

Serological studies of influenza viruses in pigs in Great Britain 1991–2

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SUMMARY

Samples from a sow serum bank representative of the pig population of Great Britain collected during 1991–2, were examined for antibodies to influenza A, B and C viruses, using viruses which had been isolated from a variety of hosts. For influenza A viruses there was evidence of the continued circulation of ‘classical swine’ H1N1 virus (26% seroprevalence), and human H3N2 viruses (39%) which are antigenically most closely-related to A/Port Chalmers/1/73 virus. In addition antibodies were detected to A/swine/England/201635/92 (8%), a strain of H3N2 virus which appears to have arisen by antigenic drift from conventional H3N2 swine strains. Specific antibodies (2%) were detected to an H1N1 virus (A/swine/England/195852/92) related most closely to avian H1N1 strains. In tests with human H1N1 and H3N2 viruses, excluding isolates from pigs, the highest seroprevalence was detected to the prevailing strains from the human population. Serological tests with avian H4 and H10, human H2, equine 1 and 2 influenza A viruses were all negative. Seven pigs seropositive by haemagglutination-inhibition, virus neutralization and immunoblotting assays for antibody to influenza B virus, were randomly distributed geographically suggesting that influenza B viruses may be transmitted to pigs but fail to spread. The seroprevalence to influenza C viruses was 9.9% indicating that these viruses are widespread in pigs. These results provide further evidence that the pig can be infected by a number of influenza viruses, some of which may have significance in the epidemiology of human influenza.

INTRODUCTION

Influenza, a disease of widespread occurrence, represents a significant problem in pigs throughout the world. Influenza A viruses, predominantly subtypes H1N1 and H3N2 are enzootic in pigs [1], occasionally being associated with respiratory disease. Classical swine H1N1 viruses became re-established in Great Britain in 1986 following introduction via imported pigs [2]. In 1987, H3N2 viruses were associated with clinical disease in pigs in Great Britain for the first time [3], although seropositive pigs had been detected as early as 1970 [4]. These viruses also circulate in pigs in continental Europe, but concurrently with ‘avian’ H1N1 virus which has become widespread since its first isolation in 1979 [5], and largely

replaced classical H1N1 viruses. In Great Britain in 1992, an H1N1 virus distinguishable antigenically from, but closely related to, the European avian-like viruses was associated with a large number of respiratory epizootics in pigs [6]. Pigs therefore serve as major reservoirs of H1N1 and H3N2 influenza viruses and appear to be involved frequently in interspecies transmission of influenza viruses. Although these strains appear to have a limited capacity to spread from pig to human, their maintenance in pigs and the frequent introduction of new viruses from other species [7–9] may be important in the generation of pandemic strains of human influenza.

Influenza B viruses are only known to infect humans, whilst influenza C viruses have been isolated only from humans, pigs [10] and dogs [11]. The question of whether animals, particularly pigs, serve as a reservoir for these viruses still remains unresolved. In the present study we tested a sow serum bank representative of the national pig population of Great Britain, for antibodies to influenza A, B and C viruses. In addition to determining the prevalence of antibodies to influenza virus subtypes known to be circulating in pigs, we looked for serological evidence of other strains or subtypes of influenza virus originating from other species of animals and birds.

MATERIALS AND METHODS

Serum samples

Samples were collected at slaughter from 2000 sows of unknown age (probably 1–4 years old), during the period October 1991–February 1992. Pigs were sampled randomly and originated from all regions of England and Wales. The number of samples collected in each region was approximately proportional to the pig population. All sera were stored at -40°C prior to testing. Reference sera to the virus strains used in the study were prepared in chickens and ferrets according to standard procedures [12].

Virus strains

A total of 20 influenza A viruses (Table 1) derived from pigs, humans, birds and horses; one strain of influenza B virus (B/Victoria/2/87) and one of influenza C virus (C/Paris/1/67) were used in this study. The viruses were grown in the allantoic and amniotic cavities of 10-day-old embryonated hen's eggs. After incubation at 35°C for 3 days, the fluids were harvested, clarified by centrifugation at 2000 g for 10 min and tested for haemagglutination activity using 1% (v/v) fowl erythrocytes. Viruses were aliquotted and stored at -70°C prior to use in serological assays.

