Chapter 3
Newcastle Disease Studies
in
Australia

Newcastle Disease Virus in Australia

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K.F. MEYER, a veterinary surgeon who made important contributions to the study of many infectious diseases in people and animals, was insistent that we recognise the distinction between infection and disease. Infection he defined as a harmless host-parasite relationship and disease as an altered state in the host resulting from this relationship (Meyer 1953). Let us recognise that distinction now. Newcastle Disease does not occur in Australia at present, and we recall very few episodes of this disease in the past. However, many of our poultry flocks are infected with avirulent strains of Newcastle Disease virus. A consideration of Newcastle Disease virus in Australia will involve a short account of outbreaks of disease and a much longer account of our attempts to understand and exploit the harmless viruses that infect our poultry. There has been no detailed review of the subject since that of Westbury (1981).

Newcastle Disease in Australia

Victorian Outbreaks (1930-31 and 1932-33)

Newcastle Disease was not recognised until 1926. In that year or the following year it was described in Java, the U.K. (near Newcastle), in Korea, India, Sri Lanka and the Philippines. The disease then spread widely to infect most of the countries of the world, especially those in which intensive poultry industries were developing. The origin of velogenic Newcastle Disease has not been clarified. It may have been present in poultry populations for some time, coming to notice only as more intensive poultry husbandry was practiced. On the other hand it may have been a virus newly introduced to domestic poultry from a reservoir in free-living birds, possibly in Asia. Certainly it was soon the most important viral disease of poultry and it has maintained this preeminent position for more than 50 years.

Australia was an early casualty during the first pandemic of Newcastle Disease. In 1930 outbreaks occurred in Victoria, mostly in the Melbourne metropolitan area. Only two of the outbreaks were on farms having more than 500 birds. The average mortality was 70% but some farms reported mortality rates of 100% or nearly 100%. The outbreak was controlled in about 4 months by quarantine and slaughter. A similar disease was encountered in the same region in 1932 and was again controlled by quarantine and by slaughter of birds on affected premises. It was suggested that poultry carcasses placed in cold storage during the first outbreak were responsible for the second outbreak.

Albiston and Gorrie (1942) reported the outbreaks in detail. They isolated the causal virus, which is usually referred to in the Australian literature as the Albiston Gorrie strain and in some overseas literature as strain Victoria. The Albiston Gorrie strain has been maintained under quarantine in Australia. It was used as antigen in a serological survey (French 1964) when the Newcastle Disease status of Australian poultry was queried. Its use as a challenge strain was also permitted on one occasion, under strict security, after strain V4 was isolated in Australia. The Albiston Gorrie strain at the passage level used as a challenge strain seemed less pathogenic than the parental virus from the outbreaks of the 1930s (French et al. 1967). The Albiston Gorrie strain is now held in a new high security laboratory (Australian Animal Health Laboratory, Geelong, Victoria).

A Malaysian Connection

In 1963 Newcastle Disease again caused problems under circumstances that have not been officially explained. Retnasabapathy and Chong (1963) reported that over the previous 4 years some batches of chickens imported into Malaysia from Australia had been diseased. On arrival they were found to be lame with red legs, and mortalities of up to 25% were recorded. The reddening of the legs was associated with swelling, haemorrhage and

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infiltration with a gelatinous fluid. The immediate stimulus for the report from Ipoh was the isolation of Newcastle Disease viruses from four different batches of these chickens. The viruses were described as 'mild' because they produced the condition of 'red legs' and death in l-day-old Australian chickens but they produced no disease in 6- or 8-week old local chickens. However there was a 48-hour death time in embryonated eggs inoculated by the allantoic cavity and this would normally be associated with velogenic strains of Newcastle Disease virus.

Investigations in Australia revealed no evidence of Newcastle Disease. All the flocks and hatcheries involved in exports to Malaysia were examined, but there were no signs of 'red legs' or of classical Newcastle Disease (McIntosh 1964). Nor was the disease reproduced when chickens from these sources were placed in a compression-decompression chamber and the conditions of air transportation were simulated. French (1964) examined more than 1400 sera from export hatcheries. A few sera showed low levels of haemagglutination inhibition antibody but none of these contained neutralising antibody against Newcastle Disease virus. Any of the strains of Newcastle Disease known at that time would have been expected to induce both types of antibody. It was concluded that Australian poultry had remained free from Newcastle Disease since the outbreaks of the 1930s. No further problems associated with the importation of Australian chickens were reported.

Smuggling

Our continued freedom from Newcastle Disease is largely the result of strict quarantine regulations and strict enforcement. Importation of all live birds and eggs is prohibited as is the importation of potentially dangerous products of birds or eggs. However, instances of smuggling of various species of birds or their eggs have been recognised. Precautionary eradication programs have been necessary when the smuggling was not discovered until populations of commercial or aviary birds were considered to be at risk. On at least one occasion a strain of Newcastle Disease virus has been isolated from illegally imported birds, indicating the necessity for the strict quarantine regulations.

Eaves and Grimes (1978) reported such an isolation from a parrot illegally introduced from Indonesia. The virus was of a lentogenic pathotype, producing severe respiratory disease in 1 -day-old chicks but no disease in 5-week-old chickens. The virus was possibly a vaccine strain, for it was suspected that the parrot had been vaccinated with La Sota vaccine. The Eaves Grimes strain is now stored in a high security laboratory. Control measures following detection of this consignment

of smuggled birds included destruction of the smuggled birds and disinfection of premises. Clinical and serological monitoring of commercial poultry flocks in surrounding areas was continued for 6 months and there was no indication of spread of Newcastle Disease virus (Gee 1978).

The Future

Newcastle Disease remains the most important exotic disease that concerns the Australian poultry industry. Smuggling and the uncontrollable movements of migratory birds could easily introduce velogenic strains of Newcastle Disease virus from neighbouring countries. The cost of controlling an outbreak would be high and the cost of poultry production would be increased if the disease became endemic. The support for Newcastle Disease research by industry and government indicates the concern that this disease causes.

Infection with Avirulent
Newcastle Disease Virus in Australia

The Isolation of Strain V4

In 1966 the V4 strain of Newcastle Disease virus was isolated from the proventriculus of a broiler chicken that had a nutritional deficiency and a concurrent infection with *Staphylococcus* (Simmons 1967). The very vigorous and immediate official reaction to this isolation has not been publicly documented. Two short announcements (Anon 1966a, b) gave minimal details. The subsequent laboratory work was published and demonstrated that the V4 strain was widespread and that it lacked pathogenicity for chickens. French et al. (1967) isolated similar viruses in Victoria and New South Wales but did not describe the source of the strains. Webster et al. (1970) recovered a Newcastle Disease virus in New South Wales from poultry that were clinically normal. In Victoria similar isolations were made from the digestive tracts (rectal swabs and caecal lymphoid tissue) from clinically normal broiler chickens (Turner and Kovesdy 1974).

The Diversity of Strains

Strain V4 (known in some overseas literature as strain Queensland) is considered the type strain. However there have been many more isolations of Newcastle Disease virus made from domestic poultry in Australia. These strains vary in some properties - in heat stability of infectivity and haemagglutinin, in immunogenicity and in ability to transmit between chickens. One trait is apparently constant - a lack of pathogenicity for chickens when the viruses spread by natural routes. This lack of pathogenicity for chicks is paralleled by a low lethality for chicken embryos and very poor

cytopathogenicity for cultured avian cells.

Kim and Spradbrow (1978a) made a detailed study of V4 and 12 newly isolated avirulent strains. All were poorly pathogenic for chick embryos after allantoic inoculation, only two strains producing sufficient mortality to allow calculation of mean death times. All had thermostable haemagglutinins but infectivity was relatively heat-resistant for only four strains, including V4. Elution times varied between strains, as did the ability to agglutinate equine erythrocytes.

Epidemiology

Even now the epidemiology of the avirulent Australian strains of Newcastle Disease virus is not fully understood. Serological surveys indicate that infection in domestic poultry is widespread but not all flocks are infected and not all areas are infected. Common experience has been that when flocks become infected, as judged by seroconversion, the virus spreads quickly and most of the birds develop antibodies within a short time (Turner and Kovesdy 1974; Kim 1977). Transmission was not observed in young chicks. By contrast, a survey report by Gilchrist et al. (1976) found only a single flock with an antibody prevalence greater than 20%. Experimentally, not all avirulent Australian strains spread with equal facility and the final infection rate in any flock may be a function of viral strain. The age of the bird also seems to influence the rapidity of spread, and the epidemiology may vary between single age sheds and multi-age free-range flocks.

The method of transmission within a flock is not known. Epizootiological studies by Turner and Kovesdy (1974) supported the concept of both respiratory and enteric spread of avirulent viruses. Viruses have been isolated from the respiratory tract and the digestive tract in both experimentally and naturally infected chickens in Australia. The duration of excretion is not known. Following aerosol vaccination, V4 could be recovered from lungs for 10 days, and from caecal tonsils for 7 days, while there was a viraemia during the first few days after vaccination (Kim and Spradbrow 1978a).

In an experiment reported by Bancroft and Spradbrow (1977) vaccinated chickens or their immediate environment remained infective for other chickens for at least 4 weeks.

