Influenza A(H1N1) viruses of the 1977/78 outbreak: isolation and haemagglutination properties

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SUMMARY

During the H1N1 outbreak of 1977/8, the virus was isolated in embryonated eggs from 59 out of 76 patients (78 %) with the serologically confirmed infection. A similar isolation frequency has been achieved during a period of six H3N2 outbreaks since 1972/3. The H1N1 strains were isolated less frequently from late specimens (collected 4–6 days from the onset of illness) and more often only in the second passage compared with the H3N2 viruses. The new H1N1 strains resembled those prevalent in the 1950s with respect to their ability to agglutinate erythrocytes of certain laboratory animals and to be eluted from these, and thus differed from the H3N2 viruses.

INTRODUCTION

Isolation of influenza viruses from local outbreaks at military training centres has proved to be an important tool for influenza surveillance in Finland. Specimens from patients with an acute respiratory disease have been collected annually in a uniform manner in the centres, which are scattered throughout the country, and sent to the National Influenza Centre at the Central Public Health Laboratory (CPHL), Helsinki, for virological investigations. This procedure was followed during the first epidemic wave of the H1N1 subtype which reappeared in 1977/8. Thus, comparable data concerning the isolation of different influenza A virus subtypes and variants have been accumulated. This paper gives a brief summary of experience of the past six outbreaks since the epidemic of 1972/3. Attention will also be paid to haemagglutination properties of the H1N1 and H3N2 viruses and to the diagnostic value of the serological tests used in selection of the material proper for the comparative isolation studies.

MATERIAL AND METHODS

Specimens

Throat washings were collected from military recruits. The patients gargled with fresh fluid supplied specially by the CPHL in volumes of 5 ml per vial. The fluid consisted of Hanks balanced salt solution supplemented with glucose and sodium bicarbonate, 0·2 % of bovine serum albumin, penicillin (500 i.u./ml) and streptomycin (500 μg/ml). The specimens were sent to the laboratory without freezing or cooling and accepted for the present study if transport did not take

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more than 30 h from the time of collection. A great majority of the specimens arrived within 8 h.

A further condition for inclusion of the throat washings was that adequate paired sera had been sent to the laboratory. The initial sera were taken simultaneously with the gargling specimens and the convalescent sera from 1·5–3 weeks later. In addition, an extra serum specimen was taken in 1977/8 from 12 patients about 2 weeks after the collection of the convalescent phase sera.

During the first wave of the 1977/8 outbreak, which appeared to be caused mainly by the H1N1 subtype viruses, a total of 142 throat washings was tentatively considered adequate. The final series for the isolation studies was limited to the specimens from the 81 patients who showed a fourfold or greater rise in complement-fixing (CF) antibodies to influenza A. The specimens accepted had been collected in 23 garrisons scattered throughout the country.

The comparable final series, accumulated during the five H3N2 outbreaks since the epidemic of 1972/3, consisted of throat washings from 142 patients confirmed by the CF test to be infected with an influenza A virus. The specimens had been supplied by 21 garrisons. Haemagglutination inhibition tests (HI) were carried out systematically with serum specimens only from the three epidemics since 1975/6.

Isolation and haemagglutination tests

Chick embryos 10–11 days old were inoculated with 0·1 ml samples of throat washings by the intra-amniotic route. Five embryos were used for each sample. After three days' incubation at 34–35 °C the allantoic and amniotic fluids were harvested, pooled and tested for the presence of haemagglutinin for hen erythrocytes. Negative fluids and those with a titre of 16 or less were subjected to a second amniotic passage. The isolated viruses were identified by means of HI tests. Most of the strains, i.e. all those from the epidemic of 1977/8, were sent to the World Influenza Centre, London, where the identification was kindly confirmed (Dr J. J. Skehel and Dr G. C. Schild: information by letter).

Haemagglutination titres were determined by employing 0·1 ml of twofold dilutions of infected allantoic and amniotic fluids and equal volumes of 0·5 % suspension of erythrocytes from hen, or in certain experiments, guinea-pig, human group O or rabbit. Unless specifically stated, the plates were kept at room temperature. The pattern of agglutination was read after 90 min, except with rabbit cells which were allowed to settle for 120 min.

