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Australian infectious bronchitis viruses: identification of nine subtypes by a neutralisation test

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The antigenic relationships between 17 Australian infectious bronchitis viruses, including six vaccine viruses, were studied by a neutralisation test using a plaque reduction method in chick embryo kidney cell monolayers. The 17 viruses formed nine distinct subtypes. Antiserum to each subtype had a high titre to viruses of the same subtype and a lower titre to viruses of different subtypes. The heterologous titres of sera varied widely.

RECOGNITION of infectious bronchitis (IB) in Australia by Cumming (1963, 1967) led to the development of live IB virus vaccines. Their widespread use and changes in broiler breeds, poultry rations and housing and management, helped to reduce the prevalence and impact of the disease (Cumming 1969). Nevertheless, there have been recurrent reports of outbreaks of IB in vaccinated flocks in Australia (Chubb et al 1976) indicating the need for a study of the antigenic relationships between IB viruses involved in field outbreaks and those incorporated into vaccines.

Previous studies of antigenic relationships between Australian IB viruses have used neutralisation tests in eggs (Westbury 1970, Chubb et al 1976). This assay method has disadvantages. It relies upon a subjective assessment of the deforming or dwarfing effect of IB virus on the chick embryo and it is a quantal assay requiring large numbers of eggs to obtain precise results. In the constant serum — variable virus method of performing the test, it lacks specificity when undiluted serum is used, due to viral inhibitors commonly present in chicken sera, and it lacks sensitivity when diluted serum is used.

Neutralisation tests using a plaque reduction method in tissue cultures avoid the first two disadvantages. The IB virus N1/62, synonym T (Cumming 1963, 1967) is the only Australian isolate which has been included in studies using this method. Both Bracewell (1973) and Hopkins (1974) reported that IB virus T was distinct from the British and American isolates which they studied.

The aim of this work was to determine the antigenic relationships between 17 Australian IB viruses, including six vaccine viruses, using a plaque reduction method for neutralisation tests in chick embryo kidney (CEK) tissue cultures.

Materials and methods

The history of the 17 Australian IB viruses, the preparation of CEK monolayers and the methods and media for their use for growth and for plaque assay of IB virus have been described (Wadey and Faragher 1981).

Viruses

Each of the 17 viruses was plaque purified in specific pathogen free (SPF) CEK monolayers to eliminate contaminants and to obtain a homogeneous clone of virus for the preparation of monospecific antiserum. Single plaques were picked from stained monolayers at the highest dilution of virus and virus was grown under fluid medium for 48 hours. This procedure was repeated once. With all viruses, the fluid which was obtained after these two steps was tested and was found to be free from bacteria and fungi, *Mycoplasma* species, reticuloendotheliosis virus and avian leucosis viruses. All viruses were sensitive to chloroform by the method of Feldman and Wang (1961), were unable to haemagglutinate fowl red blood cells and had the appearance of coronavirus when examined in an electron microscope.

The purified viruses were passaged twice in SPF eggs to obtain primary and secondary stocks of virus. These were distributed in ampoules and stored at —70°C. Samples from the secondary stocks were used in all subsequent work.

Antisera

Antiserum to each of 16 viruses was prepared by inoculating virus into the trachea of sets of two or three SPF fowls. Most sets of fowls were inoculated

twice at an interval of two weeks and bled two weeks later. All fowls were housed in single-bird isolators and were inoculated and bled within a handling unit (Ellery and Withell 1975). Serum from each fowl was tested and found to be free from antibodies to *Mycoplasma gallisepticum, M synoviae, Salmonella pullorum,* infectious laryngotracheitis virus, avian influenza virus, Newcastle disease virus, herpesvirus of turkeys and avian adenoviruses and was found to contain antibody to the homologous IB virus. Sera from fowls within sets were pooled, heated at 56°C for 30 minutes and stored in small aliquots at −16°C.

