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Antigenic Variation in Strains of Avian Infectious Bronchitis Virus

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Summary

Fifteen british field strains of IBV were compared using cross serum neutralization tests in embryonated eggs with seven standard reference strains of IBV. While the British field strains were considered to form a relatively homogeneous group considerable antigenic variation did occur. It was considered that it was not feasible at this time to describe accurately a serotype classification for IBV, similar to that described for other virus groups.

1. Introduction

Immunological and serological variations between strains of infectious bronchitis virus (IBV), first postulated by Jungherr, Chomiak and Luginbuhl (1956) have been the subject of continued controversy for many years.

Initially two main "types" of virus were recognised (i) Massachusetts type of which the prototype strain was isolated by H. Van Roekel at the University of Massachusetts in 1941. (A number of common designations are in use *e.g.* IBV41, M41, strain 82828.) (ii) Connecticut type of which the prototype strain was isolated in Connecticut in 1951 (Jungherr *et al.*, 1956).

The Beaudette strain of virus originally described by Beaudette and Hudson (1937) is now a completely egg-adapted, non immunogenic strain generally considered to be of the "Massachusetts type".

Hofstad (1958) reported on 3 new strains of IBV designated 97, 104 and 609. Two of these strains subsequently referred to as Iowa 97 and Iowa 609 have been widely used as reference strain types.

In 1961 further studies with these viruses showed that cross protection test results did not agree with cross neutralization test results. In particular the Connecticut type strain, previously found to be antigenically distinct, gave some degree of cross immunity between all other strains used including the Iowa 97 and 609 strains (Hofstad, 1961).

Winterfield and Hitchner (1962) reported two new virus isolates, designated the Gray and the Holte strain, which were capable of producing nephrosis

in experimental birds and suggested that by cross neutralization tests in eggs that these strains differed serologically from the Massachusetts, the Connecticut and the Iowa strain types.

In 1964 WINTERFIELD, CUMMING and HITCHNER reported on a comparative serological study using Australian strains of virus which had been isolated from outbreaks of a disease in chickens known at that time as "Uraemia". This report indicated that the Australian isolates were related to the American isolates but that the relationship was entirely one-way and the authors also indicated that differences might exist between the strains isolated from different areas of Australia.

At this time it was becoming apparent that the IBV type classification was unsatisfactory and further isolations of serologically different virus strains by WINTERFIELD, HITCHNER and APPLETON (1964) and PURCHASE, CUNNINGHAM and BURMESTER (1966) in the United States, by VON BÜLOW (1967, 1969) in Germany and by ZANELLA, GUALLINI and MORINI (1968) in Italy only served to increase the confusion.

To date some 19 different "types" have been recorded in the literature. The criteria necessary for the designation of a new type have never been established.

In the United Kingdom it had been assumed by various workers in this field that only one "type" of IBV occurred and that this was the so-called "Massachusetts type" similar to the Massachusetts M41 strain. BERRY and STOKES (1968) lent support to this assumption and concluded that the antigenic variation in eight English strains investigated was minor and of subtype in character.

At this time BOX and BARNES (1967) drew attention to the difficulties experienced in carrying out diagnostic serum neutralization tests with only a single strain (Beaudette strain) of virus.

In order to investigate further the possibility of antigenic variations of IBV occurring in the United Kingdom, 15 British field strains of virus were compared using cross serum neutralisation tests in embryonated eggs with 7 of the so-called reference strains of virus. This paper presents the results of this investigation.

2. Materials and Methods

2.1. Viruses

The 7 reference strains of virus used and their origins are shown in Table 1. The British field strains of virus were isolated from material submitted to the Poultry Department, Central Veterinary Laboratory, Weybridge, for the diagnosis of outbreaks of disease over the last five years. One strain designated the Allen strain, and given the code letter O, was isolated from an outbreak of disease in Buckingham in 1947 and is one of the original strains of IBV (Strain A) isolated by ASPLIN (1948) who first identified infectious bronchitis of chicken in England. The British field strains were selected to give as wide a geographical distribution as possible. The fifteen strains, their passage history, the county of origin and the clinical disease with which they were associated are shown in Table 2.

During the course of the investigations the British field strains were characterised as members of the Coronavirus group (ALMEIDA *et al.*, 1968) and shown to be free from extraneous avian adenoviruses.

The Celo-Phelps strain of avian adenovirus was used as a control preparation throughout the investigation.

