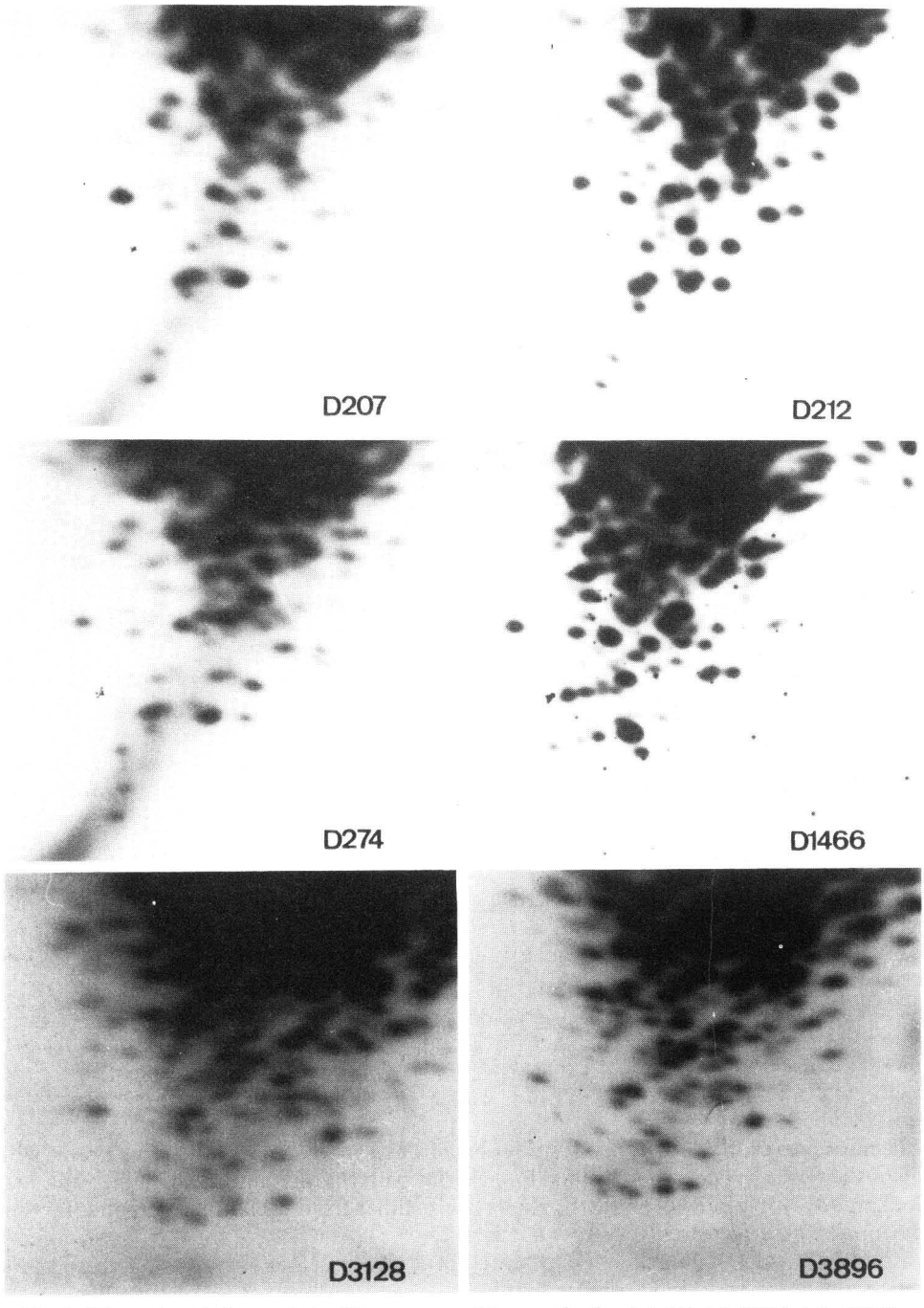


Fig. 2. Oligonucleotide fingerprints of the genomes of the genetically related Dutch IBV isolates D207, D212, D274, D1466, D3128 and D3896. Experimental details are as in Fig. 1.

The oligonucleotide fingerprint of strain M41 is shown in Fig. 3. This strain possesses no homology in its RNase T1-resistant oligonucleotides with any of the two previous fingerprint clusters as judged by visual analysis.

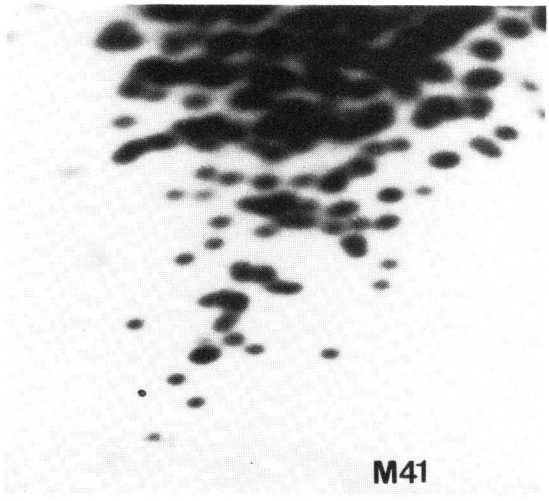


Fig. 3. Oligonucleotide fingerprint of the genomic RNA of strain M41. Experimental details are as in Fig. 1.