Haemagglutination-inhibition (HI) test

Serum antibodies to influenza viruses were detected by the HI test according to standard methods [12]. The sera were treated with 100 U/ml of receptor-destroying enzyme at 37°C overnight, inactivated at 56°C for 30 min and adsorbed with 30% (v/v) fowl erythrocytes overnight at 4°C . HI tests were done using 4 haemagglutinating units of virus and 1% (v/v) fowl erythrocytes.

Table 1. *Influenza A viruses used in the serosurveillance study*

| Virus | Subtype | Comments |
|-----------------------------|---------|-------------------------------------|
| Swine isolates | | |
| A/swine/Weybridge/117316/86 | H1N1 | GB classical prototype |
| A/swine/England/195852/92 | H1N1 | GB 'avian-like' prototype |
| A/swine/Belgium/1/79 | H1N1 | European avian-like |
| A/swine/Weybridge/163266/87 | H3N2 | GB prototype |
| A/swine/England/201635/92 | H3N2 | — |
| A/swine/England/191973/92 | H1N7 | — |
| Human isolates | | |
| A/USSR/90/77 | H1N1 | } Representative of antigenic drift |
| A/England/333/80 | H1N1 | |
| A/Chile/1/83 | H1N1 | |
| A/Taiwan/1/86 | H1N1 | |
| A/Singapore/1/57 | H2N2 | — |
| A/Hong Kong/1/68 | H3N2 | } Representative of antigenic drift |
| A/Port Chalmers/1/73 | H3N2 | |
| A/Victoria/3/75 | H3N2 | |
| A/Philippines/2/82 | H3N2 | |
| A/England/427/88 | H3N2 | |
| A/England/261/91 | H3N2 | |
| Avian isolates | | |
| A/duck/England/1196/91 | H4N2 | Prevalent H* type in birds |
| A/chicken/England/378/85 | H10N4 | — |
| Equine isolates | | |
| A/equine/Prague/1/56 | H7N7 | Equine 1 |
| A/equine/Fontainebleau/1/79 | H3N8 | Equine 2 |

* Haemagglutinin.

Virus-neutralization (VN) test

Selected HI positive sera/virus combinations were examined further using the VN test. Twofold serum dilutions prepared in Eagle's MEM (Flow Laboratories) supplemented with L-glutamine (2 mM/ml) and trypsin (10 µg/ml-Difco 1/250) were incubated in microtitre plates at 37 °C in 5% CO₂ for 1 h with 100 TCID₅₀ of tissue culture-adapted influenza virus. The plates were then seeded with MDCK cells, and after 5 days incubation at 37 °C in 5% CO₂, neutralization titres were assessed by the presence of cytopathic effect on cell monolayers and haemagglutination activity in cell culture supernatants. Neutralization titres are expressed as the reciprocal of the antibody dilution which completely inhibited virus infectivity in 50% of duplicate cultures.

Western blot analysis

The specificities of antibodies detected to influenza B virus in HI/VNT tests were examined by Western blot analysis. Influenza B virus was produced according to standard procedures (see Virus strains), concentrated by centrifugation (100000 g for 60 min) and purified by centrifugation (20000 g for 60 min) through 30/50% sucrose gradient. Purified virus was harvested and further centrifuged (100000 g for 60 min) prior to reconstitution in 0.01 M phosphate buffered saline, pH 7.2 (PBS). Viral polypeptides were separated by polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulphate

(SDS-PAGE). Purified virus was added to SDS-disruption buffer containing 5% mercaptoethanol and electrophoresed on 12% polyacrylamide gels according to the method of Laemmli [13]. The separated viral polypeptides were transferred to nitrocellulose membranes according to the method of Towbin [14]. Following transfer, the membrane was treated with 10 mM Tris/HCl pH 7.4 (wash buffer) containing 0.5% Tween 80 for 60 min at 37 °C. Membranes were reacted with sera diluted 1/50 in wash buffer, for 90 min at room temperature, washed four times (washes two and three in wash buffer containing 0.5% Nonidet P40) and gels were then reacted with protein A conjugated with horse-radish peroxidase and diluted 1/1000 in wash buffer. Following incubation at room temperature for 2 h the membranes were washed four times as before and protein bands were visualized by the addition of diaminobenzidine (0.5 mg/ml; Sigma) in PBS containing 0.0007% H₂O₂. The reactions were stopped after 4–5 min with PBS. Finally blots were dried between filter paper.