Table 1. *Seven Reference Strains of IBV Virus and the CELO Virus Strain Used and Their Origin*

Strain designation	Abbreviation	Source
Massachusetts M41	M41	Poultry Department C.V.L., Weybridge
Connecticut	Conn	Professor C. H. Cunningham, Michigan State University, U.S.A.
Iowa 97	Iowa 97	Dr. M. S. Hofstad, Iowa State University, Ames, Iowa, U.S.A.
Iowa 609	Iowa 609	
Gray	Gray	Professor R. W. Winterfield, Purdue University, Lafayette, U.S.A.
Holte	Holte	
Australian "Nephrosis"	Austral	Poultry Department, C.V.L., Weybridge
CELO Phelps	—	Professor Yates, University of Rhode Island, Kingston, Rhode Island, U.S.A.

Table 2. *The 15 British Field Strains of IBV and Their History*

Code letter	Weybridge Ref. No.	County of origin	Associated disease	Age of bird	No. of embryo passages
A	9	Surrey	Respiratory	11 weeks	4
B	48	Gloucester	Respiratory	Adult	4
C	183	Sussex	Respiratory	22 weeks	5
D	225	Hants	Nephrosis	7½ weeks	2
E	227	Nottingham	Egg Production	Adult	4
F	317	Worcester	Respiratory	Adult	3
G	265	Essex	Respiratory	3 weeks	4
H	551	Dorset	Nephrosis	5 weeks	5
I	591	Lincolnshire	Nephrosis	6 days	2
J	604	Somerset	Respiratory	2 days	2
K	690	Sussex	Respiratory	7 weeks	1
L	860	Nottingham	Egg Production	Adult	4
M	918	Northampton	Respiratory	Adult	2
N	927	Kent	Respiratory	3 weeks	2
O	Allen	Buckingham	Respiratory	?	4

Stock virus preparations were prepared by the inoculation of 0.1 ml of a 10^{-2} dilution of infected allantoic fluid, (in peptone broth to which antibiotics had been added), into the allantoic cavity of 9 to 10 day old embryonated eggs. Allantoic fluids, harvested 48 hours later, checked for sterility, were pooled and stored at −40°C in 2.0 ml aliquots until required.

Virus titrations were carried out in 9—10 day old embryonated eggs. A tenfold dilution series of the virus suspension was prepared and 0.1 ml of each dilution was inoculated via allantoic cavity into each of 5 embryonated eggs. The eggs, incubated at 37°C, were candled daily and the titration was read at 7 days post inoculation by the detailed examination of the embryo where necessary. The end points expressed as 50% embryo infective doses (EID_{50}) were calculated by the method of REED and MUENCH (1938).

Virus suspensions were considered to be satisfactory for serum neutralization tests if they had an infectivity titre of equal to or greater than $10^{5.0}$ EID_{50} per 0.1 ml.

2.2. Specific Antisera

Specific antisera to each of the virus strains were prepared in groups of 5 to 10 week old White Leghorn cross bred birds which were housed in strict isolation and which had been shown to be free from IBV antibodies.

Birds were inoculated with 2.0 ml of infected allantoic fluid given by the intranasal and intraperitoneal routes. The birds were observed daily and serum was obtained 4 weeks post inoculation. The serum obtained from each group of birds were individually titrated using the homologous virus strain and the high titre serum samples for each virus strain were pooled, inactivated at 56°C for 30 minutes and stored in 2.0 ml aliquots at −20°C until required. Pooled specific antisera were only considered satisfactory for serum neutralization tests if the sera obtained from control birds of identical batches held in a similar manner were shown to be free from IBV antibodies over the period of antiserum production.

2.3. Serum Neutralization Tests

Serum neutralization tests were carried out in 9–10 day old embryonated eggs and the tests were arranged so as to use only one virus preparation on any one day. Antisera were diluted using a twofold dilution series from 1 in 4 to 1 in 1024. 1.0 ml of a virus suspension containing 100 EID_{50} per 0.1 ml was added to 1.0 ml of each serum dilution and the serum-virus mixture was held at room temperature (ca. 22°C) for 30 minutes. 0.2 ml of each serum-virus mixture was inoculated into each of 5 embryonated eggs per dilution. The eggs incubated at 37°C were candled daily and the titration was read at 7 days post inoculation by the detailed examination of the embryo where necessary. The end points calculated by the method of REED and MUENCH (1938) were expressed as the initial dilution of serum preventing the appearance of lesions in 50% of the eggs inoculated. Full control titrations using negative serum, specific adenovirus antiserum and virus and simultaneous titration of the virus under test were always carried out.

3. Results

The detailed results of all cross serum neutralization tests carried out presented an extremely complex series of interrelationships in regard to the homologous and the heterologous serum titres. The use of one of the several techniques available for the numerical expression of serological interrelationships of virus strains, *e.g.* the use of R values as described by WENNER, ARCHETTI and DUBES (1959), was considered, but these techniques were felt to be inappropriate because of the relative inaccuracies inherent in the titration of IBV in embryonated eggs as compared to Newcastle disease virus (ALLAN and HEBERT, 1968).

