THE NEUTRALIZATION EPITOPES ON THE SPIKE PROTEIN OF INFECTIOUS BRONCHITIS VIRUS AND THEIR ANTIGENIC VARIATION

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INTRODUCTION

Avian infectious bronchitis virus (IBV) is the etiological agent of a highly contagious respiratory disease. It can cause high mortality, particulary in young chickens. Moreover, the kidneys and reproductive tract are often affected resulting in a drop of egg production in hens. In The Netherlands, infectious bronchitis (IB) used to be controlled by vaccination with the live attenuated virus strains H52 and H120, derived from field isolates (Bijlinga, 1956; Hoekstra and Rispens, 1960). Vaccination with these two strains resulted in protection against virtually all antigenic variants of the virus known at that time (Winterfield and Fadley, 1975; Winterfield et al., 1976).

However, large scale vaccination with these strains did not result in elimination of the disease. In the seventies, the intensification in poultry farming was accompanied by an increase of the IB problem. New viruses were isolated, resistant to vaccination with H52 and H120 (Davelaar et al., 1984). It became clear that IB variant viruses were involved in the new IB problems. Davelaar et al. (1984) classified these new Dutch isolates into four major serological groups (B-E) differing from the vaccine serotype A (Table 1). To protect against these newly arisen serotypes, two new strains (D274 and D1466) and strain M41 were included in the vaccine (Davelaar et al., 1983, 1984). This was no permanent solution; the IB problem occurred again in the last few years. New viruses were isolated which could not be classified into one of the existing serotypes.

The serological differences between the different IBV strains are most likely located on the spike protein of the virus, since this protein is reported to elicit neutralizing as well as hemagglutination inhibiting antibodies (Cavanagh et al., 1984). The spike protein forms the characteristic surface projections of the virions and are made up from two copies of each of the glycopolypeptides S1 and S2 derived by cleavage of a precursor (Stern and Sefton, 1982, Cavanagh, 1983ab; Cavanagh et al., 1986). Until now only a few neutralizing monoclonal antibodies are available, which all bind to the S1 subunit of this spike protein (Mockett et al., 1984; Niesters et al., unpublished data). This is in agreement with the model of Cavanagh (1983b), where S1 is on the outside of the virion and attached to the membrane by way of the S2 protein.

Table 1. Dutch IBV strains and their serotypes

Serotype	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).

Here we report on the relatedness of the circulating IBV strains of several Dutch IBV strains as measured by RNAse T1-fingerprinting of the genomic RNAs. The fingerprint data were compaired with serological data obtained by in ovo neutralization assays. Insight in the amino-acid substitutions in the spike protein that lead to a changed serotype were obtained by comparing the spike gene sequences of some related IBV strains.

RELATEDNESS OF DUTCH IBV ISOLATES

So far the classification of IBV-strains has been based on serology. The data from Table 2 show that serology does not allow a clean-cut classification of the Dutch strains in five serotypes. This becomes particularly clear when more reference antisera are used. For example, when antiserum against strain D207 is used as reference in a neutralization assay, then strain D3896 seems to form a separate serotype (Davelaar et al., 1984). When antiserum against strain D274 is used, however, the strains D207 and D3896 must be considered to belong to the same serotype as D274 (Table 1, 2). Furthermore, while anti-D3896 serum is able to neutralize D1466, anti-D1466 serum does not neutralize D3896.

The T1-fingerprint data lead to a very simple classification of IBV strains (see Fig. 1-3). Direct visual comparison of the fingerprints using the 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). However this method does not work well for genomes with less than 95% homology (Aaronson et al., 1982).

The most interesting data come from a comparison of the virus strains within each of the two T1-fingerprint clusters (Figs. 2 and 3). 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. Similarly, strains D3128 and D3896 belonging to serotypes designated D and E respectively, have nearly identical fingerprints.

On the other hand, strains that are genetically only distantly related may belong to the same serotype. Strain D1466 for instance, shows significant cross-neutralization with the genetically unrelated strain V1397 (Table 2). Also strains M41 and H52 which show no apparent genetic relatedness both belong to the Massachusetts serotype.

Table 2. Cross-neutralization titers of sera against vaccine and reference strains of IBV*

VIRUS	ANTI-SERUM 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

*: The serotype of each virus strain according to Table 1 is indicated. Homologous neutralization is in boldface. Significant cross-neutralization is underlined. Neutralization assays were preformed with preheated (30 min, 56 °C) monospecific antisera. Tenfold dilutions of the serum were mixed with an equal volume (50 ul) of 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. Titers are expressed as ^{10}log of the reciprocal antibody dilution causing 50% embryo survival at 7 days p.i.

+: neutralization titer less than 0.8.

