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Influenza Viruses: Transmission Between Species [and Discussion]

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Influenza viruses: transmission between species

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The only direct evidence for transmission of influenza viruses between species comes from studies on swine influenza viruses. Antigenically and genetically identical Hsw1N1 influenza viruses were isolated from pigs and man on the same farm in Wisconsin, U.S.A. The isolation of H3N2 influenza viruses from a wide range of lower animals and birds suggests that influenza viruses of man can spread to the lower orders. Under some conditions the H3N2 viruses can persist for a number of years in some species.

The isolation, from aquatic birds, of a large number of influenza A viruses that possess surface proteins antigenically similar to the viruses isolated from man, pigs and horses provides indirect evidence for inter-species transmission. There is now a considerable body of evidence which suggests that influenza viruses of lower animals and birds may play a role in the origin of some of the pandemic strains of influenza A viruses. There is no direct evidence that the influenza viruses in aquatic birds are transmitted to man, but they may serve as a genetic pool from which some genes may be introduced into humans by recombination. Preliminary evidence suggests that the molecular basis of host range and virulence may be related to the RNA segments coding for one of the polymerase proteins (P3) and for the nucleoprotein (NP).

Introduction

Influenza is a natural infection of man, pigs, horses, and a wide variety of domestic and wild birds. Influenza A viruses are found in each of these animals while influenza B and C viruses are confined to man. The question to be addressed here is whether influenza A viruses transmit between species and the potential significance of inter-species transmission. Influenza A viruses from lower animals and birds have been implicated in the origin of some of the pandemics of disease in man (Webster & Laver 1975; Scholtissek *et al.* 1978*b*) and outbreaks of disease in domestic avian species may originate from influenza viruses in feral (wild) species (Easterday 1975).

In man, pigs and horses, influenza takes the form of an acute respiratory infection with high morbidity and low mortality, while in domestic and wild birds, influenza may vary from an asymptomatic infection to a rapidly fatal disease with viraemia and central nervous signs of disease. In avian species one of the primary sites of viral infection is the intestinal tract (Webster *et al.* 1978).

Does inter-species transmission of influenza A viruses occur?

The simple answer is yes, but the amount of reliable documentation is still very limited. The available information suggests that transmission of influenza A viruses occurs frequently between avian species, less frequently between mammalian species and rarely between avian and mammalian species.

Transmission of influenza A viruses between mammals

Lower mammals to man

During 1976, Hsw1N1 viruses were isolated from military recruits at Fort Dix and subsequent studies showed that Hsw1N1 influenza viruses that were antigenetically and genetically indistinguishable were isolated from a man and a pig on the same farm in Wisconsin (Easterday, this symposium; Hinshaw *et al.* 1978*a*). These studies confirmed the earlier serological and virus isolation studies of Schnurrenberger *et al.* (1970) and Smith *et al.* (1976), that implicated Hsw1N1 viruses in human disease. Serological studies on slaughterhouse workers indicate that transmission of swine influenza viruses to man occurs quite frequently (up to 20% of workers in 1977 had Hsw1N1 antibodies) but in the recent past none of these incidents have resulted in an epidemic of disease in man.

Man to lower mammals

The isolation of Hong Kong H3N2 influenza viruses from pigs in Taiwan (Kundin 1970) and subsequent serological and virus isolation studies initiated by the World Health Organization has shown that each of the variants of Hong Kong influenza virus (e.g. England/42/72, Port Chalmers/1/73 and Victoria/3/75) have spread to pigs in all countries of the world. In pigs these viruses cause no overt signs of disease and until recently there was no evidence that they persisted in them. Recent studies, however, indicate that viruses similar to the A/Hong Kong/1/68 (H3N2) strain that are no longer isolated from man can still be isolated from pigs in Hong Kong (Shortridge *et al.* 1977).

The A/Hong Kong/68 (H3N2) influenza virus has also been isolated from dogs, cats, monkeys, gibbons, baboons and cattle (Easterday 1975; Fatkhuddinova *et al.* 1973). In these species the viruses usually cause no signs of disease, but in the U.S.S.R. the viruses have been reported to cause mortality in calves.

Between avian species

There is no doubt that avian influenza viruses can transmit between species: fowl plague virus (Hav1Neq1) can transmit from chickens to turkeys and kill them, but ducks are not affected.