Neutralisation test procedure

After preliminary studies, the following method was adopted for the assay of neutralising antibody to IB virus using plaque reduction in CEK monolayers. Dilutions of serum and virus were prepared in phosphate buffered saline (pH 7·4) containing 0·05 per cent bovine albumin fraction V, 1·5 per cent sucrose (Lukert 1973), 100 μg streptomycin per ml, 100 iu penicillin per ml and 2 μg amphotericin B per ml. Antiserum was diluted in a series of four-fold steps. To each tube containing 0·2 ml of diluted serum, 0·2 ml of virus containing approximately 100 plaque forming units (PFU) was added. Aliquots of 0·2 ml of virus, which was diluted a further 1:2, were added to each of 10 tubes containing 0·2 ml of diluent. These virus controls allowed an accurate estimate of the titre of virus which was added to the serum. Tubes containing 0·4 ml of diluent, as cell controls, were included in each assay. All tubes were shaken and incubated in a water bath at 37°C for 15 minutes to allow for neutralisation of virus. The CEK monolayers which were prepared from fertile eggs from commercial flocks, were coded, randomised, washed and drained before inoculation. Inoculation and adsorption of virus, overlay and incubation of CEK monolayers and formation of plaques have been described (Wadey and Faragher 1981).

Each serum was assayed against each virus in duplicate and on at least two occasions. The virus dose at the level of 50 per cent neutralisation was calculated as the mean of the plaque counts on the 10 virus control monolayers. The serum titre was calculated as the dilution of serum which reduced the virus titre by 50 per cent.

The homologous titre of each of the 16 antisera was compared with its titre against all other viruses, using Student's *t* test to evaluate the significance of the differences. Relationships between the viruses were also calculated as r values by the method of Archetti and Horsfall (1950) and were expressed as percentages.

Results

The geometric mean titres for each combination of serum and virus are shown in Table 1. Comparison of the homologous and heterologous titres showed that the viruses can be divided into nine groups which are in bold type in this table and designated A to I.

Antiserum titres against viruses within the same group were not significantly different from homologous titres ($P>0·05$) but were significantly

TABLE 1: Comparison of the neutralisation titres of antiserum of homologous and heterologous IB viruses

Serum	Virus: Vac 1	Vac 2	Vac 3	Vac 4	Vac 5	Vac 6	V1/70	Q1/76	N1/62	N2/62	N8/74	N9/74	Q1/73	V2/71	V1/71	N2/75	N1/75
Vac 1	**2·6***	**2·9**	2·0	2·1	1·8	2·0	1·9	2·0	2·1	2·3	2·4	2·1	1·9	1·9	2·0	2·1	1·8
Vac 2	**2·8**	**3·2**	2·1	1·8	1·4	1·5	2·0	1·6	1·4	1·7	1·7	1·5	1·3	1·8	1·8	1·5	NT
Vac 3	2·6	2·4	**3·6**	**3·7**	**3·2**	**3·8**	**3·5**	**NT**	2·0	2·0	1·6	1·6	2·0	1·6	1·6	NT	NT
Vac 4	2·1	2·3	**3·7**	**3·4**	**3·1**	**3·1**	**3·3**	**3·0**	2·3	2·1	1·8	2·1	2·2	1·5	2·2	1·9	1·8
Vac 5	2·0	2·2	**3·7**	**3·7**	**3·6**	**3·9**	**4·0**	**3·9**	2·5	2·7	2·3	2·1	2·6	1·8	2·3	2·6	2·2
Vac 6	2·3	2·3	**3·2**	**3·2**	**3·4**	**3·5**	**3·4**	**3·1**	2·0	2·4	1·5	1·8	2·6	1·5	2·4	2·2	2·0
V1/70	1·8	1·5	**2·8**	**2·9**	**2·7**	**3·2**	**3·1**	**NT**	1·2	1·3	1·4	1·1	1·4	1·0	1·3	NT	NT
N1/62	1·1	1·2	0·7	0·5	1·0	1·4	0·7	NT	**3·2**	**3·0**	**2·8**	0·6	0·7	1·1	0·6	NT	1·3
N2/62	1·2	1·2	0·8	0·7	0·6	1·1	0·7	NT	**3·0**	**2·9**	**2·6**	1·1	0·7	1·2	1·1	NT	NT
N8/74	1·5	1·4	1·0	1·4	1·0	0·9	0·8	1·2	**3·4**	**3·4**	**3·5**	1·2	1·3	1·5	1·5	1·4	NT
N9/74	2·6	2·5	2·2	2·2	2·0	2·0	2·3	2·1	2·6	2·5	2·5	**4·4**	2·0	2·2	2·5	2·7	1·9
Q1/73	1·7	2·2	1·8	1·8	1·7	1·5	1·7	1·4	1·9	2·0	2·3	1·4	**3·1**	1·5	1·8	1·9	1·5
V2/71	1·9	2·2	2·1	2·0	1·9	2·0	2·0	1·8	2·0	1·9	2·3	2·0	1·6	**2·8**	2·1	2·0	2·1
V1/71	1·7	1·9	1·7	1·6	1·3	1·3	1·5	1·5	1·9	1·9	2·1	1·4	1·6	2·2	**4·2**	1·5	1·4
N2/75	2·1	2·1	2·0	2·0	1·9	2·3	2·3	2·0	1·9	2·1	2·0	1·9	2·2	2·2	2·0	**3·0**	1·9
N1/75	2·3	NT	2·3	NT	NT	NT	NT	NT	2·5	NT	NT	2·3	2·3	2·3	2·4	2·5	**5·0**
Subtype	A				B					C		D	E	F	G	H	I

