Arch Virol (1994) 135: 365–382

Archives of Virology

Isolation of two H1N2 influenza viruses from swine in France

J. M. Gourreau[1], C. Kaiser[1], M. Valette[2], A. R. Douglas[3], J. Labie, and M. Aymard[2]

[1]Laboratoire Central de Recherches Vétérinaires, Maisons Alfort Cedex,
[2]Laboratoire de Virologie, Domaine Rockefeller Hospices Civils de Lyon, 8, avenue Rockefeller, Lyon Cedex, France
[3]World Influenza Centre, National Institute for Medical Research, London, U.K.

Accepted November 22, 1993

Summary. Samples collected in 1987 and 1988 in Brittany from influenza-infected swine made it possible to isolate and antigenically characterize two H1N2 recombinant viruses (Sw/France/5027/87 and Sw/France/5550/88). The former virus was cloned and reinoculated to swine to allow reproduction of the disease and reisolation of a strain similar to the original one. The serodiagnostic tests carried out on both the original sera and those from the experimentally infected animals confirmed that the virus was actually type Sw/H1N2.

Introduction

In 1980, the swine influenza virus H1N1 (SwH1N1) was isolated for the first time in France from pigs presenting multimicrobial conditions [13, 14]. A sero-epidemiological investigation initiated in 1981 showed that the virus had spread across the French territory [11, 12].

It was also in the course of 1981 that the first type A/H3N2 influenza virus, antigenically related to A/Port Chalmers/1/73, was isolated in France from apparently healthy swine [7]. This virus reappeared in 1984, in an epizootic form [25].

Since then, epidemiological surveillance allowed the isolation of numerous strains of both types. Both viruses circulate separately or jointly in the same geographical areas. H1N1 viruses are steadily demonstrated throughout the year, while H3N2 strains are isolated only episodically during outbreaks of a few months' duration, with 2–3 year intervals [19]. Antigenic analysis of all the isolated strains revealed a relative stability of the H1N1 sub-type but also, in contrast, a drifting of H and N antigens from the H3N2 sub-type. Viruses of this sub-type caused disease in a wavelike recurring pattern in 1984, 1987 and 1989/1990: antigenic drift was observed at the beginning of each new wave [19].

Several studies have shown the ability of influenza viruses to recombine [8, 45, 46]. Recombinations can be obtained both in vivo and in vitro or may occur spontaneously in nature. Avian species are a favourable medium for such recombinations [20] and therefore, many recombinants were isolated from wild birds [17, 42]. In swine, however, only two recombinant viruses have been described. They occurred in Japan: one in 1978 [29, 43] and the other in 1980 [30, 47].

In this study, we report the demonstration of two new recombinant H1N2 influenza viruses antigenically characterized by means of polyclonal and monoclonal antibodies. Only the first of the two viruses (Sw/5027/87) was thoroughly studied: cloning and experimental inoculation to the swine; comparison between excreted strains and the original strain; humoral response of naturally or experimentally infected animals.

Material and methods

Viruses

The (H1N2) Swine/France/5027/87 strain was isolated in July 1987 in nasal swab samples from diseased swine belonging to a farm located in Côtes d'Armor (Brittany).

The (H1N2) Swine/France/5550/88 strain was demonstrated in July 1988 in lung samples from a diseased swine on a farm located in Finistère (Brittany).

We used porcine, human and avian strains. Porcine origin: Swine/France/2899/82 (H1N1), Swine/France/4510/86 (H1N1), Swine/France/6608/89 (H1N1), Swine/France/3633/84 (H3N2), Swine/France/4955/87(H3N2), Swine/France/523/90 (H3N2). These were all isolated in the Laboratoire Central de Recherches Vétérinaires.

Human origin: A/Port Chalmers/1/73 (H3N2) (A/PC/1/73), A/Bangkok/1/79 (H3N2) (A/BK/1/79), A/Bangkok/2/79 (H3N2) (A/BK/2/79), A/Philippines/2/82 (H3N2) (A/Phil/2/82), A/Sichuan/2/87 (H3N2) (A/Sich/2/87), A/Shanghai/16/89 (H3N2) (A/Shang/16/89), A/Beijing/353/89 (H3N2) (A/Beij/353/89), A/Beijing/32/92 (H3N2) (A/Beij/32/92), A/Singapore/6/86 (H1N1) (A/Sing/6/86), A/Taïwan/1/86 (H1N1) (A/Twn/1/86), A/Victoria/36/88 (H1N1) (A/Vic/36/88). These were reference reagents supplied by the WHO International Collaborative Centre for Reference and Research on Influenza Virus, London.