Migrating wild birds are probably responsible for the spread of avian influenza viruses in the world, and they may also act as a source of the viruses that give rise to the sporadic outbreaks of influenza in domestic poultry. Thus an influenza virus Hav6Nav5, isolated from shearwaters (*Puffinus pacificus*) on the Great Barrier Reef off Australia (Downie & Laver 1973) has been isolated from domestic turkeys in California (where it caused overt respiratory disease) (Hinshaw *et al.* 1978*b*) from feral black ducks in Delaware and from domestic chickens in Hong Kong (K. F. Shortridge, personal communication). A similar example is that outbreaks of influenza in domestic turkeys in North Central United States usually coincide with the migration of Canadian wild ducks, and antigenically similar viruses have been isolated from both species. The evidence that these outbreaks were caused by the same virus is circumstantial and based only on antigenic similarity of viral surface proteins. In the present age of molecular epidemiology this is not sufficient evidence to conclude that the viruses are the same. To date, an identical influenza A virus has not been isolated from a wild and domestic bird in the same farmyard.

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One of the problems in establishing relations between the influenza viruses from avian species is the heterogeneity of their viral RNAs. Thus two antigenically identical viruses can show differences in their RNA migration rates in polyacrylamide gels and in their hybridization patterns. This will be dealt with in more detail later.

Transmission of influenza A viruses between avian and mammalian species

The only reported case of human infection with an avian influenza strain is with fowl plague virus (Campbell *et al.* 1970), but even this case is suspect because the patient failed to seroconvert. However, some avian viruses do have the potential to cause disease in mammals as A/tern/South Africa/61 (Hav5Nav2) and A/Turkey/England/63 (Hav1Nav3) are virulent for laboratory mice and hamsters (Uys & Becker 1967). In addition, the H3N2 viruses of man have been isolated from domestic chickens and from sea birds (Easterday 1975).

The role of influenza A viruses from lower animals and birds in the origin of new human pandemic strains

There is a considerable body of evidence to show that the H2N2 and H3N2 strains of human influenza virus may have acquired some of their genes from avian strains (Scholtissek, this symposium; Laver & Webster 1979).

It is not the purpose of this report to review this information but to stress that although the evidence is persuasive, it is circumstantial.

Gene requirement for inter-species transmission

Having established that inter-species transmission does occur, we should like to know whether there is any change in the genes or gene products of the virus that permits this to occur. Recent in-vitro studies suggest that the host range of fowl plague viruses is determined by a single gene (Almond 1977). The ability of recombinants between two strains of fowl plague virus to produce plaques in different cell cultures was associated with the gene coding for one of the polymerase-associated proteins, P3. Other studies have shown that the haemagglutinin glycoprotein molecule must be cleaved into two subunits for optimal infectivity and plaque production by the virus (Lazarowitz & Choppin 1975; Klenk *et al.* 1975).

Studies on the Hsw1N1 influenza viruses isolated from pigs and man on the same farm in Wisconsin showed that the RNA migration patterns were identical in polyacrylamide gels and that immunologically the viruses were indistinguishable (Hinshaw *et al.* 1978*a*). Other Hsw1N1 isolates from the same region were heterogeneous in their RNA migration patterns.

In the past 2 years, influenza A viruses antigenically similar to the Hsw1N1 influenza virus from man and pigs have been isolated from feral and domestic avian species (Hinshaw *et al.* 1978*c*). Since these viruses were antigenically very similar to the recent Hsw1N1 viruses from man and pigs they were inoculated intranasally into pigs to determine whether they would replicate in this species and their RNAs were studied during adaptation (table 1). On the initial passage in pigs the avian Hsw1N1 virus was isolated for only 1 day but by the third passage the virus was isolated from 2 to 5 days inclusive and by the sixth passage the virus was isolated both from the nasal passage and from the lungs. Analysis of the migration patterns of the RNAs on polyacrylamide gels showed no differences between the viruses.

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These studies are preliminary and hybridization studies are required to determine if differences can be detected. The studies do show that an avian Hsw1N1 influenza virus can be adapted to a mammalian host. From the above studies it is clear that the gene requirement for inter-species transmission has not been established, and future studies with the use of molecular hybridization and nucleotide sequencing are needed to determine which genes are involved.