For this reason and for the purposes of this communication a serum neutralization titre of 1 in 8 or greater has been expressed as a positive. In no case was a serum titre of 1 in 4 or greater recorded with control negative serum or when control avian adenovirus serum or virus was used.

The results obtained are presented in Tables 3 to 6 and the cross relationships occurring in these tables are analysed in Table 7.

The results indicate that cross relationships which are infrequent when the 7 reference strains are tested (Table 3) occur much more frequently when the British field strains are tested (Tables 4 and 6). One strain (isolate L.) shows no cross relationships with any of the 7 reference strains although it does show such relationships with 12 of the other 14 British field strains. Of the other 14 field strains examined, 5 show only a one-way cross relationship and 9 show one or more two-way cross relationships with the 7 reference strains. Of the two-way cross

Table 3. *Cross Neutralization Tests Using 7 Reference Strains of Virus, and Their Specific Antisera*

Virus strains	Antisera							Positive totals
	M41	Conn	Iowa 97	Iowa 609	Holte	Gray	Austr.	
M41	⊕	−	−	+	−	−	−	2/7
Conn	−	⊕	−	−	−	−	−	1/7
Iowa 97	−	−	⊕	−	−	−	−	1/7
Iowa 609	−	+	−	⊕	−	−	−	2/7
Holte	−	−	+	−	⊕	−	−	2/7
Gray	−	+	−	+	−	⊕	+	4/7
Austral.	−	−	−	−	−	−	⊕	1/7
Positive totals	1/7	3/7	2/7	3/7	1/7	1/7	2/7	

⊕ = Homologous reaction.

Table 4. *Cross Neutralization Tests Using 7 Reference Strain Viruses and Specific Antisera to 15 British Field Isolates*

Virus strain	Antiserum to British field isolate															Positive totals
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	
M41	+	+	+	+	+	+	+	+	−	−	−	−	−	+	+	10/15
Conn.	+	−	−	+	+	−	+	+	+	−	−	−	−	+	+	8/15
Iowa 97	+	−	−	+	−	+	−	+	−	−	+	−	−	+	+	7/15
Iowa 609	+	+	−	+	+	+	+	+	+	+	−	−	−	+	+	11/15
Holte	+	−	+	+	−	+	+	+	−	−	+	−	−	+	+	9/15
Gray	+	−	−	+	−	+	−	+	+	+	+	−	+	+	+	10/15
Austral.	+	−	−	+	−	+	−	+	−	−	−	−	−	+	+	6/15
Positive totals	7/7	2/7	2/7	7/7	3/7	6/7	4/7	7/7	3/7	2/7	3/7	0/7	1/7	7/7	7/7	

Table 5. *Cross Neutralization Tests Using Specific Antisera to 7 Reference Strain Viruses and 15 British Field Isolates*

Antisera	Virus isolate (British field isolate)															Positive totals
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	
M41	−	⊕	−	−	⊕	⊕	⊕	⊕	−	+	−	−	−	⊕	−	7/15
Conn.	−	+	−	−	−	−	−	⊕	−	−	−	−	−	−	−	2/15
Iowa 97	−	−	−	⊕	−	⊕	−	−	−	−	−	−	−	−	−	2/15
Iowa 609	−	−	−	−	−	⊕	−	−	⊕	−	−	−	−	−	−	2/15
Holte	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	0/15
Gray	−	+	−	−	−	−	−	⊕	−	⊕	−	−	−	−	−	3/15
Austral.	−	+	−	⊕	+	−	−	−	−	−	−	−	−	⊕	−	4/15
Positive totals	0/7	4/7	0/7	2/7	2/7	3/7	1/7	3/7	1/7	2/7	0/7	0/7	0/7	2/7	0/7	

⊕ = Two-way cross relationship.

relationships shown, 6 are with the Massachussets strain, 2 are with each of the Iowa 97, the Iowa 609, the Gray and the Australian strain and only 1 with the Connecticut strain. No British field strains show a two-way cross relationship with the Holte strain (Tables 4 and 5).

When the British field strains are considered separately (Table 6) it can be seen that the specific antisera to 5 of the strains neutralized all the other strains tested while the specific antisera to one of the strains (isolate I) only neutralized 2 other strains. None of the field strains were neutralized by all of the specific antisera (Table 6) although 10 of the strains were neutralized by 12 or more of the antisera.

The summary of the cross relationships obtained is presented in Table 7.