Avian origin: Tk/France/4993/87 (H1N1), a strain antigenically similar to 1987 porcine H1N1 viruses.

All these viruses were multiplied in the allantoid of hen's eggs after 9 days of embryogenesis or, for cloning purposes, on dog kidney cells, using a continuous cell line (MDCK) in the presence of trypsin.

Sera

Only six animals from the Côtes d'Armor farm were submitted to two blood sample collections, the first at the time of nasal swab collection and the second one month later.

Several antisera were prepared in ferrets using swine, turkey and human H1N1 and H3N2 strains. An antiserum to Sw/5550/88 was also produced.

An antiserum to Sw/5027/87 was prepared in hens 10 days after intravenous and intraperitoneal inoculation of the virus (10^8 EID_{50}).

Monoclonal antibodies

Monoclonal antibodies to H and N were produced against H1N1 Sw/2899/82, H3N2 Sw/3633/84 and A/PC/1/73 viruses [4].

These antibodies were prepared by fusion of Balb/C immunized mouse splenocytes with SP2/0-A 14 myeloma cells [39]. Hybrid cells were selected using "HAT" medium [23]. Antibody-producing hybridomas were cloned by the technique of limit dilution, then inoculated to pristane-treated Balb/C mice.

Experimental animals

Landrace pigs, weighing 20–25 kg, were raised on a traditional pig farm located in the countryside near Paris. A blood test carried out some time before the experiment confirmed the absence of influenza antibodies in those animals.

Molecular analysis

The sequence was determined using a series of oligonucleotide primers [34] based on the A/BK/1/79 neuraminidase sequence [28], dideoxynucleotides and vRNA template as described previously [9]. The primers used, numbered according to the sequence of A/BK/1/79 neuraminidase cDNA [28], had the following configuration:

20-ATGAATCCAAATCAA-34; 161-AACCAAGTAATG-172;
338-CGGCTTTCTGCT-349; 741-TGACTGATGGA-751;
1070-GGCTGGGCCTTT-1081 and 1352-GTGTTTTGTGGC-1363.

Virus cloning

The virus was cloned using MDCK cells [10]. The maintenance medium was made of equal parts of indubiose, containing 1% saline, and Leibovitz medium; 5 µg/ml TPCK trypsin (Worthington Biochemical Corporation, Freehold, U.S.A.) was added to the mixture. Plaques were collected after 4 days of incubation at 37 °C; 5 successive subcultures were carried out with 6 initially selected plaques. All the strains obtained from each subculture were reinoculated to eggs for subsequent antigenic characterization.

Biological tests

Hemagglutination and hemagglutination inhibition tests were performed according to standardized procedures [32].

Neuraminidase titration and neuraminidase inhibition tests were performed according to the technique described by Aymard et al. [5].

Experimental inoculation to swine

Three pigs without influenza antibodies were confined to an isolated area maintained under negative pressurization to prevent the virus from spreading outside.

Only pigs 1 and 2 received 10^7 EID_{50} of virus by the intratracheal [27] and intranasal routes. The third animal was placed in the same area and they stayed together until the end of the experiment (1 month duration). During the first 11 days, the clinical pathognomonic signs of the disease were recorded: cough, running nose, prostration and loss of appetite. Rectal temperature was recorded three times daily during the first five

days, then once daily until the end of the experiment; besides, nasal and rectal swabs were collected twice daily for the first 11 days. The animals were sacrificed after blood sample collection at day 30.

Results

Antigenic analysis of isolated Sw/France/5027/87 and Sw/France/5550/88 viruses

Characterization of the hemagglutinin

The hemagglutination inhibition by means of ferret polyclonal sera shows that it is the same as the characterization of H1N1/swine prototype viruses circulating in France since 1981 (Table 1).

In addition, the antiserum prepared against strain Sw/France/5027/87 reacted with the hemagglutinin of the three H1N1 prototype viruses but not with that of H3N2 virus.

Neither did monoclonal antibodies to virus Sw/France/2899/82 (H1N1) exhibit any antigenic difference between the haemagglutinin of the two strains studied and that of H1N1 prototype viruses. However, we noted the absence of a response of the H76 monoclone to strain Sw/France/4510/86 (H1N1) which, in fact, proves slightly different from other H1N1 viruses isolated between 1982 and 1989. This monoclone allowed the detection of certain variants of H1N1 viruses which occurred during that period.

The two H1 swine reassortant isolates were compared with contemporary human H1N1 reference strains (Table 2). Using either ferret sera or monoclonal antibodies, the strains were easily distinguishable from the human strains and they all proved closely related to each other and to the swine H1N1 current isolates.