DISCUSSION

So far the classification of IBV strains has been based on serology. Our data show that serology does not allow a clean-cut classification of the strains in five serotypes. This becomes particularly clear when more reference antisera are used. For example, when antiserum against strain D207 is used as the reference in a neutralization assay, then strain D3896 must be considered to form a separate serotype (Davelaar *et al.*, 1984). When antiserum against strain D274 is used, however, strains D207 and D3896 must be considered to belong to the same serotype as D274 (Tables 2 and 3). Furthermore, anti-D3896 serum is able to neutralize D1466, whereas anti-D1466 serum does not neutralize D3896.

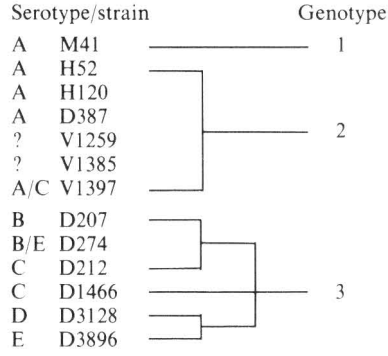
Our T1 fingerprint data lead to a very simple classification of IBV strains, not depending on reference antisera or on the particular serological method used. Direct visual comparison of the fingerprints using internal dye markers as reference points allows a quick comparison of strains having more than 50% of their characteristic oligonucleotides in common (Kew *et al.*, 1984). The method is highly reproducible, but a disadvantage is that it does not work well for genomes with less than 95% homology (Aaronson *et al.*, 1982). A comparison of our data with those of Clewley *et al.* (1981) demonstrates both these points. These authors compared serologically distantly related IBV strains by T1 fingerprinting and did not observe any similarity. On the other hand their fingerprints of strains M41 and H120 very much resemble our patterns shown in Fig. 1 and 3.

The most interesting data from our study come from a comparison of the virus strains within each of the two T1 fingerprint clusters. Fingerprint patterns of strains D207, D212 and D274, belonging to serotypes B, C and B/E, respectively, differ from each other only in a few spots indicating an almost complete identity at the level of the genome. A similar situation exists for strains D3128 and D3896 belonging to serotypes designated D and E, respectively. The two latter strains have very similar fingerprints.

On the other hand strains that are genetically only distantly related may belong to the same serotype. Anti-D1466 serum, for instance, shows significant cross-neutralization of the genetically unrelated strain V1397 (Table 3). Also strains M41 and H52 which show no apparent genetic relatedness both belong to the Massachusetts serotype. The relationships determined from T1 fingerprinting of the IBV strains are indicated in Table 4.

Our data allow conclusions to be drawn concerning the origin of new variant strains causing outbreaks of infectious bronchitis; they suggest that the strains V1259, V1385 and V1397 are

Table 4. *Schematic representation of the relationship of the IBV isolates*



mutants of the H52 and H120 viruses used for vaccination. In contrast, the IBV strains isolated in the late 1970s (D207, D212, D274, D1466, D3128 and D3896) are neither serologically nor genetically related to H52 and H120. However, the variant strains within this cluster are genetically related to each other, suggesting a common origin (see Table 4). To explain the way in which variants arise in nature, one has to consider the high error rate during replication of RNA genomes in the absence of repair mechanisms (Holland *et al.*, 1982; Steinhauer & Holland, 1986). Therefore large numbers of variants can be expected to originate in flocks vaccinated with live attenuated viruses. Although mutations will occur at random throughout the IBV genome, viruses arising from vaccine strains with altered surface epitopes will have a selective advantage over variants due to other mutations. When a mutant arises that is unaffected by the existing herd immunity it will rapidly spread through the flock. The existence of different serotypes in genetically very closely related viruses indicates that this actually happens. Thus, although the currently used attenuated vaccine strains have reduced the economic losses due to IBV infections, they might also be responsible for new variant viruses.

Finally, the data are important for the development of new vaccines. First, they are relevant to the mapping of the neutralization epitope(s) of IBV. We have recently cloned and sequenced the peplomeric protein of the M41 strain of IBV (Niesters *et al.*, 1986). This protein elicits neutralizing antibodies (Cavanagh *et al.*, 1984). Comparison of the amino acid sequence thus obtained with the sequence of another IBV strain, M42 (Binns *et al.*, 1985; Niesters *et al.*, 1986), has indicated two regions as candidates for neutralization epitopes of the protein. Similarly, comparisons of IBV strains belonging to different serotypes but with a high degree of sequence homology, as indicated in this study, will give additional information on the position of these neutralization epitopes and possibly even on the amino acid substitutions leading to conversion to another serotype. Secondly, once the neutralization epitopes have been mapped, new variant strains arising in the field can be characterized in a very short time by selective sequencing of the regions on the genome encoding these neutralization epitopes. Existing recombinant DNA vaccines can then be quickly adapted. Thirdly, alternatives for live attenuated vaccines should be developed.

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