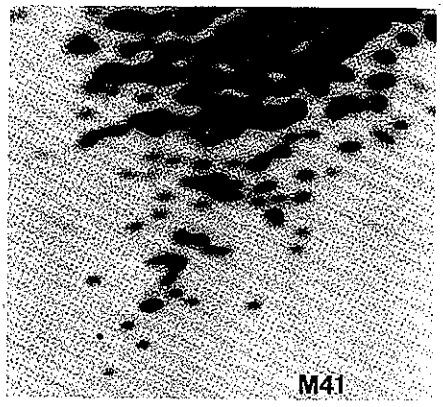


Fig 1. Oligonucleotide fingerprint of the genomic RNA of strain M41. Electrophoresis in the first dimension was from left to right, in the second dimension from bottom to top. The origin towards the bottom left-hand corner is not visible.

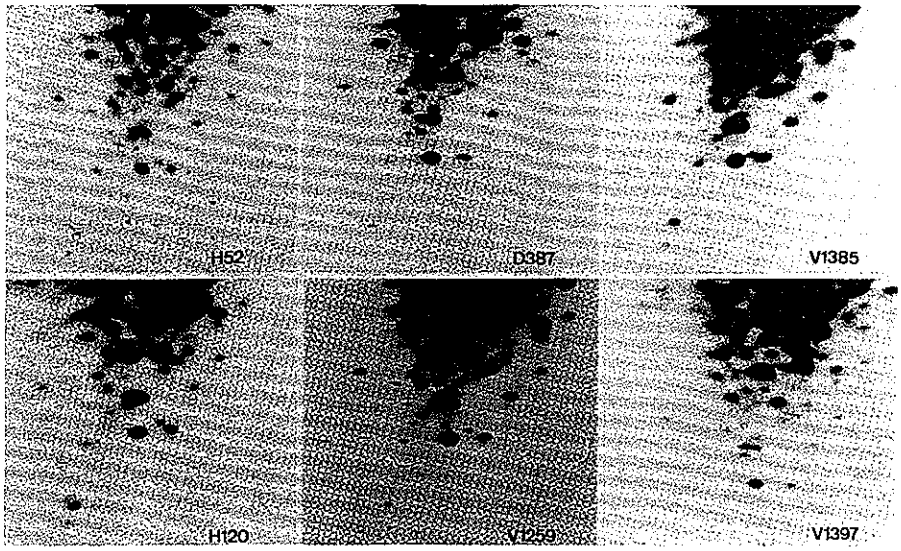


Fig. 2. Oligonucleotide fingerprints of the genome of the genetically related Dutch IBV isolates H52, H120, D387, V1259, V1386 and V1397. Experimental details are as in Fig. 1.

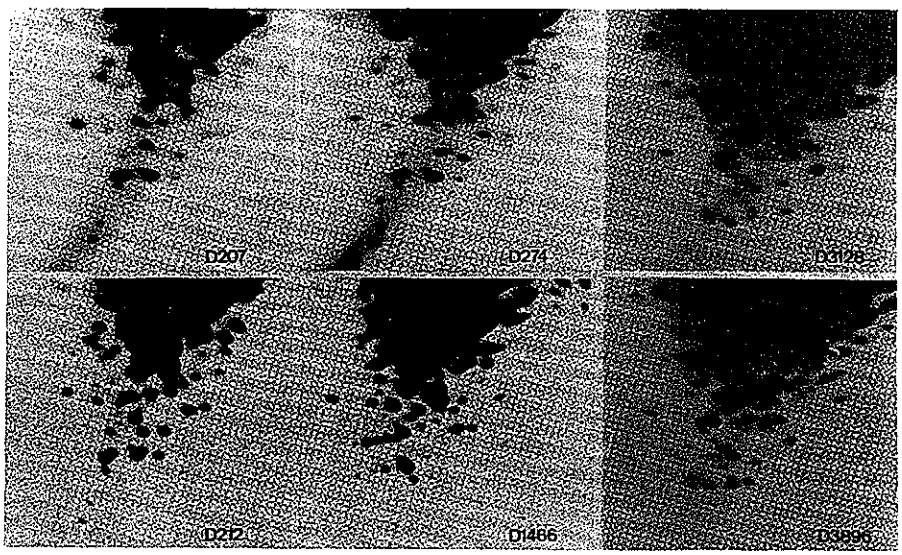
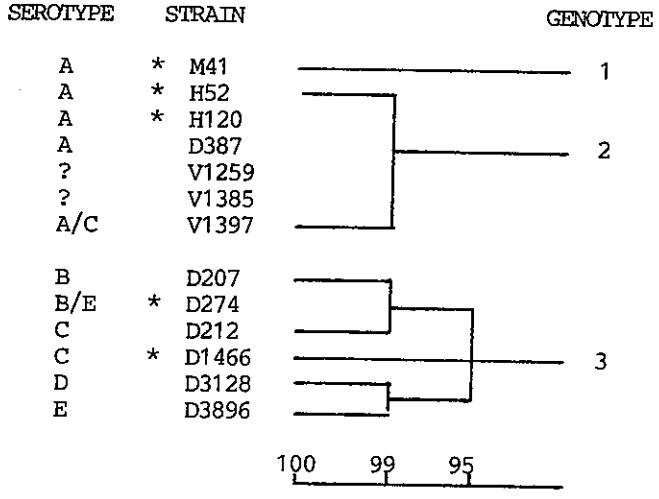


Fig. 3. Oligonucleotide fingerprints of the genome of the genetically related Dutch IBV isolates D207, D212, D274, D1466, D3128 and D3896. Experimental details are as in Fig. 1.