TABLE 1. ADAPTATION OF A/DUCK/ALBERTA/35/76 (HSW1N1) TO PIGS

passage number in pigs	virus isolation from: nasal passage (days)	virus isolation from: lungs (day 5)	differences in RNA migration
1	1	0	0
2	1, 2	0	0
3	2, 3, 4, 5	0	0
4	2, 3, 4	0	0
5	2, 3, 4	0	0
6	2	+	0

Pigs (5 weeks old) from a farm with no serological or virological evidence of influenza virus infection were inoculated intranasally with approximately $10^{7.0}$ e.i.d. of cloned virus. The virus was isolated from nasal swabs in fertile hens' eggs and infectious allantoic fluid was inoculated into the next pig. RNA analysis was done according to Bean & Webster (1978).

+, Lung consolidation on day 3.

HETEROGENEITY OF THE RNAS OF INFLUENZA VIRUSES

There is an increasing body of evidence which suggests that influenza viruses are heterogeneous. Antigenically distinct subpopulations of influenza viruses have been isolated from individual samples (Kendal *et al.* 1977) and genetic dimorphism has been described in Hsw1N1 influenza viruses (Kilbourne 1978). As mentioned above, our studies on the RNAs of most of the Hsw1N1 influenza viruses isolated from man and pigs in 1976/7 showed differences in the migration patterns of their RNAs in polyacrylamide gels.

To determine if heterogeneity of RNA is a general phenomenon among influenza A viruses, a number of avian isolates were studied. The viruses were analysed by polyacrylamide gel electrophoresis and competitive hybridization techniques to determine if influenza A viruses of a given antigenic subtype were homogeneous or heterogeneous in their RNAs. Haemagglutination inhibition assays established the antigenic identity of a group of Hav7Neq2 influenza viruses isolated from ducks from 1963 to 1977. Formamide acrylamide gels showed differences in the RNA migration patterns of these viruses and variations occurred in all of the segments.

RNA–RNA hybridization analysis confirmed the differences between the RNAs of the Hav7Neq2 influenza viruses (table 2) and hybridization with individual RNA segments suggested that genetic variations occur throughout the genome of influenza viruses.

In the above studies, individual isolates of Hav7Neq2 influenza viruses from different Canadian wild ducks were heterogeneous in their RNAs. A possible mechanism for the development of heterogeneity will be discussed below. The results do suggest that it is very unlikely that the A/USSR/90/77 (H1N1) influenza virus that has been shown to be antigenically and genetically similar to viruses isolated from man in 1950 (Nakajima *et al.* 1978; Scholtissek *et al.* 1978*a*; Kendal *et al.* 1978) has been sequestered in an animal population.

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TABLE 2. A COMPARISON OF GENETIC RELATEDNESS AMONG HAV7NEQ2 AVIAN INFLUENZA VIRUSES BY COMPETITIVE HYBRIDIZATION

	percentage RNase resistance with competing RNA from			
competing (RNA/μg)	Alb/88/76 (homologous)	Alb/78/76	A'b/75/76	Ukr/1/63
0.01	72	82	83	90
0.05	51	63	73	89
0.10	29	67	57	78
0.50	13	43	63	70
1.00	10	37	37	74

Tritium labelled viral RNA from A/duck/Alb/88/76 was annealed with an excess of homologous complementary RNA in the presence of varying amounts of unlabelled competing viral RNA from either the same virus or another strain. The efficiency with which the unlabelled RNA competes with the labelled RNA for the complementary sequences is a measure of their relatedness. Hybridization was in 0.35 M NaCl, 0.035 M sodium citrate, 50% formamide at 75 °C (7 °C below the melting temperature). Under these stringent conditions, only very closely related sequences will compete. Labelled viral RNA and complementary RNA were obtained from infected Madin–Darby canine kidney cells.