Table 6. *Cross Neutralization Test Using 15 British Field Isolates and Their Specific Antisera*

Virus isolate	Antiserum to British field isolate														Positive totals
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	
A	⊕	−	−	+	−	−	−	+	+	−	−	−	−	+	+	6/15
B	+	⊕	+	+	+	+	+	+	−	+	+	+	+	+	+	14/15
C	+	+	⊕	+	−	+	−	+	−	+	−	−	−	+	+	9/15
D	+	+	+	⊕	−	+	+	+	+	+	+	+	+	+	+	14/15
E	+	+	+	+	⊕	+	+	+	−	+	−	−	−	+	+	11/15
F	+	−	+	+	−	⊕	+	+	−	+	+	+	+	+	+	12/15
G	+	+	+	+	+	+	⊕	+	−	+	+	+	−	+	+	13/15
H	+	+	+	+	−	+	+	⊕	−	−	+	−	−	+	+	10/15
I	+	−	+	+	+	+	−	+	⊕	+	+	+	+	+	+	13/15
J	+	−	−	+	+	+	+	+	−	⊕	+	+	+	+	+	12/15
K	+	+	+	+	−	+	−	+	−	−	⊕	−	−	+	+	9/15
L	+	+	−	+	−	+	+	+	−	+	+	⊕	+	+	+	12/15
M	+	−	+	+	+	+	+	+	−	+	+	+	⊕	+	+	13/15
N	+	+	+	+	−	+	+	+	−	+	+	+	+	⊕	+	13/15
O	+	−	−	+	+	−	+	+	−	−	−	−	−	+	⊕	7/15
Positive totals	15/15	9/15	11/15	15/15	7/15	13/15	11/15	15/15	3/15	11/15	11/15	9/15	8/15	15/15	15/15	

⊕ = Homologous reaction.

Table 7. *Analysis of Tables 1 to 4 Showing Cross Relationships*

Viruses and antisera tested	Reciprocal cross neutralization test results			
	No. of reciprocal tests	No. showing 2-way cross relationship	No. showing 1-way cross relationship	No. showing No. cross relationship
Reference strains (Table 3)	21	0 (0%)	6 (28.6%)	15 (71.4%)
Reference strains British Isolates (Tables 4 and 5)	105	15 (14.3%)	51 (48.6%)	39 (37.1%)
British Isolates (Table 6)	105	53 (50.5%)	47 (44.7%)	5 (4.8%)

4. Discussion

One of the difficulties associated with the titration of IBV in embryonated eggs is that since not all strains regularly produce death of the embryos, and unlike Newcastle disease virus no alternative accurate reading technique such as

the determination of presence of haemagglutination is available for IBV, titration end points are determined by a subjective examination of the embryos for the presence of characteristic lesions. The possible operator variation in this respect was controlled in these studies by only one person (REG) reading all titrations.

Since the production flock supplying the birds and embryonated eggs for this investigation could not be shown to be completely free of IBV or of strains of avian adenovirus suitable controls were included at all stages. These controls were included in the serum and stock virus preparation and in the serum neutralization tests. The results of these control tests did not indicate the presence of any contaminating extraneous agents and no effect was detected as being due to the presence of maternally derived antibody in the embryonated eggs.

For the various reasons outlined above it was decided to represent the cross relationships detected between various strains by the acceptance of a serum neutralization titre of 1 in 8 or greater as indicative of a positive reaction and any conclusions drawn from the results are presented in the knowledge of this simplification.

The relatively distinct antigenic pattern demonstrable using the 7 reference strains of virus is no longer apparent when the British field strains are included in the examination.

The relatively high percentage of cross relationships (over 95%, with 50.5% two-way cross relationships) among the British field strains compared to the figures obtained for the reference strains (28%, with 0% two-way cross relationships) and when the reference strains were compared to the British field strains (62%, with only 14.3% two-way cross relationships) would suggest that the British field strains are a relatively homogeneous group compared with the reference strains. Nevertheless, some of the British field strains do show considerable variation one from another and even with the simplification of the relationships as presented cannot be considered to be identical.

Six of the 15 British field strains examined do show a two-way cross relationship with the Massachusetts strain of virus (Table 5) and the relationships of these 6 strains to each other and to the other field strains do have considerable similarities.

However the criteria necessary for the designation of a type strain of IBV, or the identification of related strains, have never been established. In view of the complex interrelationships demonstrated by the results of these investigations it is considered that it is not feasible at this time to describe accurately a serotype classification of IBV similar to that described for other virus groups.

Further studies on alternative techniques which are necessary for the definition of IBV serotypes are currently in progress in this and other laboratories.

The 15 British field strains selected for this study do not necessarily represent proportionately the disease related strain incidence of IBV in the poultry population of this country. The results and conclusions presented are not, therefore, contradictory of a view that the predominating field strains in any one country are likely to be of a similar "type" and that non-typical strains may only be encountered infrequently (Fritzsche, Stumpel, Toucas and Billon, 1969; von Bülow, 1969).

The results of a serological examination as presented here cannot be extrapolated to indicate immunological variation or to be of any immunological significance particularly in view of the results reported by HOFSTAD (1961).

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