These data allow conclusions on the origin of new variant strains causing outbreaks of infectious bronchitis. They suggest that the strains V1259, V1385 and V1397 isolated in 1984, are mutants of the attenuated H52 and H120 strains used for vaccination. In contrast, the IBV strains isolated in the late seventies (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. The tree of relationship determined from T1-fingerprinting of the IBV strains is indicated in Table 3.

Table 3. Schematic representation of the relationship of the Dutch IBV isolates (vaccine strains are marked with an arterisk).



Percentage genomic relatedness.

COMPARISON OF AMINO ACID SEQUENCES OF THE SPIKE PROTEIN

In order to study the amino acid substitutions of the spike protein of related IBV strains, we started with sequencing of the spike gene of two serological related strains, M41 and M42-Salk, both of the Massachusetts serotype. Antibodies elicited by the laboratory strain M42 fail to neutralize M41. This indicates that these viruses have different neutralization epitopes (Chomiak et al., 1963). The comparison of the amino acid sequences of these viruses will be useful to localize epitopes eliciting neutralizing antibodies.

First strand cDNA of M41 was synthesized with genomic RNA as template and a synthethic oligonucleotide primer located at about 110 nucleotides downstream the initiating AUG codon of the membrane protein. The second cDNA strand was synthesized using the method of Gubler and Hoffman (1983). Double stranded cDNA was dC-tailed, annealed with Pst-1 cleaved, dG-tailed pUC9 (Pharmacia) and subsequently used for transformation of E.coli strain JM109 (Yanish-Perron et al., 1985). Restriction mapping of a number of clones resulted in a continuous map of 4.5 kb (see Fig. 4). Full details are

presented elsewhere (Niesters et al., 1986). cDNA clones of the spike protein of M42-Salk were obtained using polyadenylated mRNAs from infected cells as template. The complete sequence was obtained after subcloning Pst-1 fragments into the M13mp9 vector. Oligonucleotide primers were used to prime internally in long subcloned fragments.

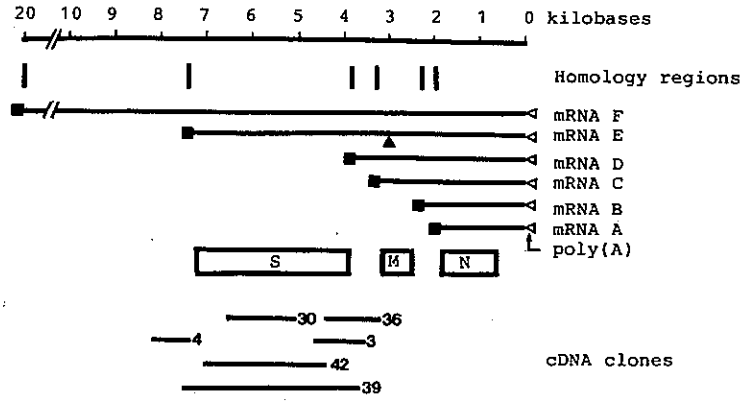


Fig.4. Genomic organization of infectious bronchitis virus. The sequence relationships between the mRNAs are indicated, as well as the positions of the clones used for the sequencing of the spike gene of strain M41. The genes for the structural genes S, M and N are indicated by boxes. Homologies present at the intergenic boundaries are indicated by vertical bars. The leader sequence present at the 5'-end is indicated (■). The position of the oligonucleotide used to prime cDNA synthesis is marked by an triangle.

We compared the amino acid sequence deduced from our cDNA sequencing with that of another M42 strain (M42-Houghton, Binns et al., 1985). All the three proteins have the same length and a 96.2% conservation of the amino acid residues. There are 29 positions where amino acid differences between S1 of M41 and the two M42 strains were observed; for the S2 proteins only 20 differences were found. Statistically significant clustering of amino acid substitutions have been found, particularly in the regions 56-69 and 117-131 of S1. In the S2 protein one cluster of changed amino acids is observed (residues 683-692) when only the M42-Houghton strain is considered. This cluster in S2 also seems to be variable between MHV, IBV and FIPV (see R.J. de Groot et al., this volume). The data are shown in Fig. 5.