PROLONGED SHEDDING OF INFLUENZA A VIRUSES IN AVIAN SPECIES

Previous studies have established that influenza A viruses are widespread in water fowl, and representatives of most of the mammalian strains have been isolated from feral ducks (Hinshaw *et al.* 1978*c*). The viruses from feral ducks replicate in the cells of the respiratory tract and of the intestinal tract and are shed in high concentration in the faeces (Webster *et al.* 1978). In their natural hosts, the viruses cause no signs of disease and are spread through the water to juvenile birds when the birds congregate before migration. To determine the period of virus shedding, young ducks were infected with A/duck/Alberta/35/76 (Hsw1N1) and faecal samples were collected and assayed for virus (table 3). All of the ducks shed virus from the 7th to the 14th day and 20% of the ducks continued shedding virus for 30 days. The prolonged shedding of influenza viruses in faecal material may explain how these viruses are maintained in nature.

TABLE 3. PROLONGED EXCRETION OF INFLUENZA VIRUS IN DUCK FAECES

		isolation of influenza virus from faecal samples	
virus strain	days after infection	percentage positive	infectivity titre ($\log_{10}$/ml)
A/duck/Alberta/35/76 (Hsw1N1)	1	0	
	2	50	
	3	85	
	4–13	100	6.30
	14	100	4.30
	15	80	
	16	80	
	21–28	80	3.96
	29	20	
	30	20	2.50
	31	0	

Pekin white ducks 1 day old were infected intratracheally with approximately 10^7 e.i.d.$_{50}$ of A/duck/Alberta/35/76 (Hsw1N1). Cloacal samples were collected daily and assayed for virus in embryonated hens' eggs.

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Possible explanations for the heterogeneity of the RNAs of avian influenza viruses

Since influenza viruses are highly variable, it is possible that heterogeneity of the RNAs might develop during the multiple rounds of multiplication required to maintain virus shedding for 30 days. To test this possibility, viruses isolated on the 10th, 21st and 30th days of shedding were cloned twice and the mobility of the RNAs examined by polyacrylamide gel electrophoresis. The mobilities of the RNAs of the original and excreted viruses were identical, suggesting that the viruses were homogeneous.

An alternative possibility to explain RNA heterogeneity in avian influenza viruses is that it might arise by genetic reassortment after multiple infection with different subtypes. To test this possibility, juvenile ducks that were shedding A/duck/Alberta/35/76 (Hsw1N1) were inoculated with a second virus, A/duck/Alberta/76 (Hav1Nav2) (table 4). Faecal samples were collected from the doubly infected ducks and viruses were isolated at limit dilution without

Table 4. Recombination between influenza A viruses in the intestinal tract of ducks

virus	days after infection	viruses isolated from faeces at limit dilution† Hsw1N1	Hav1Nav2	Hav1N1
Duck/Alberta/35/76 (Hsw1N1)	1	0		
	2	+		
	3–6	+		
Duck/Alberta/48/76 (Hav1Nav2)	7	+		
	8	+	+	
	9–17	+	+	+†
	18	+	+	+†

Juvenile Mallard ducks (*Anas platyrhynchos*) from 4 to 6 months of age were inoculated orally with approximately 10^7 e.i.d.$_{50}$ of Hsw1N2 and Hav1Nav2 influenza viruses on the days indicated. Faecal material was collected daily from the ducks and inoculated into embryonated hens' eggs at limit dilutions. The virus isolates were cloned twice and the surface antigens identified with specific antisera in serological tests (Webster & Campbell 1974).

† No selection for isolation of recombinants.