Characterization of the neuraminidase

The neuraminidase of both viruses was compared to that of several H1N1 and H3N2 viruses isolated from swine and humans in France. The antigenic characterization of these prototype viruses has been published elsewhere [7, 19]. The three porcine H3N2 viruses selected for this study (Sw/France/3633/84, Sw/France/4955/87 and Sw/France/523/90) occurred during three successive influenza epidemics (1984–1987–1990) and can be considered as representative of the other viruses isolated during the same epidemic.

The results obtained with the ferret antisera and monoclonal antibodies are reported in Tables 3 and 4.

The neuraminidase activity of both viruses is inhibited by ferret antisera to the H3N2/swine viruses isolated in 1984 and 1987 with titres similar to those obtained with the homologous viruses. A similar result is obtained with the antiserum to A/PC/1/73 (H3N2), a virus isolated in man and related with the H3N2 viruses that have affected swines in France since 1984 [7, 19]. The antisera to human H3N2 strains which occurred after A/PC/1/73 do not interact

Table 1. Hemagglutinin identification of influenza viruses Sw/France/5027/87 and Sw/France/5550/88

Viruses		Post infection ferret sera anti					Monoclonal antibodies anti Sw/2899/82 (H1N1) (ascitic fluids)				
		Sw/2899/82 H1N1	Sw/4510/86 H1N1	TY/4993/87 H1N1	Sw/3633/84 H3N2	Sw/5550/88	H16	H22	H58	H60	H76
Sw/2899/82	H1N1	*640*	40	160	<	80	3 200	6 400	12 800	3 200	> 25 600
Sw/4510/86	H1N1	160	1 280	80	<	160	3 200	3 200	6 400	1 600	< 100
Ty/4993/87	H1N1	640	160	*2 560*	<	160	3 200	3 200	12 800	3 200	> 25 600
Sw/6608/89	H1N1	640	640	1 280	<	160	800	1 600	12 800	3 200	> 25 600
Sw/3633/84	H3N2	<	<	<	*10 240*	<	<	<	<	<	<
Sw/5027/87		640	1 280	640	<	640	6 400	12 800	12 800	3 200	> 25 600
Sw/5550/88		320	320	640	<	*640*	6 400	6 400	12 800	3 200	> 25 600
Clones											
	5A	1 280	640	1 280	<	320	12 800	12 800	12 800	3 200	> 25 600
	5B	2 560	1 280	2 560	<	640	12 800	12 800	12 800	6 400	> 25 600
	5D	1 280	640	1 280	<	320	12 800	12 800	12 800	3 200	> 25 600
	5E	1 280	640	2 560	<	320	12 800	12 800	12 800	3 200	> 25 600
Post experimental infection											
	176	640	2 560	640	<	1 280	12 800	12 800	12 800	6 400	> 25 600
	178	640	2 560	1 280	<	1 280	12 800	12 800	12 800	3 200	> 25 600
	190	1 280	2 560	1 280	<	2 560	12 800	12 800	12 800	6 400	> 25 600
	193	640	2 560	1 280	<	> 2 560	12 800	12 800	12 800	3 200	> 25 600
	197	640	2 560	640	<	1 280	12 800	12 800	12 800	3 200	> 25 600
	198	640	1 280	1 280	<	> 2 560	12 800	12 800	12 800	3 200	> 25 600
		< = < 40							< = < 100		

Table 2. Hemagglutinin characterization of influenza viruses Sw/France/5027/87 and Sw/France/5550/88 using H1N1 ferret sera and MAB

	A/FT DIX/ 741/76 F 221	A/SING 6/86 F 559	A/TWN/ 1/86 Ccdc 91002	A/VIC 36/88 F 658	Sw/FR 2899/82 F 510	Sw/FR 4510/86 F 576	Sw/FR 5550/88 F 657	MAB WIC 120 A/SING/6/86			MAB FIN/51/HG/82					
								H 58	H 139	H 375	H 16	H 22	H 58	H 60	H 76	H 82
A/New Jersey/8/76	1 280	<	<	<	320	40	40	<	<	<	<	1 600	100	<	<	<
A/FT DIX/741/76	2 560	<	<	<	640	160	160	<	<	<	<	1 600	100	<	> 12 800	> 12 800
A/Singapore/6/86	<	*640*	320	2 560	40	40	40	6 400	6 400	3 200	<	<	<	<	<	<
A/Taiwan/1/86	<	160	*320*	1 280	40	<	<	6 400	200	800	<	<	<	<	<	<
A/Victoria/36/88	40	640	320	*10 240*	80	40	40	12 800	12 800	100	<	<	<	<	<	<
Sw/France/2899/82	640	<	<	<	*640*	40	80	<	<	<	3 200	3 200	12 800	3 200	> 12 800	> 12 800
Sw/France/4510/86	<	<	<	<	160	*1 280*	160	<	<	<	3 200	3 200	6 400	1 600	<	<
Sw/France/5027/87	320	<	<	<	640	1 280	640	<	<	<	6 400	12 800	12 800	3 200	> 12 800	> 12 800
Sw/France/5550/88	320	<	<	<	320	320	*640*	<	<	<	6 400	6 400	12 800	3 200	> 12 800	> 12 800
			< = < 40								< = < 100					

