Influenza Viruses in Birds: Rapid Identification by Counterimmunoelectrophoresis

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Counterimmunoelectrophoresis with an antiserum raised in rabbits against the M protein of the avian N virus proved to be particularly useful for large-scale identification of influenza A virus isolates. Of a total of 231 hemagglutinating agents isolated from 1,656 rectal swabs collected from shore and open-country birds, 158 could be identified as influenza A viruses by counterimmunoelectrophoresis, and 75 were serologically related to Newcastle disease virus by hemagglutination inhibition with an antiserum to Newcastle disease virus. Two isolates contained a mixture of influenza A virus and Newcastle disease virus; although the Newcastle disease virus particles outnumbered the influenza A virus particles in a ratio of 1,000:1, as seen by electron microscopy, the latter could be readily detected by counterimmunoelectrophoresis. This type of assay appears to be of potential use for epidemiological surveillance of influenza virus isolated from humans and animals. It combines specificity, sensitivity, and simplicity.

Since surveillance of influenza in birds or other animals involves the processing of many samples for virological examination and several different groups of viruses may be found in the avian species, there is a need to develop an assay which combines specificity, sensitivity, and rapidity for the preliminary identification of influenza A viruses. These may be identified with antisera specific to the nucleoprotein or the membrane (M) protein. In the past, identification as to type has been undertaken by complement fixation tests with anti-nucleoprotein sera (5). More recently, Beard and Helfer (1) have used double immunodiffusion for nucleoprotein antigen, whereas Dowdle et al. (3) have recommended the double immunodiffusion test for both the nucleoprotein and M-protein. Although double immunodiffusion is simple and sensitive, it requires acid precipitation of infected allantoic fluid for antigen preparation and several hours for the immunoprecipitation reaction to develop.

In the present communication we describe a counterimmunoelectrophoresis (CIE) assay for the preliminary identification of influenza A viruses isolated from rectal swabs collected from shore and open-country birds. An antiserum to the protein of an influenza avian N virus was used to distinguish influenza A viruses from paramyxoviruses. The latter were further identified by hemagglutination inhibition (HI) with Newcastle disease virus (NDV) and Yucaipa virus antisera. It is concluded that CIE offers a simple, specific, and sensitive method for the

detection of M-protein antigen in isolates from birds. The technique could potentially be expanded by testing for nucleoprotein antibodies in large serological surveys.

MATERIALS AND METHODS

Collection of specimens. During summer 1977, groups of biologists from the Canadian federal and provincial wildlife services, the Société de développement de la Baie James, and Trois-Rivières and Montreal Universities collected 1,656 rectal swabs from 78 bird species for myxoviruses isolation. These swabs were collected from birds trapped or netted in the provinces of Ontario, Québec, Nova Scotia, New Brunswick, and Prince Edward Island. Each swab was placed in 1 ml of transport medium consisting of 50% glycerol in phosphate buffer, pH 7.2. The specimens were kept at 4°C or on dry ice and sent to the laboratory within 1 week. Upon arrival they were immediately divided into two portions and stored at -70°C; one was used for virus isolation, and the other was kept for future reference.

Virus isolation. One portion of each sample, chosen at random, was thawed, and 0.2-ml volumes were inoculated into the allantoic cavity of three 11-day-old embryonated hen eggs. Gentamicin (5 µg) and fungizone (5 µg) were added to the inoculum. The eggs were incubated at 35°C for 48 h. The allantoic fluids were tested for hemagglutination activity with chicken erythrocytes. A second passage in eggs was performed in the same manner. All positive samples from this second passage were stored at -70°C for identification.

Antisera. Antisera to the paramyxoviruses NDV strain 171 (inactivated) and Yucaipa virus were prepared in roosters by intravenously injecting 1 ml of infected allantoic fluid, containing 2,000 to 4,000 hem-

agglutinating units of the respective viruses, and exsanguinating after 10 days. These antisera, as assessed by HI gave a titer of 1:160 with the homologous virus but did not cross-react with the heterologous paramyxovirus or influenza A and B viruses.

Monospecific antisera to the M-protein of the A/chicken/Germany/N/49 (Hav₂Neq₁) influenza virus were prepared in rabbits with the M-protein isolated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Purified virus was disrupted with 1% sodium dodecyl sulfate at room temperature for 30 min and subjected to electrophoresis as described previously (4) on 7.5% polyacrylamide gels (0.5 by 10 cm). The protein band, with an apparent molecular weight of 28,000, was eluted in 0.15 M NaCl overnight at 4°C and dialyzed for 1 week against several changes of saline. The resulting preparation contained 160 µg of protein per ml. Rabbits were immunized by three intradermal injections, each containing 20 µg of M-protein together with Freund complete adjuvant, at multiple sites on the back and sides at 2-week intervals. Rabbits were exsanguinated 10 days after the last injection.