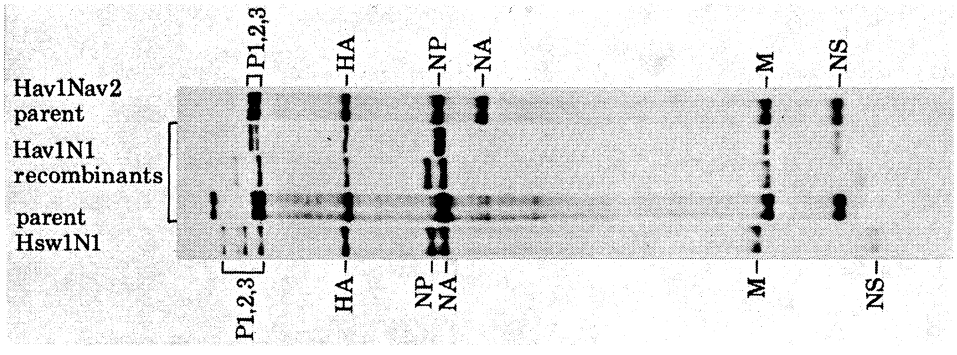

Figure 1. Electrophoretic analysis of recombinants obtained from duck intestines. Mallard ducks were infected with two avian influenza isolates, A/duck/Alb/35/76 (Hsw1N1) and A/duck/Alb/48/76 (Hav1Nav2), as shown in table 4. After recloning, RNA was extracted from purified egg-grown virus, labelled with ^{125}I and the genome RNA segments resolved by polyacrylamide gel electrophoresis (Hinshaw *et al.* 1978*a*). the slab gel (0.15 cm × 14 cm × 22 cm) contained 7 M urea, 3% acrylamide (30 g/l), *bis*-acrylylcystamine (2.5 g/l), TEMED (0.67 g/l), and ammonium persulphate (1 g/l) in 0.1 × Loening's buffer (Floyd *et al.* 1974). Electrophoresis was for 16 h at 0 °C with a constant voltage of 300 V.

any selection. Antigenic hybrid viruses (Hav1N1) were isolated from the faecal material, showing that genetic reassortment can occur readily between avian strains that replicate in the intestinal tract. The cloned parental and hybrid viruses were examined by polyacrylamide gel electrophoresis (figure 1) and showed that the antigenic hybrid viruses had acquired genes from both parents. In addition, viruses possessing surface antigens of one strain had acquired RNA segments from the other parent.

The second possibility would certainly explain RNA heterogeneity among avian strains, but whether genetic reassortment occurs so easily in nature remains to be established. Two or more different subtypes of influenza A viruses have been isolated from the same feral duck in Canada (V. S. Hinshaw, unpublished data) suggesting that conditions for genetic interaction do occur in Nature. Whether the same explanation would apply to heterogeneity among mammalian strains of influenza viruses remains to be answered. The detection of antigenic subpopulations and the description of genetic dimorphism among mammalian Hsw1N1 influenza viruses (Kendal *et al.* 1977; Kilbourne 1978) suggest that conditions exist for genetic exchange between these subpopulations.

Discussion and conclusions

From the above, it is apparent that influenza A viruses can transmit between species. Most of the evidence for inter-species transmission is based on epidemiological and antigenic studies, as the opportunities for coincidental isolation of influenza viruses from feral and domestic species of animals are limited. The genetic basis for inter-species transmission has not been fully elucidated; cleavage of the haemagglutinin molecule is important for infectivity and possession of the gene segment coding for one of the polymerase proteins is important in some strains. Heterogeneity of the viral RNAs is common in both mammalian and avian influenza A viruses, making it difficult to ascertain whether the same virus comes from different species. The studies reported above provide a possible explanation for RNA heterogeneity in avian strains, which could occur by genetic reassortment after mixed infection with different subpopulations of virus.

The isolation of Hsw1N1 influenza viruses from feral avian species that have the potential to infect pigs offers a possible explanation for the origin of some outbreaks of disease in pigs. If Hsw1N1 influenza viruses from avian sources can transmit to domestic pigs in Nature it may not be necessary to invoke either the persistence of the virus in the herd, or the lung worm–earthworm cycle to explain outbreaks of the disease in pigs each autumn. The same argument can be used to explain the yearly outbreaks of influenza in domestic poultry in North America. These outbreaks coincide with the onset of winter and the time of migration of feral ducks. The evidence that there is any relation between these events is at present completely circumstantial. It is, however, apparent that influenza viruses in feral avian species cause no overt disease. The influenza virus and the duck may have coexisted for many millions of years, and it is possible that influenza viruses of man, pigs and horses all originate from avian species.

The circumstantial evidence that the H2N2 and H3N2 influenza viruses of man may have acquired some of their genes from avian species is quite strong (Scholtissek 1978; Laver & Webster 1979) but the cyclic reappearance of influenza virus in man suggests that there may be more than one mechanism to explain the origin of human strains. It is quite apparent that there are many unanswered questions in the epidemiology of influenza viruses but the application of the molecular biological tools that are currently available should provide the necessary answers.