The mechanism of spread between sheds also needs to be explained. This does not occur as readily by indirect contact as by direct contact. Spread by contaminated food and water containers has been demonstrated experimentally (Spalatin et al. 1976), but this should not be important in commercial flocks. French et al. (1967) postulated that strain V4 might spread through the embryo. This was based on the survival of embryos inoculated, at 9 or 10 days of incubation, through the allantoic cavity and the presence of virus in the organs of chicks that were allowed to hatch from infected eggs. However, Kim and Spradbrow (1978a) showed that strain V4 was lethal when injected into the yolk sac of younger embryos, and there must be some doubt on the concept of embryo transmission. The proper experiment, with collection of fertile eggs from viraemic hens, has not been done. Latency in the avian host must be considered as cultures of both mammalian and avian cells have been shown to carry strain V4 (Spradbrow and Ford 1981).

The origin of the Australian avirulent viruses is not known. The results of the serological survey reported by French (1964) indicated freedom from infection with the velogenic, mesogenic or lentogenic viruses that were known at that time. A few sera did have low levels of haemagglutination inhibition antibody but lacked neutralising antibody. Subsequent experimental infection of adult poultry by French et al. (1969) produced similar serological profiles with low levels of haemagglutination inhibition antibody in the absence of neutralising activity. The results are consistent with the presence, even in 1964, of viruses of the V4 type.

One suggestion (Albiston 1966) was the introduction of a vaccine virus. However, strain V4 does not closely resemble any of the vaccine strains in use in the 1960s. Spread from wild birds has been suggested. Hore et al. (1973) demonstrated haemagglutination inhibition antibodies to Newcastle disease virus in sera of wild ducks in Victoria. Domestic ducks vaccinated with V4 virus have developed transient, low levels of haemagglutination inhibition antibody and virus was reisolated from birds receiving high doses of vaccines (Westbury 1981a). Turner (cited by Kim 1977) suggested a pathway involving wild ducks, domestic turkeys and then domestic chickens.

Vaccine Studies

Quarantine regulations had prevented the acquisition or testing of Newcastle Disease vaccines while Australian poultry remained free of Newcastle Disease virus. With the recognition of V4 virus, the poultry industry gained an advantage not available to other livestock industries. It was now possible to develop and test an Australian vaccine that might eventually be required to combat an exotic disease. Almost since the first recognition of V4, the poultry industry has sponsored Newcastle Disease vaccine research. Challenge experiments were still not permitted as velogenic strains could not be imported. This was not critical. Haemagglutination inhibition antibodies, measured by standard tests, are fair indications of acquired resistance. Vaccination and challenge would be possible in other countries, initially with poultry available in

other countries and eventually with Australian stock. These latter studies were possible only because of the cooperation and goodwill of the Malaysian authorities and of our Malaysian colleagues. This was valuable and valued Malaysian aid to Australia.

The first vaccine experiments were reported by French et al. (1969). These were part of the official investigation of the discovery of V4 virus, and use of the 1932 Albiston Gorrie strain as challenge virus was sanctioned. Chickens vaccinated with V4 or with another recent Australian Newcastle Disease virus were protected against challenge with the moderately pathogenic Albiston Gorrie virus.

Webster et al. (1970) conducted challenge experiments in the U.K. They used an avirulent virus from New South Wales, Australia, as both living and inactivated vaccines and challenged with the Herts strain. Both vaccines gave protection. Turner et al. (1977) conducted similar experiments in the USA. Two Australian viruses, including V4, were given oronasally, into the conjunctival sac, or by drinking water and all three methods induced protection against challenge with virulent neurotropic strains of Newcastle Disease virus. Spalatin et al. (1976) demonstrated protection in chickens not directly vaccinated with V4, but in contact with vaccinees, and also noted that protection was sometimes present before the development of detectable levels of antibody. More recent protection trials conducted overseas have involved direct comparisons between Australian avirulent viruses and recognised vaccine strains (Westbury et al. 1984a, b), The experiments indicated a lack of virulence for the V4 virus and a fair degree of immunogenicity.

Other protection trials have been undertaken in Malaysia, often on imported Australian chickens. The first trial used imported l-day-old chicks which were vaccinated with V4 vaccine and challenged with viscerotropic, velogenic Newcastle Disease virus. Vaccinated chickens, and chickens that had encountered strain V4 by contact with vaccinees, were substantially protected (Spradbrow et al. 1978). These results were confirmed by Ibrahim et al. (1980) and extended to show protection when the V4 vaccine was given by spray, aerosol or through the drinking water (Ibrahim et al. 1981). Of great interest to the Australian poultry industry was the finding that laying birds, having acquired antibody bY natural exposure in Australia to virus of the V4 type, were also substantially protected against challenge (Spradbrow et al. 1980). In the same experiment, Australian broilers from an antibody-free flock all succumbed to contact challenge.

Other vaccine experiments have been reported from Australia in which the response to vaccination is judged by the development of antibody and not confirmed by challenge. Hall et al. (1967) found that vaccine-induced antibody persisted for at least 8 months after vaccination. Adequate antibody responses are obtained after many routes of vaccination including application by aerosol and through the drinking water (Kim and Spradbrow 1978b; Kim et al. 1978; Schalkoort et al. 1979; Schalkoort and Spradbrow 1980).

Bulk supplies of V4 vaccine are held in Australia for possible use in the event of an outbreak of virulent Newcastle Disease. Policy on the use of vaccine would probably not be determined until an outbreak occurred. The company that produces the vaccine also markets it overseas in competition with established vaccines.

Other Avian Species

Viruses of the V4 type are not confined to domestic poultry. The presence of antibody in wild ducks (presumably due to infection with the V4 type virus) has been mentioned (Hore et al. 1973). Penguins in Antarctic territories have also been shown to possess antibodies, although strains of Newcastle Disease virus have not been isolated (Morgan et al. 1981). The isolations sometimes made from free-living birds are not always recorded. One published report from Western Australia (Alexander et al. 1986) detailed the isolation of 13 strains of Newcastle Disease virus from wild birds and a domestic duck. The viruses could be divided into two groups by analysis with monoclonal antibodies but all were judged to be of low virulence for chickens.

Oral Newcastle Disease Vaccine in Experimental Chickens in Australia

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THE Australian Centre for International Agricultural Research has sponsored an investigation of methods for protecting village chickens in Asia against Newcastle Disease. These studies are jointly pursued by the Veterinary Schools of the University of Queensland, Brisbane, Australia, and the Universiti Pertanian Malaysia, Serdang, Malaysia. The project arose from earlier cooperation between the two institutes that also concerned Newcastle Disease vaccines. A detailed background to these studies is given elsewhere in this volume (Spradbrow 1987). The Newcastle Disease situation in Australia is discussed in this paper.

Australia enjoys relative freedom from many of the important viral diseases of livestock. The poultry industry shares in this and Newcastle Disease has not been observed since 1933. Virulent strains of Newcastle Disease virus seem to be absent from the country, but unusual avirulent strains of Newcastle Disease virus have been present since 1966 and possibly before that. The type strain for these avirulent viruses is the V4 strain isolated in Brisbane by Simmons (1967). Strain V4 and its relatives have been widely used in vaccine experiments because Newcastle Disease is the exotic disease of most concern to the Australian poultry industry, and the importation of any avian viruses or avian viral vaccines is prohibited. The antibody response to V4 type viruses may be assessed in experimental chickens, but the use of these vaccines in commercial chickens is not condoned at present.

It has been determined that these Australian viruses produce no disease when they spread between chickens by natural routes, that the viruses do spread readily on close contact and that there is an appreciable antibody response when virus is administered by any of the routes used for conventional vaccination (for review see Westbury 1981). However, proper vaccine experiments are not possible because virulent challenge viruses are not available. This problem has been overcome by performing the challenge experiments in Malaysia, where a long series of trials demonstrated that chickens vaccinated with V4 virus were substantially protected when put in contact with virulent virus (Ibrahim et al. 1980; Ibrahim et al. 1981; Spradbrow et al. 1978; Spradbrow et al. 1980).

When the village chicken project was contemplated, V4 was considered as a candidate vaccine. Earlier work in this laboratory (Schalkoort unpublished data) had shown that it was possible to select: heat-resistant variants of V4 virus. This would overcome one of the main problems of vaccine distribution in tropical countries – the need to provide a cold chain linking the vaccine production facility with the end user if the vaccine was heat labile. V4 is known to spread readily by contact, so that vaccination of a proportion of chickens in a group might lead to protection of the whole group. V4 had already shown promise as a vaccine and a commercial Australian V4 vaccine was accepted and marketed in parts of Asia. Importantly, from our point of view, some of the experimental work could be undertaken in Australia.

The delivery of the vaccine remained a problem. The methods that were available to the commercial industry included individual intranasal and intraocular exposure of young chicks, intramuscular injection of individual mature birds and mass exposure of flocks to infected drinking water, sprays or aerosols. None of these methods was appropriate for village poultry. One of the methods we proposed in our original submission was the provision of vaccine in the food. Oral vaccines are used in human medicine (poliomyelitis vaccine) but oral veterinary vaccines were at that time still experimental (rabies vaccine for wildlife, transmissible gastroenteritis virus vaccine for adult sows). Little was known about the spread of Newcastle Disease virus by ingestion, although one Australian study had shown that contaminated carcass meat could infect

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chickens that ate it (French et al. 1967).