* Values in bold type represent a geometric mean of four or more determinations and are expressed as the common logarithm of the reciprocal of that dilution of serum which caused a reduction of 50 per cent in the plaque counts compared with the virus controls
NT Not tested

TABLE 2: Antigenic relationships between IB viruses expressed as r values* and calculated from neutralisation titres

Strain	Vac 1	Vac 2	Vac 3	Vac 4	Vac 5	Vac 6	V1/70	N1/62	N2/62	N8/74	N9/74	Q1/73	V2/71	V1/71	N2/75	N1/75
Vac 1	100	89	16	13	6	13	10	5	10	8	7	9	16	3	20	2
Vac 2		100	7	6	3	4	1	1	3	5	2	4	10	1	5	NT
Vac 3			100	158	71	89	63	1	1	1	1	4	4	1	NT	NT
Vac 4				100	79	50	71	1	2	1	2	6	4	1	6	NT
Vac 5					100	126	100	2	3	1	1	6	4	1	8	NT
Vac 6						100	100	2	4	1	1	6	4	1	10	NT
V1/70							100	1	1	1	1	3	4	1	NT	NT
N1/62								100	89	56	1	1	4	0	NT	1
N2/62									100	63	1	2	5	1	NT	NT
N8/74										100	1	3	6	1	3	NT
N9/74											100	1	3	0	4	0
Q1/73												100	4	1	10	1
V2/71													100	4	16	2
V1/71														100	1	0
N2/75															100	2
N1/75																100

* Calculated from the method of Archetti and Horsfall (1950) and expressed as a percentage
NT Not tested

different ($P<0{\cdot}05$) from heterologous titres. In most cases these differences were highly significant ($P<0{\cdot}001$).

The r values for each pair of viruses are shown in Table 2. The r value for pairs of viruses within the same group varied from 50 to 158, and for pairs of heterologous viruses from zero to 20.

The levels of the heterologous titres varied for different sera. Sera N1/62, N2/62 and N8/74 showed very low heterologous titres which were not much higher than the non-specific antiviral activity of normal serum. However all other sera neutralised all heterologous viruses but to a titre lower than the homologous titres of the sera, indicating a relationship between the viruses. These relationships are shown in Fig 1. Each block on this diagram represents the geometric mean titre of all sera of a particular group against all viruses from another group. The differences between the viruses within, for example, group A may be masked by this

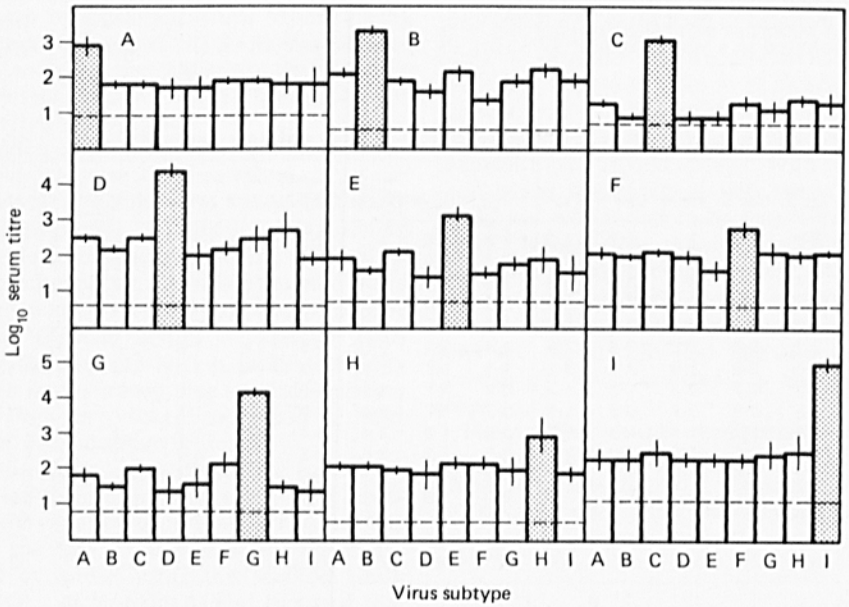

FIG 1: Relationships between nine subtypes of IB virus. Each block represents the mean titre of all sera of one subtype assayed against all viruses of another subtype. Hatched blocks represent homologous titres, the vertical bars represent the 95 per cent confidence limits of the means and the horizontal broken lines represent the mean serum titres before inoculation with IB virus

