Prevalence of antibody to influenza C virus among pigs in Hyogo Prefecture, Japan

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The prevalence of influenza C virus among pigs in Hyogo Prefecture, Japan, was investigated by serological techniques. Out of 240 sera tested, 45 (19%) showed haemagglutination inhibition (HI) to influenza C virus. Pig sera with high HI titres also scored high in neutralization tests and ELISAs. When fractionated by sucrose density gradient ultracentrifugation, the HI/ELISA reactivities corresponded to antibodies of the IgM and IgG classes. Radioimmunoprecipitation tests revealed that some, but not all, of the pig sera with high HI activities precipitated HEF glycoprotein of influenza C virus. These results suggested that the HI activities of pig sera in Hyogo Prefecture were due to the presence of antibody to influenza C virus. Sera with IgM class antibody to influenza C virus were found throughout the year. However, the question of whether or not pigs serve as a natural reservoir for human influenza C virus still remains to be solved.

Influenza C viruses cause a mild upper respiratory tract disease (Dykes et al., 1980; Katagiri et al., 1983, 1987) and are widespread in the human population (Minuse et al., 1954; Andrews & McDonald, 1955; Jennings, 1968; Homma et al., 1982; Kaji et al., 1983). Little is known, however, about the mode of transmission and maintenance of the virus in nature although some investigators have assumed that the natural reservoir of the virus is limited to man (Air & Compans, 1983). In man, primary infection occurs soon after birth and most people become seropositive by the age of 7 years (Homma et al., 1982; Kaji et al., 1983). A long-range survey in a children's home demonstrated three outbreaks of influenza C during a 2 year period and recurrent infections among infants as well as adult employees (Katagiri et al., 1983, 1987). This observation shows that influenza C virus is transmitted efficiently from human to human and can be maintained among human populations by recurrent infections.

On the other hand, Guo et al. (1983) found antibodies to influenza C virus in about 4% of abattoir pigs in Beijing, China, and succeeded for the first time in isolating the virus from pigs. They also reported that pigs could be experimentally infected with both pig and human influenza C virus isolates and that the virus was transmitted from infected to uninfected contact pigs. This finding suggested that pigs as well as man could be a natural reservoir of influenza C virus. In contrast to the above report, we have previously been unable to detect antibody to influenza C virus among pigs in Yamagata Prefecture (Ohwada et al., 1987). This was consistent with the report by Kawano et al. (1978) who could not detect antibody to the virus in sera of 792 pigs in Hokkaido. The discrepancy could result from differences in the prevalence of influenza C virus among pigs from one place to another. In the present paper, we demonstrate the presence of antibody to influenza C virus among pigs in Hyogo Prefecture, Japan. We also show that sera with IgM class antiviral antibody were found almost throughout the year.

A total of 240 sera of 6- to 8-month-old pigs were collected in an abattoir in Hyogo Prefecture, Japan, during the period of July 1981 to June 1982. Some sera were collected from pigs in 1983 and 1984. Sera were fractionated by sucrose density gradient ultracentrifugation as described previously (Yamaoka et al., 1982). Absorption of IgG from pig sera was performed according to the method of Roggendorf et al. (1980) using the Cowan I strain of Staphylococcus aureus (Absorb G; Chemo- and Serum Therapy Institute, Kumamoto, Japan).

Hyperimmune rabbit sera were prepared by intravenous inoculation of purified influenza C/Aomori/74 virus, five times at weekly intervals. The hyperimmune pig serum prepared against influenza C/Ann Arbor/50 virus has been described previously (Ohwada et al., 1987).

Three strains of influenza C virus, C/Aomori/74, C/Ann Arbor/50 and C/Hyogo/83, were grown in the amniotic cavity of 10-day-old embryonated chicken eggs.
The amniotic fluids were clarified by low-speed centrifugation and the supernatants were used for haemagglutination inhibition (HI) and neutralization (NT) tests. For ELISA and immunization experiments, virus was purified using continuous sucrose density gradient ultracentrifugation (30 to 60% w/w) at 100000 g for 3 h. To remove non-specific inhibitors prior to HI tests, pig sera were subjected to treatment with trypsin and KIO4 according to Jensen (1961).