CIE. CIE was performed on glass plates (7 by 10 cm) covered with a 2-mm layer of 1% Noble agar (Difco) in 0.025 M barbital buffer, pH 8.6. All isolates positive for hemagglutination were treated with 0.1% (final concentration) Sarkosyl NL-97 (Ciba-Geigy Corp.) and heated for 90 s at 100°C. The M-protein of influenza A viruses is remarkably heat stable and retains its antigenic activity (6). Two sets of double rows of 12 wells (2.5 mm) were punched in the agar gel, and 3 µl of each isolate was placed in the cathodally oriented wells. The anti-M-protein serum, diluted 1:4, was placed in the anodally oriented wells. A potential of 8 V/cm was applied across the gel for 1.5 h. After electrophoresis, plates were pressed, dried, and stained with Coomassie brilliant blue R250 as described previously (10).

Serological tests. The hemagglutination activity titrations and HI assays were performed by standard procedures (7). All antisera for the HI assays were treated with receptor-destroying enzyme by conventional methods (7).

Immunodiffusion tests were done in 1% Seakem standard agarose dissolved in a solution containing 0.035 M barbital, 0.050 M glycine, 0.075 M tris(hydroxymethyl)aminomethane, and 0.003 M sodium azide, pH 8.6. Precipitin lines were stained with Crocein scarlet and Coomassie brilliant blue as described previously (2).

RESULTS

Isolation of hemagglutinating agents from avian species. Of 1,656 rectal swabs examined, a total of 231 hemagglutinating agents were isolated at the first (94) or second passage (137) in chicken embryos (Table 1). Of the 78 bird species examined, 24 yielded a hemagglutinating agent. Ducks provided the greatest number of isolates. A detailed distribution of the species yielding hemagglutinating agents with their respective regional location will be re-

TABLE 1. *Number of hemagglutinating agents isolated from avian species*

Parameter	No.	% Recovery
Specimens examined	1,656	
Bird species examined	78	
Hemagglutinating agents isolated at:		
First passage	94	5.6
Second passage	137	8.2
Isolates from shore birds ^a	210	12.6
Isolates from open country birds ^b	21	1.2

^a Shore birds: wood and black ducks, mallards, teals, and Canada geese.

^b Open-country birds: thrushes, warblers, finches, sparrows, and flycatchers.

ported elsewhere (Boudreault et al., manuscript in preparation).

Identification of influenza A viruses by CIE. Because the M-protein of influenza viruses is type specific (8), preliminary identification of the hemagglutinating agents as influenza viruses was performed with an antiserum to the M-protein by CIE. The specificity of the antiserum to M-protein raised in a rabbit against the avian N virus is shown in Figure 1. In double immunodiffusion, one line of precipitate was obtained, which showed a reaction of identity with all influenza A viruses tested, whether of human, avian, or animal origin, but not with an influenza B virus. A similar reaction pattern was obtained with the same influenza viruses heated at 100°C for 90 s (data not shown).

All of the avian hemagglutinating agents isolated in chicken embryos at the first or second passage were then reacted against the antiserum to M-protein in CIE. Of the 231 avian isolates, 158 could be identified as influenza A viruses. A typical reaction pattern is shown in Fig. 2. All of the isolates were detergent disrupted and heated at 100°C for 90 s. No lines of precipitation were obtained with uninfected allantoic fluid, NDV, and the influenza B virus used as negative controls. A clear line of precipitation was obtained with the human A/USSR virus used as a positive control. The intensity of the lines of precipitation for the avian isolates were related to the amount of M-protein. Upon dilution of the A/USSR virus positive control a line of precipitation of decreased intensity and in a slightly more cathodic position was observed.

No reactions could be detected when the original swab suspensions were subjected to electrophoresis against the same M-protein antiserum.

HI assays with paramyxovirus antisera. HI assays were then performed on all of the hemagglutinating isolates with antisera to NDV

FIG. 1. Double immunodiffusion test showing reactions of "N" virus [A/chicken/Germany/"N"/49 (Hav₂Neq₁)], PR8 [A/PR/8/34 (HON1)], Duck [A/Duck/BV1/56 (Hav₂N?)], Sw [A/Sw/15/31 (Hsw1N1)], Vic [A/Victoria/(X-47)/75 (H3N2)], and B/H.K [B/Hong Kong/8/73] with an antiserum to M-protein to the "N" virus. These purified viruses were disrupted with 1% Sarcosyl NL-97.

strain 171 and Yucaipa virus. A total of 75 of the isolates were serologically related to NDV, and none were inhibited with the Yucaipa antiserum. Of these 75, 73 were negative by CIE (Table 2). Two of these isolates also reacted with the anti M-protein for influenza A virus.