representation but the very low heterologous titres of $10^{1\cdot1}$ of group C and the highest heterologous titres of $10^{2\cdot3}$ of groups D and I are evident.

Discussion

Using a plaque reduction method for reciprocal neutralisation tests in CEK monolayers to examine the antigenic relationships between 17 IB viruses, these viruses formed nine distinct groups.

No procedure has been generally accepted and adopted for the nomenclature of subdivisions of viruses which are serologically distinct.

We have analysed the antigenic relationships between 17 IB viruses by the method of Archetti and Horsfall (1950) and by the method of Kapikian et al (1967).

Archetti and Horsfall (1950) expressed the degree of relatedness between viruses by a numerical value r but did not define serotypes in terms of r values. By this method, the r values of pairs of homologous viruses, as percentages, ranged from 50 to 158. The r values of pairs of heterologous viruses ranged from zero to 20, 68 of the 94 values falling below 5, 72 below 6 and 79 below 7. If two viruses are defined as distinct when they have an r value of 20 or less, homologous when they have an r value of 50 or greater and the relationship to be doubtful when the r value falls between 20 and 50, then the 17 IB viruses can be divided into the same nine distinct groups by this method and by the *t* test as shown in Table 1.

Kapikian et al (1967) defined a candidate virus to be a distinct serotype if the homologous titre of serum to this virus is at least 20 times that of its heterologous titres and if the homologous titres of sera of all other serotypes are at least 20 times the titres of all these sera to the candidate virus. By this method, sera to six of our viruses had homologous titres greater than 20-fold higher than their heterologous titres, three had homologous titres less than 20-fold higher than their heterologous titres and seven had some heterologous titres greater and some less than 20-fold lower than their homologous titres. Because of the high heterologous titres of three of the sera, the differences between all pairs of viruses were insufficient for their definition as serotypes by the criteria of Kapikian et al (1967). Because of the cross-reactions found between the groups and their failure to meet the above criteria for definition as serotypes, these nine groups of IB viruses are designated as subtypes.

Studies of antigenic relationships between IB viruses by Bracewell (1973), Hopkins (1974), Cowen and Hitchner (1975) and Lohr (1976, 1977) described differences between viruses of a similar magnitude to those found in this study. In studies in which r values were calculated, most but not all virus pairs had r values of less than 5 per cent and, due to the high heterologous titres of some sera, the criteria of Kapikian et al (1967) were not met.

In our study, the heterologous titres were found to vary considerably with different sera, from low levels of heterologous neutralisation with sera from subtype C to high levels of heterologous neutralisation using sera from subtypes D and F. Although there has been disagreement about the relevance of serum neutralisation titres to protection (Raggi and Lee 1957, 1965, Hopkins 1969, Winterfield and Fadley 1972, 1975, Cunningham 1975, Cowen and Hitchner 1975, Johnson and Marquardt 1975), it would be expected that those viruses which elicited the highest heterologous titres in chickens would be the viruses most likely to confer protection against the widest range of subtypes of IB virus. As the two vaccine viruses in subtype A differed in their heterologous titres, the capacity to elicit a high heterologous antibody response might be a characteristic of an individual virus and not of a subtype of virus. However no such difference was observed between the viruses in subtype C. It is interesting that the six vaccine viruses, the histories of some of which are not complete, are of subtypes A and B.

No pattern was observed among the 11 IB viruses which had been isolated from outbreaks of disease. Of the three viruses in subtype C, two were isolated in 1962 and the third in 1974. In 1971, subtypes F and G caused disease in flocks in Victoria, while in 1975 subtypes H and I caused disease in flocks in New South Wales. Subtype B contains four vaccine viruses and two viruses which were isolated from affected chickens in 1970 and 1976, although both may be residual vaccine virus.

No correlation was found between subtype and plaque morphology.

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