ELISA tests to detect anti-influenza C virus antibodies were performed as described previously (Konishi & Yamaoka, 1982) with minor modifications. Purified C/Aomori/74 virus was sonicated at 28 kHz for 30 s (Nihon Seiki sonicator) before use as an ELISA antigen. As second antibodies, alkaline phosphatase (ALP)-conjugated goat anti-swine IgG (H, L chain-specific; Cappel Laboratories) and rabbit anti-swine IgM (μ chain-specific; Miles Laboratories) were used. Rabbit anti-swine IgM antibody was conjugated with ALP (Type VII-s; Sigma) in our laboratory (Yamaoka et al., 1982) according to the method described by Engvall & Perlmann (1972).

The radioimmunoprecipitation (RIP) test was performed as follows. MDCK cells infected with C/Ann Arbor/50 virus and labelled with [35S]methionine were treated with 0.025 M-Tris-HCl pH 8.0, containing 0.5% Triton X-100, 0.5% sodium deoxycholate and 0.005 M-NaCl at 4 °C for 30 min, and centrifuged at 18000 g for 10 min to obtain cell lysate (Saleh et al., 1979). Cell lysates were mixed with test sera and the resultant immune complexes precipitated with Protein A-Sepharose CL-4B beads (Pharmacia) followed by SDS–PAGE according to Laemmli (1970). After electrophoresis gels were treated with 2,5-diphenyloxazole (Enhance; New England Nuclear), dried and exposed to X-ray films (Fuji) at −80 °C.

Forty-five out of 240 sera (19%) exhibited HI activity, titres ranging between 1:32 and 1:512. Sera with high HI activities possessed high titres of both NT and ELISA activities (data not shown).

Ten sera showing both HI and NT activities were fractionated by sucrose density gradient ultracentrifugation, and HI and ELISA titres of fractions were determined. Two representative results are shown in Fig. 1 (a, b). In Fig. 1 (a), HI activities were distributed among two peaks with the highest titres at fractions 8 and 14. ELISA tests using ALP-labelled goat anti-swine IgG (H and L chain-specific) as a second antibody confirmed these results. Since the ALP-labelled antibody cross-reacted with L chains of the IgM molecules, it was likely that the first peak corresponded to antibody of the IgM class and the second peak to that of the IgG class. Only the first peak was observed when goat anti-swine IgM (μ chain-specific) was used as a second antibody. Fig. 1(b) shows that HI activity was detected only in the second peak corresponding to IgG class antibody. The HI activities of the second peak were abolished completely by absorption of the samples with the Cowan I strain of S. aureus (data not shown). These results indicated that the HI activities in the pig sera were due to antibodies to the virus. Among the 10 sera tested, six contained anti-influenza C virus antibody of both IgM and IgG classes whereas four sera contained only specific IgG antibody.

For comparison, two piglets were intranasally inoculated with influenza C/Ann Arbor/50 or C/Hyogo/83 viruses and the humoral antibody response was analysed. The sera obtained 8 days post-infection (p.i.) had antibody activity in both the IgM and IgG fractions, whereas the sera obtained 55 days p.i. had antibody only in the IgG fraction.

Fig. 2 shows the monthly distribution of HI titres in the pig sera including the 10 sera described above which were analysed further. HI-positive sera were found every month, the monthly prevalence ranging between 5 and 35%. HI IgM antibodies were detected throughout the year.

To identify the viral components to which the antibody in pig sera was directed, RIP tests were performed using a [35S]methionine-labelled lysate of
Fig. 2. Monthly distribution of HI antibody to influenza C virus in pig sera in the period of July 1981 to June 1982. HI antibody titres were determined using C/Aomori/74 virus as antigen. Each symbol represents the serum HI titre of each individual pig. Six sera possessing antibodies of both the IgM and IgG classes (tested by HI and ELISA) and four sera possessing IgG antibody alone are marked with symbols * and +, respectively.