The oral vaccine proved to be a successful approach and studies at both centres have concentrated on this aspect of the project. As in the earlier Malaysian and Australian studies, the Australian work was concerned with antibody responses and the natural history of the avirulent virus. These studies have been facilitated by the availability of chickens that are not vaccinated against Newcastle Disease and that are not exposed to virulent Newcastle Disease virus. The Malaysian work took every advantage of the opportunity to test immunity by challenge.

The following series of experiments investigate the response of chickens to V4 vaccines given orally. The results indicate some strategies that might be useful in the development of oral Newcastle Disease vaccines.

Materials and Methods

Experimental Chickens

Chickens were produced at the University Farm or purchased from commercial sources. No Australian commercial chickens received Newcastle Disease vaccine and no chickens had contact with virulent virus. Some commercial farms are infected with viruses of the V4 type which produce relatively low levels of antibody. Consequently, young chicks in Australia have either no naturally derived anti-Newcastle Disease antibody or low titres of antibody that are usually undetectable by 2 weeks of age. Chickens were housed as indicated later and always had access to water and commercial feed.

Titration of Virus

Titrations were performed in embryonated eggs after 9 or 10 days of incubation. Tenfold dilutions of virus were inoculated into the allantoic cavity in volumes of 0.1 or 0.2 ml and 4 or 5 eggs were used for each dilution. Because V4 virus causes few mortalities in chick embryos, 50% embryo lethal doses cannot be calculated. Instead, infected eggs were harvested after 4 days incubation and allantoic fluid was examined for Newcastle Disease virus haemagglutinin, using chicken red blood cells. Fifty percent embryo infectious doses (EID_{50}) were calculated by the method of Reed and Muench (1936).

Haemagglutination Inhibition (HI) Test

The microtest procedure developed by Allen and Gough (1974) was used. In this test serial twofold dilutions of serum are allowed to react with four haemagglutinating units of Newcastle Disease virus. Residual haemagglutinating activity was detected by the addition of a 1% suspension of chicken red blood cells. Serum dilutions, haemagglutinin and red blood cell suspensions were mixed in volumes of 0.025 ml. Controls included chicken anti-Newcastle Disease antiserum of known titre and chicken serum free of antibody. Homologous W4) Newcastle Disease virus was used as antigen.

Vaccine

The vaccine consisted of diluted allantoic fluid infected with the V4 strain of Newcastle Disease virus derived from a commercial vaccine (Arthur Webster Pty Ltd, Sydney). The titre of each vaccine was determined by inoculation of embryonated eggs.

Faecal excretion of vaccine virus delivered into the crop – Experiment 1 Four groups, each consisting of five 5-week-old chickens were used. Three groups of chickens were given respectively $10^{5.4}$, $10^{6.4}$ and $10^{7.4}$ EID_{50} of virus. The virus was introduced into the crop by a plastic tube attached to a syringe. After introduction of the virus in a volume of 1 ml, a further 1 ml of phosphate-buffered saline was given. This minimised contamination of the oro-pharynx during removal of the tube and ensured delivery of the whole dose. The fourth group of control chickens received only phosphate-buffered saline.

Each bird was housed individually in a wire-floored cage placed over a sheet of plastic. Each day for 10 days after vaccination the total faecal output of each chicken was collected and stored at –70°C. The faecal samples were later thawed, weighed and homogenised in two parts of phosphate-buffered saline (w/v). A portion of the homogenate was further diluted 1 part in 4 (v/v) in a diluent of antibiotics (gentamycin 250 µg/ml, streptomycin 500 µg/ml, penicillin 10 000 IU/ml) in phosphate-buffered saline. After centrifugation to remove gross particles the final homogenate was titrated in embryonated eggs.

Each chicken was bled and HI antibody titres were recorded before vaccination and 2, 4,9 and 14 days after vaccination.

Antibody dose response to vaccine virus delivered into the crop – Experiment 2 Five groups, each containing eight to twelve 3-week-old chickens were used.

The birds were housed in wire-floored cages with four birds in each cage. The vaccine was delivered by tube to the crop, as in the previous experiment. The grouts received no virus or $10^{6.2}$, $10^{7.2}$, $10^{8.2}$ and $10^{9.2}$ EID_{50} of virus per bird respectively. Thirteen days after initial vaccination, half the vaccinated birds in each group received a dose of $10^{6.2}EID_{50}$ virus to the crop.

HI antibody titres were determined before vaccination and at 5, 9, 13, 20 and 26 days after initial vaccination. Faecal excretion of vaccine virus was not measured.

Antibody response to food vaccine and spread to contact birds - Experiment 3 Chickens were vaccinated when 6 weeks of age. The vaccine was applied to food pellets (Betalay Pellets, Whitewings, Brisbane) by shaking in a conical flask immediately before presentation to the chickens which had been fasted for 16 hours. One millilitre of vaccine was allowed for each 10 g of pellets and sufficient pellets were supplied to allow 20 g for each chicken. These were consumed within 20 min. The nominal dose of vaccine per bird (assuming equal consumption by each bird and no inactivation of virus on the pellets) was $10^{7.7}$ EID_{50}. Twenty birds received the food vaccine and a further 11 contact controls were introduced after the vaccine had been consumed. The birds were run together on a concrete floor. Four unvaccinated control birds were kept in a separate room. Thirteen days later the contact birds were removed, the vaccinees received a similar dose of vaccine on food pellets, and the contact birds were reintroduced to the room.

HI antibody titres were measured before vaccination and 7, 13, 20, 27 and 33 days after original vaccination. Faecal excretion of vaccine was not measured.

Antibody dose response to food vaccine - Experiment 4 Pellets containing various doses of vaccine were prepared as described above and given as a single dose to four groups of nine 9-week-old chickens. Each group was kept in a separate room, on a concrete floor. The nominal dose for each group was $10^{5.2}$, $10^{6.2}$, $10^{7.2}$ and $10^{8.2}$ EID_{50} per bird respectively. Four unvaccinated control birds were kept in a separate room for 5 weeks. They were then transferred to the room containing the chickens that had received the highest dose of vaccine.

HI antibody titres were determined before vaccination and 6, 14, 21, 28, 35 and 42 days thereafter. Cloacal swabs were taken from the chickens receiving the highest dose of vaccine on one occasion, 9 days after vaccination.

Results

Faecal Excretion of Vaccine Virus Delivered into the Crop

Virus was not recovered from prevaccination faecal samples, nor at any time from control birds. The major evidence of faecal excretion was on days 6 and 7 post-vaccination in the two groups receiving the highest doses of virus. In the group receiving $10^{6.4}$ EID_{50}, three of five birds were positive on each day and in the group receiving $10^{7.4}$ EID_{50} all five birds were positive each day. There were a few isolations earlier in the experiment as shown in Table 1. However the titres of virus were apparently very low - only a proportion of the eggs inoculated with undiluted homogenates yield haemagglutinating

TABLE 1. Isolation of Newcastle Disease virus from total faecal samples collected daily from chickens receiving V4 vaccine by tube to the crop.

Dose of vaccine (EID_{50})	No. of chickens	Days after vaccination					
		3	4	5	6	7	8
Nil	5	0^a	0	0	0	0	0
$10^{5.4}$	5	0	0	0	0	0	0
$10^{6.4}$	5	0	0	0	3	3	0
$10^{7.4}$	5	0	2	1	5	5	0

[a] Number of chickens excreting virus.

agents. The haemagglutinating agents were inhibited by anti-Newcastle Disease antiserum.

The haemagglutination inhibition antibody responses are shown in Table 2. Only with the highest dose rate ($10^{7.4}$ EID_{50}/bird) was there an appreciable antibody response within 14 days. Prevaccination sera contained no detectable antibodies.

TABLE 2. Haemagglutination inhibition antibody titres (geometric mean titres) in chickens receiving various amounts of V4 Newcatle Disease vaccine by dosing into the crop.

Dose of vaccine (EID_{50})	No. of chickens	Days after vaccination				
		0	2	4	9	14
Nil	5	$<1^a$	<1	<1	<1	<1
$10^{5.4}$	5	<1	<1	<1	<1	<1
$10^{6.4}$	5	<1	<1	<1	1.6	<1
$10^{7.4}$	5	<1	1.5	1.8	2.6	2.4

[a] $\log_2$.

Antibody Dose Response to Vaccine Virus Delivered into the Crop

The results of the response to a single vaccination are shown in Table 3. Prevaccination sera contained no antibody and there was an obvious antibody response with doses higher than $10^{7.2}$ EID_{50}/bird. Increased dose rates gave a higher and more rapid response. The second vaccination produced no marked increase in HI antibody titre.

Antibody Response to Food Vaccine and Spread to Contact Birds

Birds given vaccine on food pellets developed high levels of HI antibodies within 2 weeks, as did unvaccinated chickens in contact with them (Table 4). Control birds had low levels of HI antibody that remained fairly uniform throughout the trial. Antibody titres increased approximately eightfold in the birds that were revaccinated and in birds in direct contact with them.

TABLE 3. Haemagglutination inhibition antibody titres (geometric mean titres) in chickens receiving various amounts of V4 Newcastle Disease virus by dosing into the crop.

Dose of vaccine (EID$_{50}$)	No. of chickens	Days after vaccination					
		0	5	9	13	20	26
Nil	12	<1[a]	1	1.2	1.7	ND[b]	ND
10$^{6.2}$	12	<1	1.3	1.9	2.5	ND	ND
10$^{7.2}$	8	<1	1.4	2.6	2.5	2.3	2.5
10$^{8.2}$	8	<1	2.0	3.5	3.7	4.5	3.8
10$^{9.2}$	8	<1	2.4	5.1	4.4	5.2	4.4

[a] log$_2$.
[b] Not done.