RESULTS

The influenza A virus strains used in the study were selected because they are antigenically distinguishable in HI tests with reference polyclonal antisera and are representative of the major groups of viruses known or considered likely to infect pigs. However, due to antigenic similarities between some viruses of the same subtype, detected by cross HI tests, further discrimination was required for the interpretation of data. Seropositive pigs were those showing a positive HI titre (> 10) to the virus. Homologous reactors are those pigs with the highest titre to the specified virus when tested against a range of viruses of the same subtype.

Serological responses to influenza A viruses

A total of 1193 (59.7%) pigs were serologically positive to one or more strains of influenza A virus used in this study. This included 498 (24.9%) pigs which were both seropositive to influenza A viruses isolated originally from pigs and humans.

Serological responses to swine H1N1 and H3N2 viruses

The summarized results of HI tests with swine H1N1 and H3N2 viruses are shown in Table 2. The majority of pigs which were seropositive to H1N1 viruses had specific antibodies to classical H1N1 viruses. No pig had homologous antibodies to a prototype strain of the European 'avian-like' H1N1 viruses. Some low cross reactivity occurred in HI tests between H3N2 viruses of swine origin and early human H3N2 viruses. A majority of pigs seropositive to swine H3N2 viruses had specific antibody to the prototype strain but a significant number (166 = 8%) had specific antibody to a recent virus (A/swine/England/201635/92) which has minor antigenic differences detectable by HI tests. A total of 277 (14%) pigs was positive serologically to both swine H3N2 and swine H1N1 viruses, while 994 (49.7%) pigs had antibodies to at least one strain of swine influenza.

Serological responses to human H1N1 and H3N2 viruses

The results of HI tests with human H1N1 and H3N2 viruses are summarized in Table 3. The majority of pigs which were seropositive to human H1N1 viruses had specific antibodies to A/Taiwan/1/86 'like' viruses. Serum from nine pigs had

Table 2. Results of haemagglutination inhibition tests on 2000 sow sera collected in Great Britain using swine influenza A viruses as antigens

| Influenza A virus | Number (%) | |
|-----------------------------------|--------------------|----------------------|
| | Seropositive pigs* | Homologous reactors† |
| A/swine/Weybridge/117316/86(H1N1) | 522 (26) | 480 (24) |
| A/swine/England/195852/92(H1N1) | 311 (16) | 31 (2) |
| A/swine/Belgium/1/79(H1N1) | 90 (5) | 0 (0) |
| A/swine/Weybridge/163266/87(H3N2) | 769 (39) | 482 (24) |
| A/swine/England/201635/92(H3N2) | 455 (23) | 166 (8) |

* Seropositive pigs are those showing a positive titre > 10.

† Homologous reactors are those pigs with the highest titre to the specified virus when tested against a range of viruses of the same subtype and are a subset of seropositive pigs.

Table 3. Results of haemagglutination inhibition tests on 2000 sow sera collected in Great Britain using human influenza A viruses as antigens

| Influenza A virus | Number (%) | |
|----------------------------|--------------------|----------------------|
| | Seropositive pigs* | Homologous reactors† |
| A/USSR/90/77(H1N1) | 6 (< 1) | 0 |
| A/England/333/80(H1N1) | 33 (2) | 13 (< 1) |
| A/Chile/1/83(H1N1) | 5 (< 1) | 0 |
| A/Taiwan/1/86(H1N1) | 93 (5) | 85 (4) |
| A/Hong Kong/1/68(H3N2) | 127 (6) | 0 |
| A/Port Chalmers/1/73(H3N2) | 346 (17) | 15 (< 1) |
| A/Victoria/3/75(H3N2) | 248 (12) | 44 (2) |
| A/Philippines/2/82(H3N2) | 221 (11) | 16 (< 1) |
| A/England/427/88(H3N2) | 426 (21) | 183 (9) |
| A/England/261/91(H3N2) | 67 (3) | 35 (2) |

* Positive titre > 10.

† Homologous reactors are those pigs with the highest titre to the specified virus when tested against a range of viruses of the same subtype and are a subset of seropositive pigs.

indistinguishable HI titres to virus strains from both 1980 and 1986. All pigs seropositive to human H1N1 viruses had lower or no reactivity in HI tests with A/swine/England/191973/92 (H1N7). A broad spectrum of reactivity occurred in HI tests with human H3N2 viruses. All 426 samples positive in HI tests with A/England/427/88 were tested using the VN test with this virus (data not shown). A total of 183 (9.2%) sera was positive in the VN test, confirming the calculated number of homologous reactors in the HI test. In HI tests a greater number of pigs were seropositive having specific antibodies to a recent strain (A/England/427/88) of H3N2 virus. Serum from 34 pigs had indistinguishable HI titres to several strains of human H3N2 virus. A total of 170 (8.5%) pigs had homologous antibodies to H3N2 viruses isolated from both pigs and humans, whilst 391 (19.6%) pigs had homologous antibodies to H3N2 and H1N1 viruses originating from humans.