Serology

CF antibodies to S antigen of influenza A virus were determined with a microtitre technique (Casey, 1965) as described previously in detail (Pyhälä, Aho, Kantanen & Koistinen, 1976). The principle presented by Robinson & Dowdle (1969) was followed in HI tests (Pyhälä & Aho, 1975). Epidemic influenza A viruses, selected each year from the strains isolated in Finland, were used as antigens. The sera were pretreated with cholera filtrate (Philips-Duphar, B.V. Holland) to remove non-specific inhibitors.

RESULTS

It was predictable that the condition of a diagnostic rise in CF antibodies to influenza A would eliminate a number of influenza A infections from the final series. During the H3N2 epidemics, the lack of rise could be associated with a relatively high titre of these antibodies, directed to the S antigen of the virus, in the acute phase sera of the patients (Pyhälä & Kleemola, 1976). During the H1N1 epidemic of 1977/8 the proportion of such cases was especially high. The 142 patients in the preliminary series included 61 patients who did not respond with a diagnostic rise in CF antibodies to influenza A. In CF tests about a third of the patients showed serologic evidence of infection by some other respiratory pathogen: adenovirus (15 cases), *Mycoplasma pneumoniae* (5 cases) or RSV (1 case). No influenza viruses could be isolated from any of these 21 patients. The virus was isolated in 15 of the remaining 40 patients, who also failed to show a diagnostic rise in CF antibodies to influenza A. All the strains isolated were of the H1N1 subtype. The acute phase titres of the CF antibodies of the 15 patients reached a geometric mean of 13; a twofold rise was detected in 8 patients with an acute phase titre of 8–32.

In 1977/78, when the H1N1 and H3N2 subtypes were both circulating simultaneously, there were certain difficulties in using HI serology to determine the subtype responsible for the infection detected by CF. The difficulties were presumed to be caused by a poor and slow primary response to the H1 haemagglutinin. In patients responding with a diagnostic rise in CF antibodies, a fourfold or greater rise in HI antibodies to the epidemic H3N2 virus, and/or to the variant prevalent in 1976/7, was considered to be a sufficient indication of an H3N2 infection. On the other hand, the lack of a diagnostic rise in the H3N2 antibodies was regarded as sufficient evidence for an H1N1 infection.

The division seemed to be proper and correct because of the following findings:

(1) A fourfold or greater rise in HI antibodies to the epidemic virus was detected in all of the 65 patients who had been infected during the H3N2 outbreaks in 1975/6 and 1976/7 and responded with a diagnostic rise in CF antibodies to influenza A. (2) Patients in the age group in question, infected with H3N2 viruses, did not respond with antibodies to H1N1 viruses (Pyhälä, 1978). (3) During the mixed epidemic of 1977/8 the lack of a significant rise in H3N2 antibodies was associated with an H1N1 infection in a great majority (76 out of 81) of the patients with a diagnostic rise in CF antibodies. In the paired sera from these 76 patients, a response of HI antibodies to H1N1 subtype viruses was demonstrable in only 38 cases (50 %). However, in the third serum specimens, available from 12 patients, a response was detected in 11 cases. On the other hand, none of the 5 patients who responded with H3N2 antibodies had a rise in antibodies to H1N1 viruses. These 5 patients were all from the same garrison.

During the epidemic of 1977/8 virus was isolated from 59 of the 76 patients (78 %) considered by serological tests to be infected with H1N1 subtype viruses. In HI tests carried out for subtype determination, all the strains appeared to be like A/USSR/90/77(H1N1). The isolation was successful in two of the five patients

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Table 1. *Virus isolation by outbreaks from patients with serologically confirmed influenza*

			No. of isolated strains	
Season	Subtype	No. of patients	First passage	First and second passage in all
1972/3	H3N2	41	29 (71%)	31 (76%)
1973/4	H3N2	25	16 (64%)	18 (72%)
1974/5	H3N2	11	8 (73%)	8 (73%)
1975/6	H3N2	41	30 (73%)	32 (78%)
1976/7	H3N2	24	22 (92%)	24 (100%)
1977/8	H3N2	5	1 (20%)	2 (40%)
Total	H3N2	147	106 (72%)	115 (78%)
1977/8	H1N1	76	43 (57%)	59 (78%)

considered to be infected with H3N2 subtype viruses. Both of the viruses isolated were found to be like A/Texas/1/77(H3N2).