TABLE 4. Haemagglutination inhibition antibody titres (geometric mean titres) in chickens receiving 10$^{7.7}$ EID$_{50}$ of V4 Newcastle Disease virus on food pellets and in chickens in contact with the vaccinated chickens.

Group	No. of chickens	Days after vaccination					
		0	7	13[b]	20	27	33
Isolated control	4	1.7[a]	3.0	2.7	2.3	3.0	2.5
Food vaccine	20	1.6	3.9	6.9	9.5	8.2	7.5
Direct contact	11	1.5	2.7	6.0	9.0	8.5	8.0

[a] log$_2$.
[b] Day of revaccination.

Antibody Dose Response to Food Vaccine

The results are shown in Table 5. There was a high HI antibody response to the vaccine regardless of dose, with peak response 2-4 weeks after vaccination. There were low levels of antibody in the chickens before vaccination and these were fairly constant in the control birds throughout the experiment. Virus was recovered from two of the nine cloacal swabs collected 9 days after vaccination.

The control birds developed high levels of antibody when placed with the vaccinated birds 5 weeks after vaccination.

TABLE 5. Haemagglutination inhibition antibody titres (geometric mean titres) in chickens receiving various amounts of V4 Newcastle Disease virus on food pellets.

Dose of vaccine (EID$_{50}$)	No. of chickens	Days after vaccination						
		0	6	14	21	28	35	42
Nil	4	1.2[a]	2	ND[b]	2.2	2.2	1.5	ND
10$^{5.2}$	9	2.3	2.3	5.3	7.2	7.0	5.2	5.6
10$^{6.2}$	9	2.0	3.1	7.0	6.1	6.0	4.3	5.1
10$^{7.2}$	9	2.2	5.1	7.3	8.0	8.1	5.3	5.5
10$^{8.2}$	9	1.8	4.8	5.5	6.2	6.9	4.6	4.5

[a] Log$_2$.
[b] Not done.

Discussion

We have tried to investigate, under controlled laboratory conditions, some factors that influence the response of chickens to 'oral' Newcastle Disease vaccine. We have fed the vaccine on food pellets in a manner that mimics the field vaccination procedure. Because this precludes any calculation of individual doses, we have also introduced accurately measured doses of vaccine into the crop, a procedure that is not exactly equivalent to either water or food vaccination. These experiments, and others reported elsewhere in this volume, have yielded information on antibody responses and the spread and persistence of V4 virus in groups of chickens. New questions of relevance to the vaccine program have arisen: how does V4 infect, how is it excreted and how long does it persist in a chicken? One preliminary observation should be made, although it serves only to confirm the results of 20 years' experimentation with V4: V4 is a safe vaccine and causes no discernible harm to vaccinated chickens.

When chickens were kept in cages, individually or in small groups, a dose response to V4 vaccine was demonstrated (Table 3). Higher doses of virus produced higher levels of antibody. When chickens were placed together on a solid floor, the dose responsiveness was lost (Table 5), and antibody responses were similar in groups of chickens whose doses of vaccine differed by a factor of 1000. A likely explanation is that vaccine virus is excreted by some route and that all the chickens are reinfected. Thus, although the original doses of vaccine varied between groups the challenge from the environment was similar for all groups. There is probably a similar explanation for the antibody response in control chickens placed in direct contact with chickens vaccinated with food vaccine. Both groups developed similar levels of antibody but the antibody response in the control group occurred about 1 week later than in the vaccinated group. The lack of prevaccination antibodies in the caged chickens may have influenced these results.

V4 virus appears to persist for some time in small groups of experimentally vaccinated chickens or in their environment. In one trial (experiment 4), control chickens, introduced to a group of vaccinated chickens 5 weeks after vaccination, became infected, as indicated by the development of antibodies. A similar persistence of V4 virus has been shown for vaccinated chickens kept on litter (Bancroft and Spradbrow 1977).

Little is known about the oral infectivity of strains of Newcastle Disease virus. Infection is believed to be usually by the respiratory route (Hanson 1978), and living vaccines are delivered by routes that ensure contact with cells of the respiratory tract.

However, French et al. (1967) showed that V4 virus in carcass meat could infect chickens when included in their rations and natural faecal-oral transmission of V4 virus has been postulated. This could occur, for example, when replacement chickens are placed on used litter in a shed, although the eventual rapid spread of V4 in flocks could indicate respiratory transmission (Turner and Kovesdy 1974). Recent experience in the U.K. with a strain of Newcastle Disease virus adapted to pigeons also shows that oral infection is sometimes important. This virus has spread to domestic poultry in feed contaminated by infected pigeon droppings, but there are few respiratory signs and little lateral spread in populations of chickens (Alexander et al. 1984, 1985).

Faecal excretion of virulent strains of Newcastle Disease virus has been demonstrated, as has oral transmission. Kohn (1955) found 800 000 50% infectious doses of virulent Newcastle Disease virus in each gram of faeces in chickens undergoing fatal infection, making this material a richer source of virus than infected mucous membranes. Much more virus was required to infect by the alimentary route than by other routes but, even so, 25 mg of faeces would infect a chicken when ingested. The source of the faecal virus is not known. It is unlikely that virulent Newcastle Disease virus is adapted to grow in enterocytes, as are some viruses that cause infection confined to the alimentary tract. Possible sources of virus are the intestinal collections of lymphoid tissues that seem to be a target for Newcastle Disease virus, blood shed from haemorrhagic lesions and bile.

Viruses of the V4 type also reach the faeces. No lesions are found in V4 infections to indicate the source of virus, which has been recovered both from cloacal swabs and from caecal lymphoid tissues. Infection of enterocytes might be suggested as a source of V4 virus, but our total faecal collections from orally infected chickens indicated that faecal excretion of V4 occurred inconsistently and at a low level. The delay of maximal excretion until days 6 and 7 after feeding does not suggest a simple pattern of ingestion followed by infection of enterocytes and release into faeces. The delay is consistent with a period of viraemia and access to the intestine by a secondary route, possibly through lymphoid tissue.

It would be unwise to assume that the target cells for the oral Newcastle Disease vaccine must be the cells of the upper or lower intestine, as they are with enterovirus, rotavirus or coronavirus vaccines. Shuaib et al. (1985) made experimental oral Newcastle Disease vaccines which were given by tube in volumes great enough to fill the entire intestinal tract. The problems they recognised were those of acidity and of high trypsin levels, which they

measured at 0.35 mg/ml in duodenal secretions. Before Newcastle Disease vaccines or methods of delivery are designed to resist a highly acidic environment and proteolytic enzymes, it should be established that the vaccine virus is required to survive transit through the intestine. The actual target cells may be in the upper digestive tract and the problem of access for the vaccine virus may not be difficult. The target cells should be identified.

The chickens used in the experiments have come from various sources. Some groups have had low levels of antibody before vaccination (experiment 3, experiment 4) and these have responded well to vaccination. Others (experiment 1, experiment 2) have lacked detectable antibody before vaccination, and the antibody response in these birds has been slight. It is possible that chickens in the later experiments had had some slight exposure to avirulent Newcastle Disease virus and we were measuring a secondary immune response. Where there has been no previous exposure to avirulent Newcastle Disease virus, it seems that two doses of vaccine are required to ensure an adequate serological response.

What will happen in the field? In isolated villages where the poultry population has not encountered Newcastle Disease virus, two doses of vaccine will be required initially. If the vaccine virus persists in the environment, chickens will encounter small amounts of virus and will develop low levels of antibody. This will probably act as a primary vaccination so that a single administration of a large dose of virus in a vaccine will produce a good serological response. We should probably aim to produce populations of village poultry in which V4 virus is endemic and in which immunity is boosted by occasional high doses of vaccine. The present situation seems to be one in which populations of village chickens are completely susceptible and outbreaks of Newcastle Disease destroy virtually the entire population. If these disastrous outbreaks can be prevented there will always be poultry in a village and endemicity of the vaccine virus might be achieved.

These experiments, and those conducted in Malaysia and presented elsewhere in this volume, indicate that some type of food vaccine will be of great benefit to the village chicken industry. Indeed it is probably not too early to consider problems that might arise from a highly successful vaccination program — including effects on the premium now paid for kampong chickens and eggs and the size of the chicken population that can survive by scavenging in the environs of a village. However, problems in relation to vaccination do remain, and a major one is the large-scale production and presentation of the vaccine. Is a coated food pellet the best solution? Is there a place for a dust vaccine

similar to that described by Kraft and Baumer (1975). 'Concerning Newcastle Disease' wrote Hanson (1974) 'nothing is more controversial than the question of how well immunity can be induced and assessed...' We expect that the vaccination of village chickens will provide further controversies.

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Oral Newcastle Disease Vaccine:
What is the Initial Site of Replication?

J.L. Samuel *

THE ACIAR-sponsored project for vaccination of free-range chickens against Newcastle Disease is concentrating on ways of administering the vaccinating virus – the Australian lentogenic V4 strain – to chickens in feed. In using this route we are, of course, assuming that the V4 virus, when ingested, will invade the birds' tissues, thus setting up an infection and promoting the production of protective antibodies. It might be protested that this can be assumed without question, since administration of vaccines in drinking water is recognised as effective in commercial poultry. However, the structure of the avian oropharynx makes it uncertain whether virus taken in drinking water in fact makes its entry via the digestive system or via the upper respiratory tract.