Serological responses to other influenza A viruses

All HI tests with equine influenza (equi 1 and equi 2), H4 and H10 subtypes from birds and H2N2 from humans were negative.

Table 4. *Detection of antibodies to influenza B virus by VN test and Western blotting in pig sera known to be positive by HI test*

| Serum No. | Test | | |
|-----------|-----------|-----------|---------------|
| | HI titre* | VN titre† | Western blot‡ |
| 473 | 80 | 80 | ++ |
| 792 | 80 | 20 | ++ |
| 1273 | 40 | 40 | ++ |
| 1376 | 80 | 40 | + |
| 1772 | 160 | 160 | ++ |
| 1824 | 40 | 40 | ++ |
| 1868 | 160 | 160 | ++ |
| 1972 | 160 | 160 | ++ |

* Haemagglutination inhibition titres expressed as the reciprocal of the dilution of serum inhibiting four haemagglutinating doses of virus.

† Virus neutralization titres expressed as the reciprocal of the dilution of serum which completely inhibited virus infectivity in 50% of duplicate cultures.

‡ Western blot. + = clear positive reaction with HA1 and NA. ++ = clear positive reaction with HA1/2, NA and M.

Serological responses to influenza B virus

In HI tests with B/Victoria/2/87, eight sera were positive, with titres ranging from 40–160. These samples were examined further in VN tests and by Western blotting with B/Victoria/2/87 (see Table 4). With all of these sera analyses by Western blotting reactions detected proteins with relative molecular mass (M_r) 71 K (HA1) and 54 K (NA) together with two proteins of M_r between 26 K and 30 K, believed to be HA2 and M (matrix), which reacted with 7 of 8 sera.

Serological responses to influenza C virus

In HI tests with C/Paris/1/67, 198 (9.9%) sera were positive, with titres in the range 10–320. Eight representative sera with both positive and negative HI titres were tested by VN for antibodies to strain C/Paris/1/67, results obtained showed complete correlation between the two tests.

DISCUSSION

The prevalence of pigs seropositive for classical swine H1N1 influenza virus in the national pig population has remained high since the first appearance of the virus in Great Britain in 1986. Then, a serological survey of pigs associated with outbreaks of respiratory disease, found that 48% were seropositive [2]. A subsequent survey in 1990 using sera collected from 1000 healthy sows found 23% seropositives (Brown, unpublished data) compared to the current prevalence of 24%. Similar observations of continued circulation have been reported from Germany [15], Japan [16] and USA [17]. Viruses closely related to classical swine H1N1 appear to be endemic in pigs in Great Britain but are only occasionally associated with outbreaks of disease.

The inference from results with avian-like H1N1 influenza viruses is that these

viruses were not present in pigs in Great Britain until early 1992 when the first isolations of virus were made [6]. The small number of serological reactors detected to these viruses supports this observation because the majority of serum samples used in this survey were collected in late 1991.

Swine H3N2 influenza viruses related most closely to the human strain A/Port Chalmers/1/73 continued to circulate widely, as indicated by 24% seropositives detected in the survey. The survey in 1990 in Great Britain detected 43% seropositives (Brown, unpublished data). Other surveillance studies in pigs in Europe have detected 28–62% seropositive to 'swine' H3N2 viruses [18, 19]. Some influenza H3N2 viruses isolated in our laboratory since 1992, appeared to have a lower reactivity in both HI and NI tests with antibody to the prototype swine isolate suggesting some antigenic differences in the surface glycoproteins. In order to investigate the prevalence of these viruses in the national pig population we incorporated a representative strain (A/swine/England/201635/92) into our panel of viruses and examined the results in conjunction with those from the prototype swine H3N2 virus. We were able to detect 8% specific seropositives to recent viruses suggesting they appear to be established in pigs in Great Britain. These viruses have apparently evolved due to antigenic drift as they are most closely related to the conventional swine isolates and in HI and NI tests are quite distinct from recent human strains. Such variations in the surface antigens of swine H3N2 viruses have been reported from Belgium and France since 1984 [18, 20]. These viruses appear to be evolving in a different lineage to those of human viruses, and pigs therefore present a reservoir of virus which may in the future infect the human population. Furthermore, 14% of pigs had been infected with both swine H3N2 and H1N1 viruses, leading to a potential for the reassortment of influenza virus genes following co-infection, as occurred in Japan in 1978 [21], Italy since 1985 [22] and France in 1987/8 [23].