The strains isolated during the five H3N2 outbreaks since 1972/3 closely resembled the strains prevalent at those times elsewhere in Europe. Thus, in 1972/3 the strains appeared to be similar to A/England/42/72(H3N2), in 1973/4 and 1974/5 closely related but not identical to A/Port Chalmers/1/73(H3N2), and in both 1975/76 and 1976/77 like A/Victoria/3/75(H3N2).

A summary of virus isolations from throat washings of the patients serologically confirmed to be infected with influenza A is given in Table 1. During the first five epidemics of the H3N2 subtype the isolation percentage ranged from 72 to 100. In the total series the H1N1 and H3N2 strains were isolated at exactly the same rate (78%). A second amniotic passage was, however, required more often by H1N1 than by H3N2 subtype viruses ($P < 0.01$) for successful isolation. Fig. 1 compares the isolation results of H3N2 and H1N1 subtypes in relation to the collection time of the throat washings. The haemagglutinin titres of the H1N1 isolates tended to be lower than those of the H3N2 isolates from 1975/6 (Table 2).

A total of 86 H1N1 strains and 6 H3N2 strains isolated in CPHL during the 1977/8 outbreak were tested for haemagglutinin activity to hen, guinea-pig and human group O erythrocytes. A majority of the H1N1 strains (72%), but none of the H3N2 strains, exhibited a higher haemagglutinin titre with guinea-pig and human erythrocytes than with hen cells; a comparison of the titres against hen and guinea-pig erythrocytes is presented in Fig. 2.

As compared with representatives of H3N2 viruses from three outbreaks, the H1N1 strains from the outbreak of 1977/8 and the 1950s were characterized by low or no titres against rabbit erythrocytes when tested at room temperature (Fig. 3). At 4 °C, however, titres up to 128 were obtained. In addition, virus heated for 30 min at 56 °C acquired an enhanced haemagglutination titre even at room temperature. During propagation of two of the new H1N1 strains through ten passages in eggs, the characteristic of low or no titres against rabbit erythrocytes was found to be almost unaltered. On the other hand, great differences in agglutinability were detected between rabbit erythrocytes taken from different
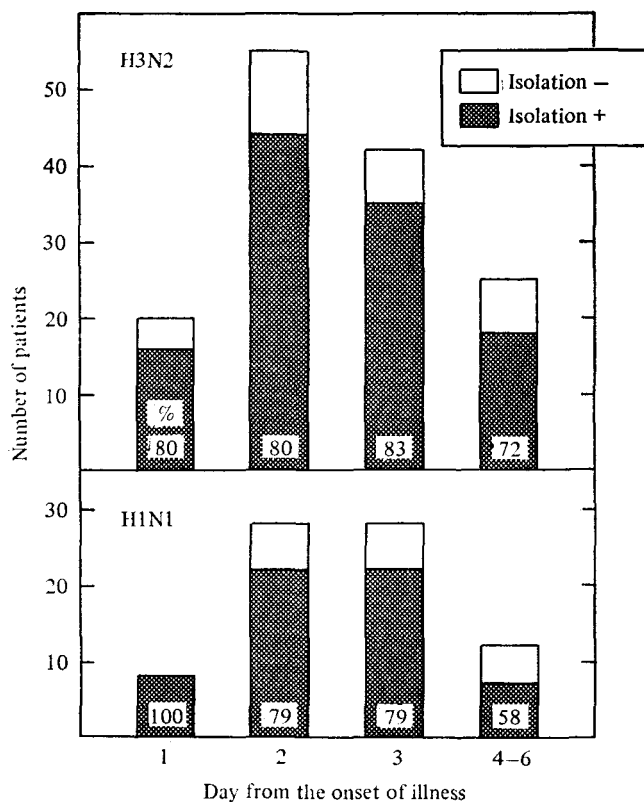
Fig. 1. Dependence of isolation results of H3N2 and H1N1 subtype viruses on the collection time of the throat washings. The figure summarizes results from the six H3N2 outbreaks since 1972/3.