Several workers have shown that Newcastle Disease virus can infect via the digestive tract. Kohn, in 1955, produced the disease by administering a velogenic strain directly to the crop by stomach tube, though he found that higher doses were needed than for infection by respiratory routes. Shuaib et al. (1985), using the V4 strain among others, administered by crop-tube enough virus-containing fluid to fill the entire gastrointestinal tract, and produced marked rises in antibody titres when large infecting doses ($10^9 EID_{50}$) were used. In an experiment that mimicked more closely a real situation, Alexander et al. (1984) mixed virus from an outbreak into pigeon faeces and added this to feed; when chickens ate the contaminated feed, some showed clinical signs and all seroconverted and excreted the virus in faeces. Using the V4 strain, French et al. (1967) produced antibody titres in 4 out of 16 chickens fed the minced carcasses of birds that had been given the virus intramuscularly. As reported elsewhere in this volume, workers in this current project, in both Australia and Malaysia, have confirmed that the V4 vaccine does indeed infect chickens when taken in the feed, as is evidenced by rises in haemagglutination inhibition (HI) antibody titres, by excretion of virus a week after ingestion, and by resistance to challenge with virulent virus.

What is still unknown, however, is what form this infection takes: where, along the digestive tract, the virus enters the tissues; where it is replicated; and whence it is shed back into the lumen. Cheville et al. (1972), using fluorescent antibody staining, demonstrated Newcastle Disease viral antigen in the intestinal epithelium of young chickens that had been orally dosed with the virus; with a lentogenic strain (Ulster) the antigen was confined to the anterior small intestine, whereas velogenic strains produced antigen, and lesions, in the jejunum, caecum and colon. However, as tissues were not examined until the third or fourth day after dosing, there was no guarantee that the virus at these sites represented a primary infection. Nevertheless, it is important to know at what level of the digestive tract the primary infection takes place. Shuaib et al. (1985) spoke of the need to incorporate trypsin inhibitors and pH buffers in oral vaccines, in order to protect the virus against the deleterious effects of gastrointestinal secretions. They based this suggestion on the assumption that infection occurred via the wall of the intestine: i.e. that a successful vaccine must survive passage through the proventriculus, gizzard and duodenum. However, whether this assumption holds true for the V4 strain is not known. We have therefore begun a series of experiments, still in progress, to determine the fate of a vaccinating dose of V4 in the hours and days immediately following its delivery to the crop.

Methods and Results

For the first experiment, seven adult male bantam game chickens were used: members of a free-range flock in which the epidemiology of the V4 virus was being studied (see Samuel, this volume). None of the birds had previously been dosed with V4 virus

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and, although they were in Contact with crop-vaccinated birds, all had low titres of HI antibody to V4: 2 logs (base 2) or less. They were not fasted before being dosed with virus.

The virus used was the same as that used in our vaccination trials on commercial breeds of chickens (see Spradbrow and Samuel, this volume): allantoic fluid infected with V4 vaccine strain supplied by Arthur Webster Pty Ltd. Each bird received, directly to the crop by stomach tube, 1 ml of undiluted allantoic fluid ($10^{9.2}$EID$_{50}$), followed by 2 ml of phosphate-buffered saline (PBS) to ensure complete delivery. The birds were then kept in wire cages until they were killed, by intravenous injection of barbiturate, at intervals after dosing of 1, 2, 3.5, 4.5, 5.5, 23 and 23.5 hours. From 3.5 hours on, they had feed and water available.

Immediately after the death of each bird, samples of the walls and contents of the gastrointestinal tract were taken at various sites: crop; proventriculus (wall only); gizzard (contents only); duodenum; mid-part of small intestine; distal end of small intestine; each caecum-wall from the proximal end including the 'tonsil,' and contents from the distal end; and rectum. Instruments were sterilised between each sample. Samples of contents from the gizzard and from intestinal sites were taken either directly or, if not sufficiently copious, by flushing with approximately 1 ml of PBS (with added antibiotics; penicillin, streptomycin, gentamycin). To sample the crop we emptied out recently ingested food, flushed the crop with 1 ml of PBS which was then reaspirated, and finally rubbed the inner surface with a cotton wool swab and placed this in the PBS. All samples were stored at –70°C until they were processed. In addition a piece of wall from each site was fixed in 10% formalin for routine histopathological processing.

When samples were processed for viral content, 1 ml of PBS with antibiotics was added to each of the contents samples that had not already been diluted. Samples were then shaken vigorously on a vortex mixer and centrifuged at 2500 rpm for 30 min. Crop samples had the swabs removed after vortexing. The supernatant was inoculated to the allantoic cavity of lo-day embryonated eggs: 0.3 ml to each of three eggs per sample. Allantoic fluid was harvested on the fourth day and tested for haemagglutinating activity against chicken red blood cells. The samples of wall were each washed in five changes of sterile physiological saline, followed by five changes of PBS. They were then ground with sterile sand in PBS with antibiotics, at a rate of 1 ml PBS per 100 mg of tissue. The resulting suspension was centrifuged, and tested in eggs, as for the contents samples.

The results are shown in Table 1. Virus was recovered from the crop contents of all seven birds, and from the crop wall of all but one of them: the bird was killed at 4.5 hours. For each but one of these contents samples, and for four of the wall samples, all three inoculated eggs were infected, indicating a fairly high number of virus particles in the sample. Virus was also recovered from the wall of the proventriculus in all seven birds, but from the gizzard contents of none of them. No other wall samples were positive, but virus was found in the contents of the duodenum in two birds (killed at 1 and 3.5 hours), of a caecum in one (23 hours) and of the rectum of another (3.5 hours). The contents

TABLE 1. Recovery of V4 virus from digestive tract of chickens after administration of $10^{9.2}$ EID$_{50}$ by tube to crop.

Sampling site	Contents (C) or Wall (W)	Hours after administration of virus						
		1	2	3.5	4.5	5.5	23	23.5
Crop	C	+ [a]	+ [a]	+ [a]	+	+ [a]	+ [a]	+ [a]
	W	+ [a]	+ [a]	+ [a]	–	+ [a]	+	+
Proventriculus	W	+ [a]	+	+	+	+	+	–
Gizzard	C	–	–	+	–	–	–	–
Duodenum	C	+ [a]	–	+	–	–	–	–
	W	–	–	–	–	–	–	–
Mid small intestine	C	–	–	– [b]	–	– [b]	– [b]	– [b]
	W	–	–	–	–	–	–	–
Distal small intestine	C	–	–	–	–	–	–	–
	W	–	–	–	–	–	–	–
Caeca	C	–	–	–	–	–	+ [c]	–
	W	–	–	–	–	–	–	–
Rectum	C	–	–	+	–	–	–	–
	W	–	–	–	–	–	–	–

[a] All three inoculated eggs yielded virus.
[b] All three inoculated eggs contaminated by bacterial growth.
[c] Only one caecum yielded virus.

of the mid-part of the small intestine, in four of the birds, produced bacterial growth in all three of the eggs inoculated. The only abnormality seen in histological sections was the presence of nematodes in the duodenal wall of one bird.

These results prompted the question of whether the high recovery from crop contents was due to the different method of sampling: i.e. swabbing the surface rather than collecting material from the lumen. Therefore an experiment was carried out in three birds to see whether swabbing of mucosa gave a better recovery at other sites also. This time, the birds used were females of a commercial layer breed, 6 weeks old, each with a HI titre of 1 $\log_2$. The virus used was from the same batch as in the previous experiment, and dosing and handling were as before, with one bird killed 1 hour after dosing and two at 23 hours. Samples were taken from the same sites as before, including both proventriculus and gizzard, except that the caeca were sampled at the proximal ends near the tonsils. At each site, contents were gently wiped away and a cotton wool swab, pre-wetted in PBS, was rubbed over the mucosal surface, without enough pressure to cause visible damage. Each swab was then placed in 1 ml of PBS with antibiotics, and stored at −70°C until processing; this was carried out as for the samples of crop contents in experiment I. No samples of wall were taken.

Two of the birds, one killed at 1 hour and one at 23, yielded virus by this method from the crop and the proventriculus, all other sites being negative. No virus was recovered from the third bird.

A third experiment is now in progress to extend the time of observation, with birds killed from 5 hours to 4 days after dosing with V4. Not all the samples have yet been processed, but results so far obtained confirm the crop and the proventriculus as the sites from which virus is most frequently recovered, the latest recovery to date being from the crop contents 50 hours after dosing.

Discussion

More investigations will have to be carried out before conclusions can be drawn about the main site of invasion of V4. However, from these preliminary observations some statements can be made. V4 virus is capable of invading — or of adhering to very tenaciously — the walls of the crop and of the proventriculus. It can survive on the crop epithelium for 50 hours following administration. And it can reach the rectum from the crop within 3.5 hours.

It is likely that the poor recovery of virus from the intestines was due at least in part to the action of digestive enzymes. It is possible, too, that invasion of intestinal walls was taking place, but by numbers too low to be detected by our method. It is planned to perform immunocytochemical staining on the formalin-fixed tissues, in the hope that this may prove a more sensitive method of detecting virus. This method could also give information as to the exact location of the virus: whether adherent to or within epithelial cells, as observed by Cheville et al. (1972), or deeper in the wall, for example in lymphoid tissues.