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Discussion

E. D. Kilbourne (*Mount Sinai School of Medicine, New York, U.S.A.*). Earlier in this meeting, I speculated that in view of the constraints placed on replication by the haemagglutinin itself, the list of candidate 'pandemic' antigens in Nature may diminish as we learn more about their structure and biology. This speculation was strengthened by the data presented by Professor Rott, suggesting a need for an appropriate gene constellation as well as a proteolytically cleavable haemagglutinin in determining the pathogenicity of avian influenza viruses. Therefore, even should a putative human influenza virus – avian influenza virus recombinant derive all but the glycoprotein genes from the human parent, it may still be incapable of infecting man as a potential pandemic virus. Studies are needed of the capacity of viruses bearing avian haemagglutinins to infect human cells.

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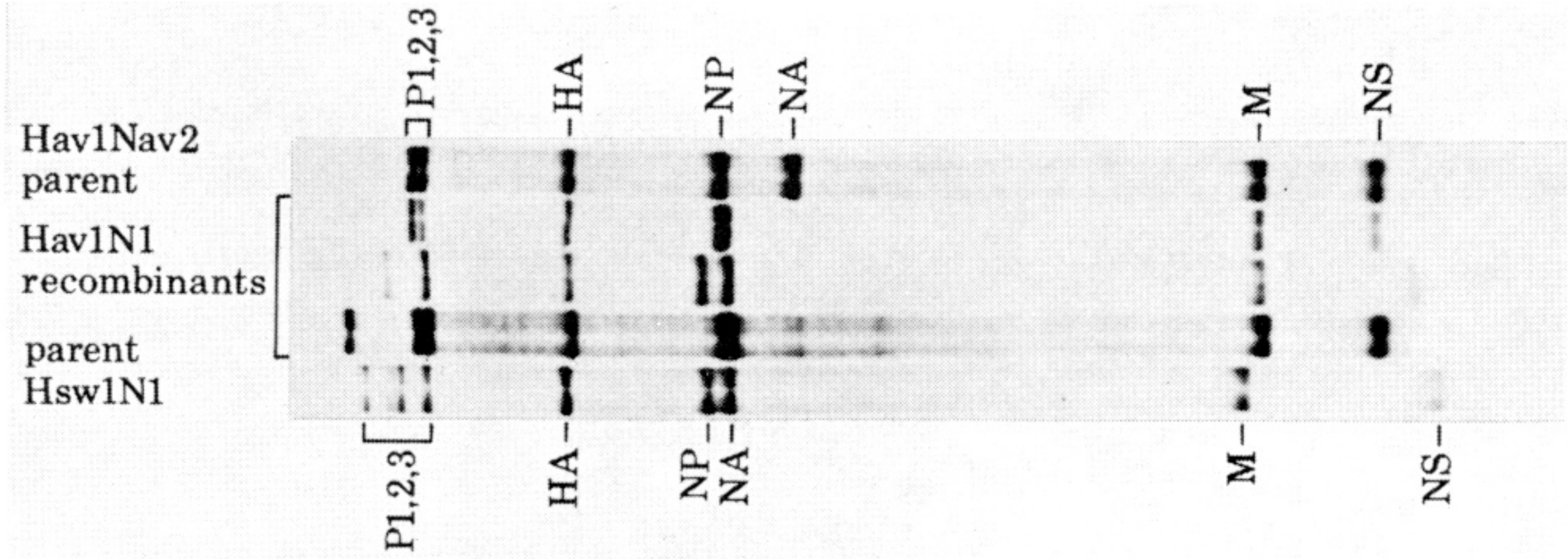

FIGURE 1. Electrophoretic analysis of recombinants obtained from duck intestines. Mallard ducks were infected with two avian influenza isolates, A/duck/Alb/35/76 (Hsw1N1) and A/duck/Alb/48/76 (Hav1Nav2), as shown in table 4. After recloning, RNA was extracted from purified egg-grown virus, labelled with ^{125}I and the genome RNA segments resolved by polyacrylamide gel electrophoresis (Hinshaw *et al.* 1978*a*). the slab gel (0.15 cm × 14 cm × 22 cm) contained 7 M urea, 3% acrylamide (30 g/l), *bis*-acrylylcystamine (2.5 g/l), TEMED (0.67 g/l), and ammonium persulphate (1 g/l) in 0.1 × Loening's buffer (Floyd *et al.* 1974). Electrophoresis was for 16 h at 0 °C with a constant voltage of 300 V.