Sera from positive animals reacted in HI tests with human H1N1 viruses predominantly with the prevailing strain in the human population (A/Taiwan/1/86). These findings are consistent with previous studies where antibodies in pigs to human H1N1 viruses were recognized in relation to H1N1 influenza epidemics in the human population [2, 15, 16, 24]. These viruses apparently fail to persist in the pig population, although seropositive animals to a strain of human H1N1 virus which had circulated 30 years earlier have been reported [25]. However, the recent detection of an H1N2 influenza A virus in pigs in Great Britain, whose haemagglutinin is related most closely to that of a human H1 virus from the early 1980s, suggests antigenically-related viruses may persist after reassortment with a swine influenza virus [26]. As with human H3N2 viruses, after a period of adaptation in the pig they may become pathogenic for pigs [27]. We were unable to detect any pigs with antibodies to an H1N7 influenza virus isolated from pigs in Great Britain in 1992. Natural and experimental infection of pigs with this virus [28], and experimental infection of pigs with avian influenza viruses [29] induced no or only low levels of detectable humoral antibody, which suggests that serosurveillance may not be suitable for the detection of some reassortant or 'new' influenza viruses in pigs.

Not unexpectedly, the reactivity of sow sera in HI tests with human H3N2 viruses was often broad ranging. The interpretation of these results, with the

objective of determining which H3N2 strain infected the sow from which the serum was collected, was not always easy, and resulted in indeterminate identification of the probable infecting strain for a number of pigs. The serological response of different pigs following infection with the same strain may be variable. However, as a rule, the serum of an individual pig infected with an H3N2 virus should show high HI titres against the infecting strain and closely related strains but much lower titres to virus strains that are poorly related antigenically. As with human H1N1 viruses, H3 seropositive pigs reacted predominantly with a recent human virus, A/Eng/427/88 (H3N2). It would appear that the prevailing H3N2 strains in the human population are transmitted to pigs, but fail to persist. Serological studies in pigs in Japan have detected strain specific antibodies to recent human H3N2 viruses independent of these viruses producing epidemics in the human population [16]. However, the detection of seropositive pigs to current human H3N2 viruses following H3N2 influenza epidemics in humans have been well described [2, 15, 30]. Interestingly, we were able to detect individual pigs which had apparently been infected with both recent human H3N2 viruses used in the current survey in addition to the prototype swine H3N2 strains. It would appear therefore, that due to antigenic dissimilarity between these viruses, there may be no protection against cross infection, and raises the possibility that in time these later strains may become adapted to pigs, with implications for infection of a susceptible human population.

There was no evidence for the presence of other influenza A virus subtypes in pigs. It has been suggested that the next human pandemic strain may emerge from pigs and that conventional serosurveillance using H1 and H3 subtypes may not detect such an event [31]. We investigated infection of pigs with H4 subtype from birds because this is the predominant subtype in waterfowl, and an H10 subtype isolated from birds because the host range of these viruses includes mammals [32].

Influenza B viruses have only been recorded in humans. There is no substantiated evidence for the presence of these viruses in other animals, although serosurveillance studies in Hungary detected a small number of seropositive pigs in HI tests, following a human epidemic, and experimental transmission of influenza B virus to young pigs suggested that pigs are susceptible to infection [33]. In Japan serosurveillance studies detected a small number of seropositive horses and one pig in HI tests [34]. In the present study serum samples from eight pigs were positive in both HI and VN tests, with good titre correlation. The specificity of these reactions was confirmed by Western blotting, which revealed antibody directed against influenza B glycoproteins. The locations of seropositive pigs were spread across England and Wales, suggesting that transmission of influenza B virus to pigs in a random event, most probably from humans. It would appear that these viruses do not spread within the pig population.

The results with influenza C virus indicate that these viruses are widespread in pigs in Great Britain. However they have not been associated with clinical disease in Great Britain or elsewhere, although influenza C viruses have been isolated from pigs in China, and transmitted experimentally between pigs [10]. Serosurveillance studies in China and Japan, using sera collected from fattening pigs at slaughter detected 3–19% seropositives to influenza C virus respectively [10, 35] compared to 9.9% in the present study.

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