It should not be surprising, however, if the crop and/or proventriculus do prove to be sites of primary invasion. V4 virus multiplies in the proventriculus, and has been recovered from it in chickens that have been infected by the intracerebral route (French et al. 1967), via the palatine cleft or by contact with infected birds (Hall et al. 1967). If infection does indeed occur via the upper digestive tract, then there should be less need to protect the vaccine from the effects of digestive juices, either by incorporating protective substances in the pellets or by trying to ensure that chickens are not fasted before being fed the vaccine.

Rates of recovery of the virus in the third experiment have been lower than in the earlier trials, and it is believed that this may be because, although the chickens were selected as having an HI titre of 2 logs, circulation of virus apparently occurred in the flock in the last week before they were removed for the trial, and the titres of all of them rose by one log. If this is indeed the reason, it sheds an interesting light on a possible mechanism of immunity: resistance to primary invasion of digestive tract epithelium, perhaps mediated by secretory IgA.

Further experiments will be needed to extend the scope of these observations: for example the duration of infection within the crop and proventriculus, the spread of the virus from these organs to sites of secondary replication, and the site of shedding into the gut lumen. It is also planned to repeat the trials using vaccine incorporated in feed rather than delivered to the crop, and to compare results in birds with no detectable antibodies to those in birds with 'protective' titres. The answers to all these questions should be important in planning the most efficient use of a feed vaccine, both for protection of vaccinated birds and for maximum spread of infection to contacts.

Acknowledgments

Epidemiology of the V4 Strain of Newcastle Disease Virus in a Free-Range Flock of Chickens

J.L. Samuel *

THE difficulties that stand in the way of vaccinating village, or 'backyard,' poultry against Newcastle Disease may be summed up as a problem of dispersal. That is, the vaccines that have been produced in the past are best suited for use in commercial poultry farms, where large numbers of birds of one age are kept together, conveniently for either rapid individual inoculation (by injection or eye-drop) or for mass-dosing (via drinking water or aerosol spray). Such products and such methods of application are not suited to a population that is scattered all over the country in small free-ranging flocks, into which new, susceptible birds are continually being introduced by hatchlings or purchases. Getting the traditional vaccines into all these birds in an effective form involves: first, keeping the vaccines cold while they are transported to the many remote areas where the birds are; second, catching and handling every bird; and third, repeating the whole exercise sufficiently often to ensure that every bird, including new hatchlings, gets at least two doses.

As described in other papers, the first of these problems has been addressed by the development of heat-resistant strains of virus, and second by the application of the virus to feed pellets, which are readily eaten by the birds. Both these developments have used the V4 strain of Newcastle Disease virus: an Australian, avirulent strain which has been shown to protect chickens against challenge with virulent Newcastle Disease and which spreads from bird to bird, apparently by the faecal-oral route (Westbury 1981). It is this latter property — of spreading between birds — which it is hoped may go some way towards solving the third problem: that of the need for frequent administration of vaccine to ensure that every bird is fully protected. Even with the relative ease of transport and administration offered by the heat-resistant, feed-incorporated vaccine, it is obvious that the less frequently the vaccine needs to be given, the cheaper the program will be. If, once introduced to a flock, the V4 virus were to continue circulating, then newly hatched chicks, or birds that had missed out on vaccination, for example through failing to get any of the feed, would become infected and thus acquire immunity. In this case it would be necessary to vaccinate only at widely-spaced intervals, perhaps annually. In addition, the vaccine virus might spread to wild birds, or to flocks belonging to owners who were not participating in the vaccination program, and thus the pool of susceptible birds, and the risk of epizootics of virulent disease, would be reduced.

In order to test the likelihood of this attractive possibility, we have established a small flock of free-range chickens, introduced V4 virus to some of the birds by the oral route, and have studied the development and duration of antibodies in the flock over more than 2 years.

Management of Experimental Flock

The experimental flock consists of bantam game chickens, kept under conditions which simulate, to some extent, the husbandry of village chickens in Malaysia. They are confined within a paddock, 30 m, open to the sky and with a number of trees growing in it. Until December 1986, the paddock also contained up to 10 sheep. Feed, consisting of commercial poultry pellets, is continuously available in a small shelter shed, which also houses water containers, roosts and nesting boxes. Most of the chickens retire into the shed at night, but some roost outside in the trees. Natural brooding and rearing of chicks is allowed every few months, and occurs mostly in the nest-boxes. Young chicks are provided with a crumble feed, but are not creep-fed. Apart from occasional treatment for ectoparasites there are no measures taken to control parasites or infectious diseases; however, with the exception of a single case of Marek's disease, most deaths have

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been due to injury or, in young chicks, starvation and possibly predation.

Methods for Serological Tests and Cloacal Swabbing

Blood samples for serology are taken from the wing vein. Tests for haemagglutination-inhibition (HI) antibody are carried out following the microtitre method of Allen and Cough (1974). Titres are expressed as logs to the base 2. Two control antisera, one with a known titre of 5 and one with no detectable HI antibodies, are run with each batch of sera tested. For each serum sample, a control test is performed without viral antigen in order to screen for natural agglutinins. These have proved to be present in a number of samples and prevent the reading of the HI test at low titres (they are diluted out after one or two doubling dilutions, so that high HI titres are not obscured). Absorption with washed red blood cells at 4°C for 30 min removes these haemagglutinins. Inactivation of serum samples at 56ºC for 30 min was tried for several weeks but this appeared to increase the number of samples with haemagglutinins; for example, in one batch of 21 samples, tested before and after heat treatment, the number with haemagglutinins was increased from 4 to 15 by the treatment. The practice of heat inactivation has therefore been discontinued.

Cloacal samples for culture are obtained with cotton-wool swabs, moistened before use with phosphate-buffered saline (PBS). Each swab is placed in 1 ml of PBS with antibiotics (penicillin, streptomycin, gentamycin) and stored at -70°C. For processing, a sample is shaken vigorously on a vortex mixer, then the swab is removed, solid material separated by centrifugation, and 0.2 ml of the liquid inoculated into the allantoic cavity of each of three 10-day embryonated eggs. Four days later the allantoic fluid is tested for haemagglutinating activity against washed chicken red blood cells.

Initial Introduction of V4 to the Flock

In October 1984, the original flock of 13 chickens (3 roosters and 10 hens) was given the V4 strain of Newcastle Disease virus in feed. The virus was diluted in palmolein (palm oil) and mixed with cooked rice, which the chickens ate readily. The dose per bird was not recorded. For the next 14 months there was no further administration of virus. No new birds were introduced to the flock either from outside or by hatchlings, until late December 1985, when a total of 65 chicks were hatched from three hens.

On 20 January 1986, blood samples were taken from all the birds and serum HI titres to Newcastle Disease virus (V4 strain) were determined. Titres of the 13 adult birds ranged from 1 to 3 logs (Geometric Mean Titre (GMT) 1.8) and of the 15 chicks from 0 to 2 (GMT 1 .0). Four of the chicks were immediately removed from the flock and reared in isolation. The GMT of these four birds remained higher than 1.0, with individual titres ranging from 1 to 3, for a further 10 weeks, after which it dropped below unity, with no individual greater than 1. This is a much longer period of persistence than would be expected if the titres had represented maternal antibody (Kim et al. 1978). Thus it seems that the chicks hatched in December 1985 had become infected with Newcastle Disease virus, which in turn suggests that the V4 virus introduced 14 months earlier had persisted within the flock of 13 birds. However, since the enclosure was open to access from wild birds, the possibility of a new introduction from this source cannot be ruled out. It is also possible that, rather than persisting in the chickens themselves, the virus had been maintained elsewhere in the environment, for example in soil or in invertebrates.

On 20 January 1986, immediately after the blood sampling and the removal of four of the chicks, 12 of the remaining birds (5 chicks and 7 adults) were dosed with V4 virus by stomach tube directly into the crop. The virus, which was derived from freeze-dried vaccine (Arthur Webster Pty. Ltd.), had been subjected to three cycles of selection for heat resistance at 56°C; it had a 50% infectivity titre in egg-bit culture of $10^{7.6}$/ml. Each bird received 1 ml of the allantoic fluid, followed by 1 ml of PBS to ensure that all the fluid was flushed through the stomach tube. The untreated birds (six chicks and six adults) were then allowed to mix with the vaccinated birds as before.

Cloacal swabs were obtained from all birds in the paddock on the second, fourth and sixth days after this inoculation, and weekly thereafter for 5 months, but no virus was recovered. Samples for serology were taken weekly until July 1986 and then at intervals of 2-4 weeks. By the 6th day after inoculation, all 12 of the treated birds showed a marked rise in HI titre, with a range from 5 to 9 and a GMT of 6.7. This initial rise was followed by a slight fall, down to 4.9, and then by a slower climb back to 6.8 by the fifth week. There was then a gradual fall, so that by 100 days the GMT was 4.5, with a range from 3 to 6.