Table 2. *Distributions of haemagglutinin titres of newly isolated H1N1 and H3N2 strains when tested with hen erythrocytes*

Subtype	Epidemic	No. of strains agglutinating in titres of:					
		16	32	64	128	256	512
H3N2	1975/6	—	1	—	11	14	4
H1N1	1977/8	3	9	11	8	9	3

animals. The erythrocytes used in the experiments were taken from a rabbit which in a preliminary test had been shown to possess unreactive cells.

Some differences were detected in the elution of H1N1 and H3N2 viruses from erythrocytes. After the agglutination titres were read, the plates were left at room temperature and their duplicates at 4 °C. The agglutination pattern read again after an interval of 8 h indicated that it was possible to elute the H1N1 but not the H3N2 viruses from hen erythrocytes at room temperature. On the other hand, both subtypes failed to elute from human group O erythrocytes. The elution of H1N1 viruses from hen erythrocytes failed at 4 °C and, after the virus was heated (56 °C, 30 min), also at room temperature. This elution pattern was found to be a common characteristic of the new H1N1 strains and those prevalent in the 1950s.
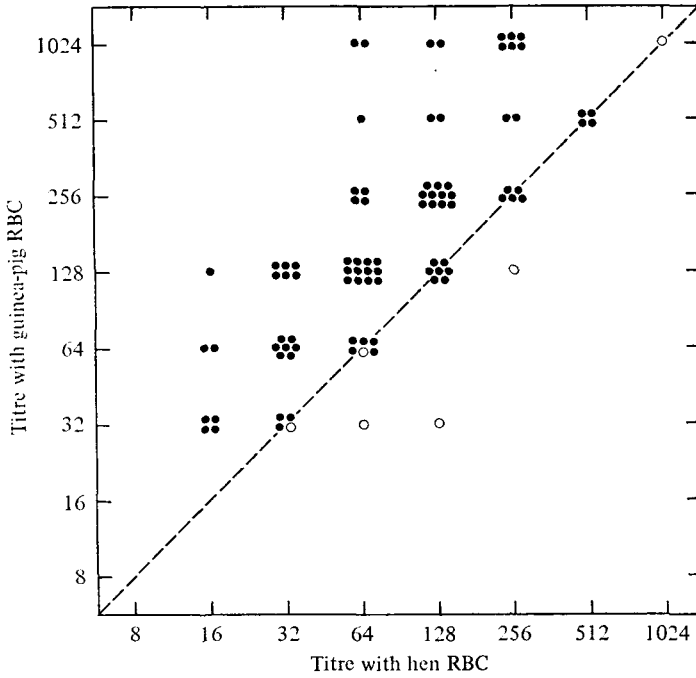
Fig. 2. Haemagglutinin titres of H1N1 (●) and H3N2 (○) strains isolated in 1977/8 when tested with hen and guinea-pig erythrocytes.
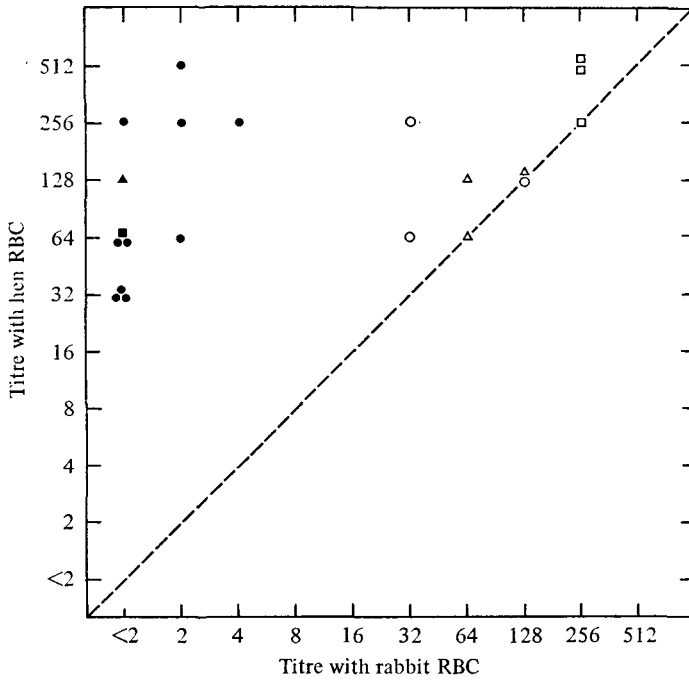