LOCATION OF NEUTRALIZATION EPITOPES

So far as is known, only antibodies directed against the S1 protein are able to neutralize virus infectivity (Mockett et al., 1984; Niesters et al., unpublished data). Thus amino acid substitutions in S1 are likely to be responsible for antigenic differences between the various strains of IBV. Our

Fig.5. Differences between the M41 and M42 strains are shown by vertical bars. Characteristic features, like signal peptide, cleavage site between S1 and S2 and membrane anchor sequence are indicated. Glycosylation sites are indicated (●), as well as the extra glycosylation site present on the M41 spike protein (O).

hypothesis is that at least one of the two clusters found in S1 reflects the antigenic differences between the M41 and M42 virus. However, we do not know how many neutralization epitopes are present on S1, nor can we exclude their presence on S2. Up to now, only two neutralizing monoclonal antibodies recognizing the same epitope are available. Presently, we are testing a larger panel of neutralizing monoclonal antibodies in order to determine the exact number and position of their epitopes.

MAPPING OF NEUTRALIZATION EPITOPES

We are using several strategies to determine the amino acid sequence recognized by the neutralizing monoclonal antibodies. Firstly, several synthetic peptides are being tested for their capacity to elicit neutralizing antibodies in rabbits as well as in chickens.

Secondly, short DNAse-1 generated DNA fragments from the gene of the spike protein were ligated into the SmaI site of the cro-lacZ gene of the bacterial expression vector pEX (Nunberg et al., 1984; Stanley and Luzio, 1984). The resulting expression product consists of 15-100 amino acid long spike peptide fragments fused to the cro-beta-galactosidase hybrid protein. Fig. 6. shows typical results of screening this bank with antibodies to IB virions. Sequencing of these positive clones will give us information on the localization of the epitopes recognized.

Thirdly, comparison of sequence data will help us to define those places on the spike protein with the highest mutation frequency. We started to sequence M41 and M42. They are the best characterized IBV strains and give high yields of virus. Comparison with other strains will give a more complete picture, especially when closely related field isolates of different serotype (Table 3) are included.

IMPLICATION FOR VACCINE STRATEGY

Vaccination against IBV is only partially successful since new variants not covered by the vaccine strains used continuously emerge. How do these variants arise in nature? Probably as a result of the high error rate during replication and lack of repair mechanisms (Holland et al., 1982; Steinhauer and Holland, 1986). Therefore large numbers of variants can be expected to

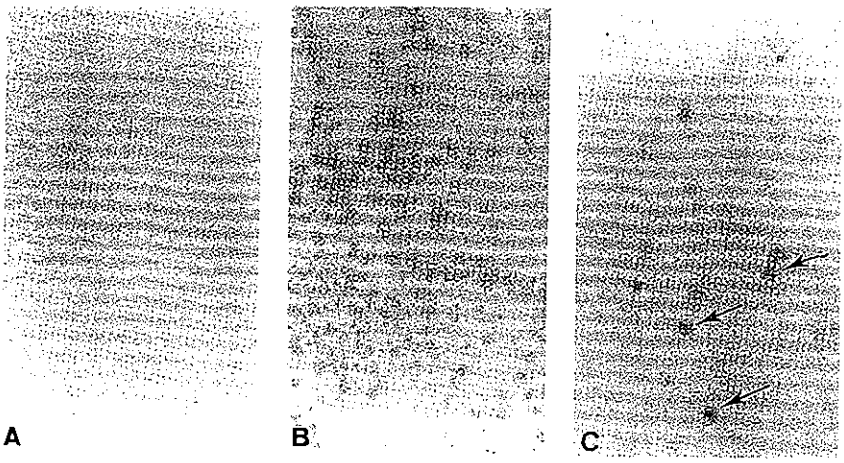


Fig.6. Immunological screening of a random IBV spike protein library subcloned in pEX. Colonies were transferred to nitrocellulose filters. (A) Screening of pEX without insert with a polyvalent antiserum. (B) pEX with a 1.3 kb PstI insert of the spike protein was screened with a polyvalent antiserum. (C) Screening of the random spike protein library with a polyvalent antiserum. Some positive clones are indicated with an arrow.

originate in flocks vaccinated with live attenuated viruses. Although mutations will occur at random in the entire 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 H52 and H120 have reduced the economical losses due to IBV infections, they might also be responsible for new variants viruses (see Fig. 2).

Our goal is to develop a new generation of vaccines, which at least in itself cannot give rise to new IBV variants. In principle, this can be achieved by vaccination with a bacterial or eukaryotic expression product of our cDNA. However, vaccination with an expression product will not protect against variant strains arising in the field. Therefore, additional information is needed on the localization of neutralization epitopes. Once the neutralizing 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 of the genome encoding these neutralization epitopes. Existing recombinant DNA-vaccines can then be quickly adapted.

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