The titres of the 12 in-contact, unvaccinated birds showed little change from 2 weeks, but at 3 weeks the GMT had risen from 1.5 to 2.1, with one bird having a titre of 5. The GMT reached a peak of 3.0 at 6 weeks, and the highest individual titre recorded was 7. Thereafter, however, titres fell, so that by 100 days the GMT was 0.5 and the highest titre 2. There seems little doubt, from this response, that

the virus had passed from the inoculated birds to the others in the flock. The most likely mode of spread was by faecal shedding, even though this was not detected in the cloacal swabs; probably the shedding was intermittent, and the amount of virus low. It also seems that circulation of the virus was responsible for the biphasic rise in antibody titre of the vaccinated birds, the initial exposure being closely followed by a second. It was of interest that for both the vaccinated and the in-contact birds, the GMT of the adults was consistently higher than that of the chicks. Again this suggests that the virus had been circulating in the original flock so that, at the time of vaccination, the adults had had more exposure than the chicks and therefore mounted a greater antibody response.

Acquisition of Infection by Later Hatchings of Chickens

Further clutches of chicks were hatched in early March, mid-April and August 1986. Clutch size, excluding early deaths, ranged from 6 to 10. Serum samples were taken from chicks regularly from the age of 2-4 weeks. Because of the hens' practice of shared brooding it was impossible to know the parentage of the chicks, but some of the mothers were birds with high titres. Four chicks that were artificially hatched and reared in isolation had a GMT of 1.5 at 3 weeks old; this fell to 0.3 by 5
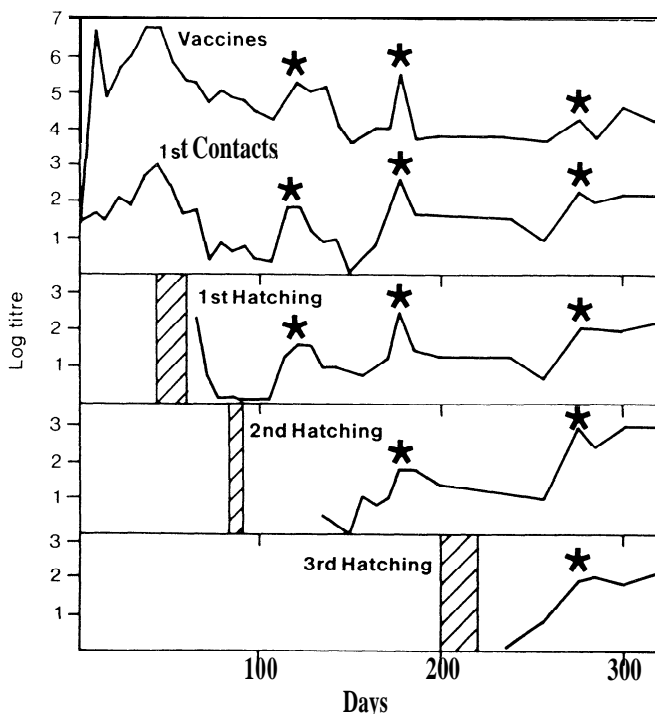
weeks and then to 0, and can be assumed to be maternal antibody 1

In each of the three clutches that hatched during 1986, a similar pattern was observed for HI antibody (Fig. 1). By 30 days, any maternal antibody had been lost and there were no titres higher than 1. Then at around 60 days titres rose, with individual titres going as high as 3 and the GMT peaking between 2 and 2.5. Apparently simultaneously the titres of the older birds in the flock rose also, the GMT of each group (vaccinees, in-contacts, previous clutches) rising by at least 1 log and sometimes by 2 or more. It seems that, as maternal antibody to V4 falls in a batch of chicks and they become susceptible, one or more of them acquires infection with the virus. In this naive host, it is assumed, the virus is rapidly multiplied and thus in a short time spreads to infect the entire flock.

In this way, the HI antibody titres of the birds, which otherwise show a tendency to fall gradually, are maintained. In the case of the group that was originally dosed with vaccine, this has meant that in over a year the GMT has never fallen lower than 3.7. One year (362 days) after vaccination, the GMT was 4.4, with a range from 3 to 7. The contact birds, both the original in-contact group and the three groups of later hatchings, had GMTs of 2.0 or 2.1, with range of only 2 to 3.

Implications for Vaccination Schedules

In spite of the fact that some of the initial in-contact birds showed high titres shortly after the administration of the vaccine, these titres quickly fell, and from the time of seroconversion of the March group of hatchlings, the original in-contact birds have had titres no higher than those of the younger adults in the flock. In this time, the GMTs have seldom risen above 2.5, and individual titres have generally been 3 or less. As 3 is regarded as the minimum titre that will ensure protection against most strains of virulent Newcastle Disease (Allan and Gough 1974a; Balla 1986), this means that only the birds that were vaccinated directly can confidently be regarded as protected. A titre of less than 3 does not necessarily indicate a susceptible bird, and it is possible that, after the several encounters that the birds have had with the V4 virus, they would in fact be able to withstand natural challenge with a viscerotropic velogenic strain: for example through the action of intestinal IgA. Unfortunately, however, it is impossible to test this by challenging the birds within Australia. It must be assumed, therefore, that these birds are not fully protected, and therefore that, in order to ensure protection of the whole flock, every bird would need to be vaccinated at least once with a high dose of the virus. Once the virus has been established in the

Fig. 1. HI titres (log$_2$) of bantams from day of crop-dosing with V4; also shown is seroconversion of chickens hatched at intervals after dosing of vaccinees. Cross-hatched areas show period of hatching of each batch of chicks.

flock, it should be necessary to vaccinate birds only once in order to obtain long-lasting protection, since the low level of antibody already acquired will ensure a prompt and marked rise in titre, as was seen in our crop-vaccinated birds.

It may be noted that these results are at variance with those obtained with commercial chickens (see Spradbrow, this volume), in which it was found that in-contact birds rapidly developed titres as high as those of vaccinated birds. The latter experiments, however, were carried out in birds crowded together on damp concrete which had no exposure to sun or wind, conditions which would favour the survival and spread of excreted virus. In the bantams' environment, on the other hand, there were few places with good protection from sun and wind (the trees being all Australian natives, and the shed open mesh on three sides), the soil was well-drained, and in addition the weather over the period of the study was unusually dry. In many of the Southeast Asian countries one might perhaps expect a situation somewhere between these two.

Summary

V4 virus, introduced in the feed to a free-range flock of 13 bantam chickens, persisted for 14 months although no new birds were introduced.

When V4 virus was introduced directly to the crops of chickens with low levels of antibody, HI titres rose and remained at protective levels for at least a year.

Chickens in contact with crop-vaccinated birds experienced a rise in HI antibodies but titres quickly dropped to less than protective levels.

Chickens hatched into a flock into which V4 had been introduced, seroconverted at about 8-9 weeks of age, apparently amplifying the virus and thus boosting the antibody levels of the entire flock.

Unvaccinated chickens in an infected free-range flock had antibody titres which were generally lower than the minimum recognised as indicating protection against virulent Newcastle Disease. Thus, to ensure protection of an infected flock, every bird should receive at least one dose of vaccine.

Production and Quality Control of Newcastle Disease Vaccine (V4 Strain) in Australia

P.D. Claxton and I. Leonard *

ARTHUR Webster Pty. Ltd. is a private Australian company that has been manufacturing a wide range of veterinary vaccines for nearly 60 years. The company has had a long association with the Poultry Industry both in Australia and Southeast Asia. A number of our products, including Newcastle Disease V4 vaccine, are available in Asia and the company currently produces about 85% of all vaccines used by the Australian poultry industry.

Our involvement in this present project commenced last year when we were asked if we would be interested, and had the capability, in producing heat-resistant NDV4 vaccine on a commercial scale. The company has for some years produced NDV4 lyophilised vaccine for commercial sale in Southeast Asia, and has held a reserve stock of at least 10 million doses of NDV4 vaccine for emergency use by the Australian poultry industry in the event of an outbreak of virulent Newcastle Disease in the country. Being satisfied that we had the capability to produce NDV4 vaccine we set out to select a -heat-resistant strain along the lines described by Latif (this volume). We have now achieved this and have successfully produced a heat-resistant Newcastle Disease V4 (HRNDV4) vaccine which is undergoing extensive testing to confirm its suitability as a vaccine. We are confident that we have the capability to produce HRNDV4 vaccine in the quantities that may be required as a result of this consultation, and the adoption of HRNDV4 for the vaccination of village flocks. The difficulties which need to be resolved relate to the method of presentation of such a vaccine (i.e. in bulk quantities, liquid or lyophilised) and the delivery system to the chickens. I am sure solutions will be found for both of these problems.

We have been asked to describe the production of NDV4 vaccine in Australia, and in particular to comment on the quality assurance and quality control measures adopted for this and all our poultry virus vaccines.

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Vaccine Production

In Australia, strict controls are supplied to the production of vaccines, particularly poultry viral vaccines. These controls ensure that each product that is sold to the public is registered and conforms to the standards that are laid down. Each vaccine must be manufactured in premises and by methods that conform to the Code of Good Manufacturing Practice. This code ensures that vaccines for use in animals (and people) are produced under clean-room conditions and by procedures that prevent cross-contamination, etc.

Successful commercial production of a viral vaccine requires a constant product. This can be achieved with virus vaccines by eliminating as many variables as possible. This is usually achieved by adopting the following practices: (a) genetically purify vaccine virus; (b) use of a seed-lot system; (c) use of standard media and procedures; and (d) ensure freedom of seeds and substrates from extraneous microorganisms.