C/Ann Arbor/50-infected MDCK cells (Fig. 3). Some of the pig sera precipitated HEF glycoprotein (lanes 5, 6 and 10). Some sera with relatively high HI titres (Fig. 3, lanes 3, 7 and 9) did not precipitate HEF glycoprotein. The membrane (M) protein of the virus was precipitated only with the hyperimmune rabbit serum (lane 1). The difference between rabbits and pigs in the precipitating activities of their hyperimmune sera may be due to differences in the immune response during natural infection or immunization. Nucleoprotein (NP) precipitated with rabbit hyperimmune serum migrated more slowly than that precipitated with pig sera (Fig. 3, lanes 1 and 2). This phenomenon is reproducible but not explained at present.

The present study demonstrated that about 19% of pig sera obtained at an abattoir in Hyogo Prefecture had HI activity against influenza C virus. This HI activity is thought to be due to specific antibody for the following reasons: (i) HI activities were proportional to NT and ELISA titres; (ii) HI activities co-separated with IgM and IgG antibodies when analysed by sucrose density gradient ultracentrifugation; (iii) HI activities observed in IgG fractions were completely abolished by treatment with the Cowan I strain of S. aureus; (iv) pig sera with HI activity precipitated the HEF glycoprotein of the virus, although no strong correlation was observed between HI titres and HEF-precipitating activities. The overall results suggested that a considerable number of pigs in Hyogo Prefecture were infected with influenza C viruses and that the infection among pigs occurred in farms throughout the year.

Previously we found HI activities in sera of pigs bred in Yamagata Prefecture, the northern part of Japan. Those HI activities were considered to be due to non-specific inhibitors, since HEF-precipitating antibody was not detected by RIP tests (Ohwada et al., 1987). Subsequent studies, however, revealed that sera of pigs experimentally infected with influenza C virus did not precipitate detectable amounts of HEF glycoprotein, although they were shown to contain specific antibodies as determined by HI, NT and ELISA tests (unpublished results). Similarly, we found that sera of rabbits immunized once with the virus precipitated only a small amount of HEF glycoprotein, although the HI activities reached high levels, and that the HEF-precipitating activity of immune sera increased with increasing times of immunization (unpublished results). It is possible, therefore, that some pigs with HI activities in Yamagata Prefecture had low levels of antibody against influenza C virus. In our present study, sera obtained from some pigs clearly precipitated the HEF glycoprotein (Fig. 3, lanes 5, 6 and 10). These pigs might have experienced recurrent or prolonged infection with the virus.

The discrepancy between HI activity and the HEF-precipitating activity of sera could be due to the possibility that the affinity of antibodies for detergent-treated antigen is weak compared with that for antigens in the native form. With prolonged duration and boosting after primary immunization the immune response would become stronger (Siskind & Benacerraf, 1969).

It is possible that infection of livestock with influenza C virus in Japan originated from man. Pigs in farms near densely populated areas may have a higher probability of
infection with influenza C virus. Guo & Desselberger (1984) reported that influenza C virus isolates from pigs in China were genetically different from those isolated from man. But no contemporary human influenza C virus isolate obtained in Beijing was available for comparative study. Influenza C virus can be successfully maintained in the human population without animal reservoirs because of frequent recurrent infections and prolonged shedding periods of over 1 month (Katagiri et al., 1983, 1987). Comparative sequence analysis of eight human and three porcine influenza C virus HEF genes led to the conclusion that at any one time influenza C viruses of different evolutionary pathways co-circulate and that therefore it cannot be decided whether or not pigs are a true animal reservoir for influenza C virus infections in man (Buonagurio et al., 1985). Further study is underway in relation to the question whether pigs or other animals are reservoirs of human influenza C virus. Virus isolation from pigs in abattoirs in the Hyogo Prefecture is being attempted, and should give us further clues to answer the question.

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