Evidence for mixed populations of influenza A viruses and paramyxoviruses. On the basis of the specificity of the CIE as shown in the above studies, it was suspected that the two isolates of doubtful classification could be a mixture of influenza and paramyxoviruses. These were further investigated serologically and by electron microscopic examination. The undiluted isolates were absorbed with an excess of NDV antiserum at 20°C for 1 h and overnight at 4°C and tested for residual hemagglutinating activity. A reciprocal hemagglutination activity titer of 8 was found for both isolates.

When examined by electron microscopy, the paramyxoviruses could be distinguished from orthomyxoviruses by the characteristic morphology of their nucleocapsids (Fig. 3). The paramyxoviruses and orthomyxoviruses were present in a ratio of a 1,000:1 in both isolates. This provides additional evidence for the sensitivity and specificity of the CIE assay for the influenza A M-protein.

Examination by electron microscopy and correlation with CIE. A total of 40 isolates which were positive for influenza A viruses by CIE were also examined by electron microscopy. There was complete agreement between electron microscopy and CIE for influenza viruses.

DISCUSSION

These studies have shown that CIE of detergent-disrupted viruses with an antiserum to M-protein of influenza A virus is a satisfactory and simple assay for preliminary identification of influenza virus isolates from birds. The antiserum to M-protein cross-reacted with influenza A viruses of different subtypes and of different human, animal, or avian origin, but not with influenza B virus, in double immunodiffusion. In CIE, it did not react with uninfected allantoic fluid, NDV, and other paramyxoviruses. It was thus found suitable for large-scale identification of influenza A viruses among hemagglutinating agents isolated from birds.

Of 231 hemagglutinating agents isolated in rectal swabs from 1,656 apparently healthy ducks and open-country birds, 158 could be identified by CIE as influenza A viruses, whereas 75 were serologically related to NDV. The latter were identified by HI assays with an antiserum to NDV. Two of the isolates were of doubtful classification in that both reacted with the anti-M-protein for influenza A virus by CIE, and with NDV antiserum by HI. Electron microscopic examination confirmed the occurrence of mixed viral populations in which orthomyxoviruses were greatly outnumbered by paramyxoviruses. Initial identification by HI with specific antisera to influenza A viruses would not have revealed these mixed populations, as the influenza population would have been diluted out. Mixtures of ortho- and paramyxoviruses isolated from wild

FIG. 2. CIE with an antiserum to M-protein (α -M) of the "N" virus and the hemagglutinating agents isolated from birds. Negative controls were B/H.K (B/Hong Kong/8/73), A.F (uninfected allantoic fluid), NDV, and the positive control was A/USSR (A/USSR/92/77 (H1N1)).

TABLE 2. Correlation between CIE for the influenza A virus M-protein and HI for paramyxovirus identification

No. of isolates	CIE with influenza A anti-M-protein		HI with antiserum to:			
			NDV		Yucaipa virus	
	Positive	Negative	Positive	Negative	Positive	Negative
231	158 (68.3%)	73 (31.6%)	75 (32.4%)	156 (67.5%)	0 (0%)	231 (100%)

FIG. 3. Electron micrographs of a mixed population of paramyxovirus and influenza virus isolated from the cloaca of a mallard duck. (a) Nucleocapsid of the paramyxovirus is seen (upper right-hand corner) together with an influenza virus (lower left-hand corner). Insert shows a higher magnification of the influenza virus. (b) Characteristic morphology of the nucleocapsid of the influenza virus is shown together with some paramyxovirus nucleocapsid material (arrows). Bars, 100 nm.

birds have been reported previously (9). One of these was recovered from a Canada goose, whereas the other was isolated from a black duck.

The percent recovery of hemagglutinating agents after one passage in eggs was 5.6% (94), and a second blind passage in eggs permitted the detection of an additional 8.2% (137). Of these, 98.9% (93) and 47.4% (65), respectively, were influenza A viruses, as detected by CIE with the anti-M-protein of the avian influenza "N" virus. No reactivity with this antiserum could be demonstrated with the original rectal swab suspensions. It would be of some interest to test for the presence of influenza viruses in bird feces, which would be expected to contain more virus particles.

More definitive identification is being explored with reference antisera to the hemagglutinin and neuraminidase antigens of influenza A subtypes. The aims are to determine whether any avian subtypes are prevalent in the eastern part of Canada and whether new subtypes are emerging. This testing would also provide some information on the occurrence of influenza viruses in birds and their epidemiological and antigenic relationship to influenza viruses of man.

The CIE with antiserum to M-protein from an influenza A virus provides an additional method for the rapid identification of influenza virus isolates from birds, and possibly from man. It rapidly separates influenza A viruses from influenza B and noninfluenza viruses. Over 280 samples can be tested on the same day. The test combines specificity, sensitivity, and simplicity and appears to be adequate for epidemiological surveillance of influenza in man and animals.

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