Genetic Purification of Vaccine Virus

A candidate vaccine virus whether from a diseased bird or from an inapparent infection may be genetically heterogeneous. To produce a consistent vaccine the virus used should be genetically homogeneous and it is usual therefore to genetically purify the candidate virus. This can be achieved in one of two ways: by plaque purification, or limiting dilution. In each case the procedure is repeated with the virus being multiplied from the selected theoretical one infective virus unit. The subsequent pool of purified virus is then tested for bacteriological purity, safety and freedom from adventitious viral agents (Fig. 1).

Seed-Lot Systems

If the purified candidate virus satisfies all of these criteria, a seed-lot system is established. This seed-lot system permits production of a virtually unlimited amount of virus from the original isolate. In the case of NDV4 the seed-lot was established as follows:
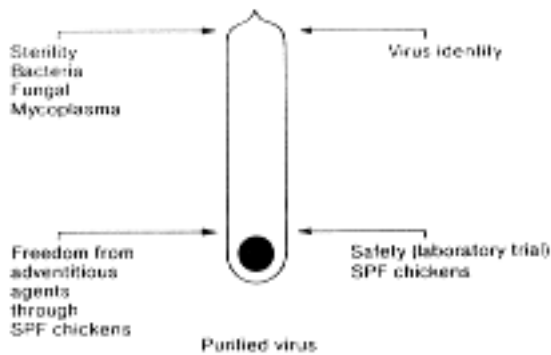
Fig. 1. Potential vaccine virus isolate.

(i) *Master Seed* — Prepared by inoculating purified virus into embryonated specific-pathogen-free (SPF) eggs, harvesting after 2 days, sterility testing, dispensing into ampoules which were stored at –70°C.

(ii) *Primary Seed-Lot* — Prepared by passaging Master Seed virus in SPF eggs, harvesting, testing and storing as before.

(iii) *Secondary Seed-Lot* — Prepared from primary seed-lot in SPF eggs and processing as before.

(iv) *Working Seed* — Prepared from secondary seed-lot in SPF eggs and processed as before. In addition it was tested for virus purity.

Standard Media and Procedures

Vaccine is prepared in SPF eggs from the working seed by a bulk and batch system which is tested for freedom from contaminants and virus titre at each stage.

All raw materials are certified to specification by Quality Assurance staff and and all sterility tests are validated. The production flow chart is presented in Fig. 2.

Freedom of Seeds and Substrates from Extraneous Microorganisms

SEEDS

As mentioned above, Master Seeds through Working Seeds are checked by detailed standard procedures for freedom from bacterial, mycotic and mycoplasmal contaminants.

SUBSTRATE

The substrate for NDV4 vaccine is embryonated SPF eggs derived from Webster's own SPF flock. All of Webster's poultry vaccines are grown on substrate derived from SPF eggs. The use of SPF substrate *ensures* that vaccines will be free of bacterial contaminants and all the known viral pathogens of poultry. Webster's SPF flock consists of 3000 SPF laying hens which are housed in a large self-contained building supplied with HEPA filtered air under positive pressure to exclude bacterial and viral agents. It is a totally closed flock with replacements being bred in the building, The unit is under permanent quarantine: all goods entering the building must be sterilised and personnel must shower in and out, wearing special clothing within the building,
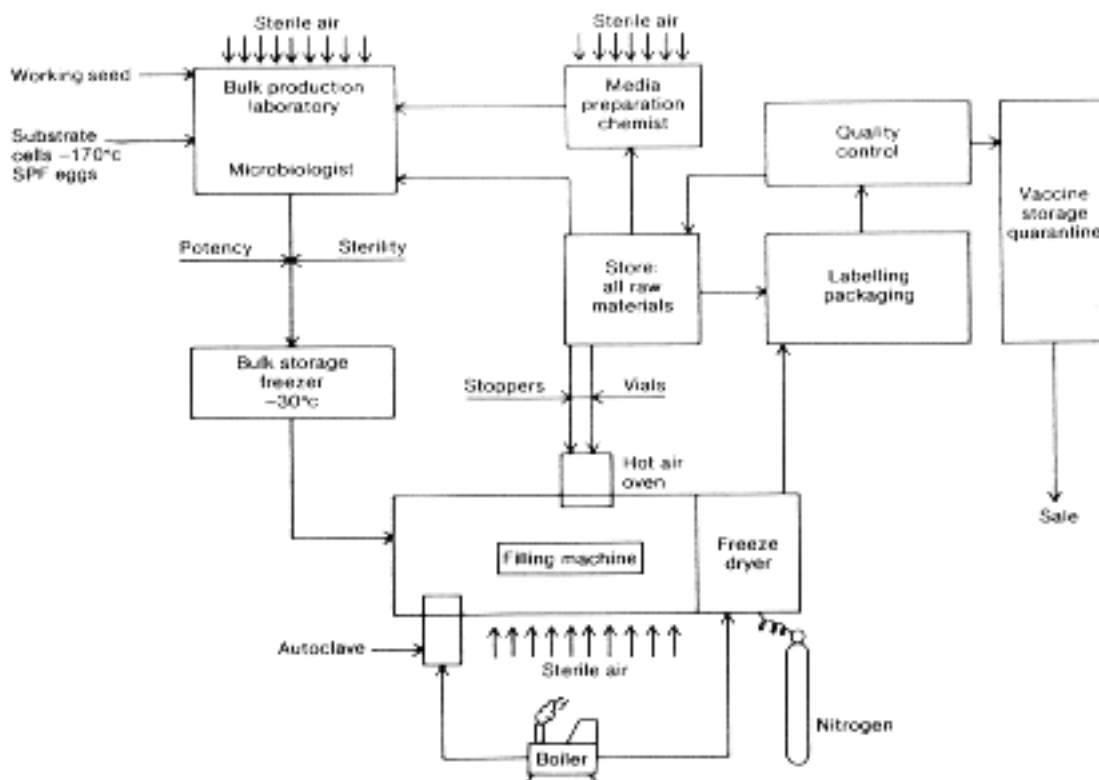

Fig. 2. Avian vaccine production.

Quality Control in Finished Product

Sterility Tests — Extensive sterility testing is carried out on each batch of finished vaccine. Approved methods are employed and the results are interpreted according to defined standards — ensuring a quality product. Tests are conducted to determine freedom from bacterial, mycotic and mycoplasmal contamination (Fig. 3).

Fig. 3. Quality control of avian vaccines.

TABLE 1. Specific-pathogen-free poultry flocks.

Agent	Test
Viral	
Avian encephalomyelitis virus	Embryo susceptibility
Adenovirus	Agar gel precipitin (AGP)
EDS76 virus	Haemagglutination
Newcastle Disease virus	HA
Reovirus	AGP, IFA
Marek's HVT virus	AGP
Marek's Disease virus	AGP
Infectious Bursal Disease virus	AGP, ELISA
Infectious Bronchitis virus	AGP, IFA, HA
Infectious Laryngotracheitis virus	Serum neutralisation
Reticuloendotheliosis virus	IFA
Avian Influenza virus	AGP
Haemorrhagic enteritis virus	AGP
Leucosis virus	COFAL, ELISA
Bacterial	
Mycoplasma gallisepticum	Agglutination
Mycoplasma synoviae	Agglutination
Pullorum Disease	Agglutination
Salmonella sp.	Isolation (feed, water, faeces)

Identity - In the case of NDV4 this test invo!ves inoculating IO-day-old chickens and embryos with vaccine neutralised with monospecific Newcastle Disease antiserum. There should be no evidence of infection; and birds vaccinated with unneutralised vaccine should develop specific antibodies.

Chick Inoculation Test

This test is designed to determine whether the vaccine is free from all other viral agents. SPF chickens must be used. These are inoculated with 10 doses of vaccine and maintained in an isolation unit. Fourteen days after vaccination three-quarters of the birds are reinoculated with the vaccine employing 10 doses of vaccine by five different routes. The birds are then carefully observed for 21 days and bled, the sera being tested for the diseases mentioned in Table 1.

Heat-Resistant NDV4

This vaccine has been produced by subjecting ampoules of NDV4 Master Seed to 56ºC for 6 hours rather than the 4 hours mentioned by Aini Ideris (this volume). Virus was harvested, passaged and treated three times in this way. The resultant virus preparation was used to prepare a HRNDV4 Master Seed, and HRNDV4 vaccine has been prepared from this master seed using the same procedure as for NDV4.

Thermostability of HRNDV4

We have demonstrated that the HRNDV4 is considerably more thermostable than NDV4. At 56°C HRNDV4 lost under 3 $\log_{10}$ of titre over 6 hours whereas NDV4 lost 7 $\log_{10}$ titre over the same period. At 50°C HRNDV4 lost under 1 $\log_{10}$ titre in 1 hour and less than 3 $\log_{10}$ after 6 hours. These results are consistent with those reported by Aini Ideris and Latif Ibrahim (this volume).

We believe, therefore, that we have a HRNDV4 vaccine with similar characteristics to the Malaysian vaccine. Spradbrow and Samuel (this volume) are now employing this vaccine in their work in Australia.

We are in a position to produce this vaccine on a large scale once testing is complete. We could supply, economically, bulk high-titre SPF-derived vaccine which could then be processed down or applied to pellets or other feed by a central laboratory in the recipient country. This would enable the recipient country to utilise the vaccine in the most appropriate way.

Such bulk vaccine would be supplied in effective refrigerated or dry ice containers and would certainly be available for field trials in each country later this year. We would welcome the opportunity to take part in the project in this way.