Fig. 3. Haemagglutinin titres of representatives of H1N1 strains isolated in 1953 (▲), 1956 (■) and 1977/8 (●), and of H3N2 strains isolated in 1974/5 (△), 1975/6 (□) and 1977/8 (○) when tested with hen and rabbit erythrocytes.

DISCUSSION

Great differences have been described concerning the isolation of influenza A viruses of different subtypes. Unlike the H2N2 strains (Hoyle, 1968), H3N2 viruses have been readily isolated in embryonated eggs (Coleman & Dowdle, 1969; Douglas, 1975; Ivanova *et al.* 1976) and recovered in some cases for 7–10 days after the onset of illness (Dowdle & Schild, 1975). The H1N1 subtype viruses were also readily isolated in small series from the 1940s and 1950s (Nagler, van Rooyen & Sturdy, 1949).

Because of the uniform procedure followed over the years at CPHL, Helsinki, a meaningful comparison of the isolation of H1N1 and H3N2 viruses was possible. The H1N1 subtype viruses were isolated as readily as the H3N2 viruses in serologically confirmed infections. For the high isolation rate (78 %) a second passage was, however, required more often by H1N1 than by H3N2 viruses. The results suggest that the H1N1 viruses may not be shed after the third day of the disease as frequently as the H3N2 viruses. If this is so, it might have a restricting effect on the spread of H1N1 epidemics. On the other hand, the recovery of the H1N1 viruses, in contrast to the H3N2 viruses, from all the specimens collected on the first day of the disease may be due either to the absence of specific antibodies in the secretory immunoglobulins, known to be a typical situation in primary infections (Zalan *et al.* 1973), or to differences in the average incubation period.

With respect to their ability to agglutinate erythrocytes of certain laboratory animals and to be eluted from these, the new H1N1 strains resembled the old ones prevalent in the 1950s and differed from the recent H3N2 viruses. Thus, the newly isolated H1N1 strains showed evidence of the O phase, which is characterized by higher haemagglutination titres with guinea-pig and human group O than with hen erythrocytes (Nagler, Burr & Gillen, 1951; Coleman & Dowdle, 1969). The failure of the H1N1 strains to agglutinate rabbit erythrocytes at room temperature, the ability to agglutinate these at 4 °C and after treatment with heat (Chu, 1948), as well as the rapid elution from hen but not from guinea-pig and human group O erythrocytes can be interpreted as a characteristic of the N1 neuraminidase.

Sialic acid residues are involved in early interactions between influenza viruses and their host cells, although the composition of virus receptors on the host cells may differ significantly from that on erythrocytes (Schulze, 1975). It has been suggested that partial cleavage of *N*-acetyl-neuraminic acid molecules of the receptors by neuraminidase allows a more effective contact between paramyxoviruses and the cell (Wassilewa, 1977). It is tempting to speculate that differences between influenza A virus subtypes in their ability to agglutinate erythrocytes of different animal species and to elute from these might reflect some differences between the subtypes in their interaction with host cells and even in the host range. If this is so, the ability of influenza A virus to cause an epidemic in populations of a given animal species might depend, among other things, on a proper combination of haemagglutinin and neuraminidase components. It is worth noting that the reservoir of newly returned H1N1 subtype viruses is unknown, and that

the number of influenza A virus subtypes known to have been responsible for epidemics in man is still very restricted.

REFERENCES

CASEY, H. L. (1965). Standardized diagnostic complement fixation method and adaptation to micro test. II. Adaptation of LBCF method to micro technique. *Public Health Monographs* **74**, 31–34.

CHU, C. M. (1948). Agglutination of red blood cells of different animal species by influenza and Newcastle disease viruses. *Journal of Hygiene* **46**, 239–246.

COLEMAN, M. T. & DOWDLE, W. R. (1969). Properties of the Hong Kong influenza virus. 1. General characteristics of the Hong Kong virus. *Bulletin of the World Health Organization* **41**, 415–418.

DOUGLAS, R. G., JR (1975). Influenza in man. In *The Influenza Viruses and Influenza* (ed. E. D. Kilbourne), p. 406. New York, London: Academic Press.

DOWDLE, W. R. & SCHILD, G. C. (1975). Laboratory propagation of human influenza viruses, experimental host range, and isolation from clinical material. In *The Influenza Viruses and Influenza* (ed. E. D. Kilbourne), p. 263. New York, London: Academic Press.

HOYLE, L. (1968). The influenza viruses. In *Virology Monographs*, p. 201. Wien, New York: Springer-Verlag.

IVANOVA, N. A., TAROS, L. YU, GRINBAUM, E. B., RUMEL, N. B., KAMFORIN, L. E. & LUZYANINA, T. YA. (1976). Results of virological and serological studies of three influenza A Hong Kong epidemics in Leningrad. *Journal of Hygiene, Epidemiology, Microbiology and Immunology* **20**, 186–194.

NAGLER, F. P., BURR, M. M. & GILLEN, A. L. (1951). The influenza virus epidemic in Canada during January-February 1951. *Canadian Journal of Public Health* **42**, 367–74.

NAGLER, F. P., VAN ROOYEN, C. E. & STURDY, J. H. (1949). An influenza virus epidemic at Victoria Island, N.W.T., Canada. *Canadian Journal of Public Health* **40**, 457–65.

PYHÄLÄ, R. (1978). Antibodies to influenza A viruses of the H1N1 subtype: age-dependency and effect of recent infections with H3N2 viruses. *FEMS Microbiology Letters* **3**, 165–7.

PYHÄLÄ, R. & AHO, K. (1975). Serum HI antibody and protection against influenza: a follow-up survey at community level of three epidemics caused by different H3N2-variants. *International Journal of Epidemiology* **4**, 127–9.

PYHÄLÄ, R., AHO, K., KANTANEN, M.-L. & KOISTINEN, J. (1976). Virus antibody levels and IgA deficiency. *Scandinavian Journal of Immunology* **5**, 1093–6.

PYHÄLÄ, R. & KLEEMOLA, M. (1976). The value of complement fixation and haemagglutination inhibition tests in the diagnosis of influenza A. *Acta virologica* **20**, 66–9.

ROBINSON, R. Q. & DOWDLE, W. R. (1969). Influenza viruses. In *Diagnostic Procedures for Viral and Rickettsial Infections* (ed. E. H. Lennette and N. J. Schmidt), pp. 426–7. New York: American Public Health Association.

SCHULZE, IRENE T. (1975). The biologically active proteins of influenza viruses: the haemagglutinin. In *The Influenza Viruses and Influenza* (ed. E. D. Kilbourne), p. 78. New York, London: Academic Press.

WASSILEWA, L. (1977). Cell receptors for paramyxoviruses. *Archives of Virology* **54**, 299–305.

ZALAN, E., PUKITIS, D., RHODES, A. J. & LABZOFFSKY, N. A. (1973). Interference of influenza-specific IgA antibodies present in throat washings with isolation of the virus. *Archiv für die gesamte Virusforschung* **42**, 307–10.