Table 3. Neuraminidase identification of influenza viruses Sw/France/5027/87 and Sw/France/5550/88

Viruses	Post infection ferret sera anti									Monoclonal antibodies anti N2				
	Sw/2899/82 H1N1	Sw/3614/84 H1N1	A/CHILI/1/83 H1N1	A/SIN/6/86 H1N1	Sw/3633/84 H3N2	Sw/4955/87 H3N2	Sw/5550/88 H1N2	A/PC/1/73 H3N2	A/TEX/1/77 H3N2	Anti A/PC/1/73	Anti Sw/3633/84			
										NC1	NC17	NC22	NC34	NC42
Sw/2899/82	*2 560*	6 400	<[a]	160 p[b]	<	<	<	<	<	<[c]	<	800 p	<	<
A/SIN/6/86	–	–	–	10 240	<	<	<	<	<	<	<	<	<	<
A/PC/1/73	<	<	<	<	2 560	160	40 p	*10 240*	160 p	25 600	<	1 600	3 200	<
A/SIC/2/87	–	–	–	–	–	–	–	–	–	<	<	<	<	<
Sw/3633/84	<	<	<	<	*2 560*	640	320	1 280	<	<	1 600	25 600	1 600	1 600
Sw/4955/87	<	<	<	<	1 280	*1 280*	960	640	<	<	1 200	12 800	2 400	800
Sw/523/90	<	<	<	<	320	160	<	320 p	40 p	<	<	6 400	1 600	<
Sw/5027/87	160 p	160 p	<	160 p	640	640	1 280	640	<	<	800	12 800	2 400	1 600
Sw/5550/88	<	<	<	<	1 280	640	2 560	2 560	<	<	800	12 800	3 200	1 600
Sw/5027/87 Clones														
5A	<	<	<	160 p	640	640	640	640	<	<	400	10 000	1 600	800
5B	<	<	<	40 p	640	320	640	640	<	<	400	10 000	1 600	800
5D	<	<	<	160 p	640	640	1 280	2 560	40 p	<	800	10 000	1 200	800
5E	<	<	<	160 p	640	640	960	640 p	<	<	800	3 200	1 600	800
Infection post experimental														
176	<	<	ND	<	640	640	1 280	640	<	<	400	12 800	3 200	800
178	<	<	ND	<	640	640	1 280	640	<	<	800	10 000	1 600	800
190	<	<	ND	<	640	640	960	1 280	<	<	800	6 400	3 200	800
193	<	<	ND	<	640	640	960	160	<	<	800	25 600	3 200	800
197	<	<	ND	10	640	640	1 280	640	<	<	1 600	10 000	3 200	800
198	<	<	ND	20	640	1 280	960	640	<	<	800	10 000	6 400	800

[a] < = < 10

[b] p: partial inhibition. The inhibition does not exceed 50% up to the dilution indicated here
The partial inhibition is never observed in the homologous antibody response but appears in the cross neuraminidase inhibition test

[c] < = < 100

Table 4. Neuraminidase characterization of H1N2 swine strains

Reference strains	Dilution test	Monoclonal antibodies					Post infection ferret sera										
		A/PC/1/73 MRC 11	Sw/3633/84				Sw/ 3633/84	Sw/ 4955/87	Sw/ 5550/88	A/PC/1/73 MRC 11	A/SICH/ 2/87	A/SHANG 16/89	A/BEIJ/ 353/89 x 109	A/BEIJ/ 32/92	Sw/ 4510/87	Sw/ 6578/89	A/VIC/ 36/88
		NC1	NC17	NC22	NC34	NC42	H3N2 Sw		H1N2 Sw			H3N2			H1N1 Sw		H1N1
Sw/3650/84 H3N2 Sw	1/40	<	400	25 600	1 600	1 600	*1 280*	640	480	1 280	p	160	<	p	<	<	<
Sw/4955/87 H3N2 Sw	1/30	<	400	9 600	800	800	320	*480*	640	960	20	p	<	<	<	<	<
Sw/684/92 H3N2 Sw	1/60	<	<	25 600	1 600	<	960	640	40	2 560	p	80	<	<	<	<	<
Sw/5027/87 H1N2 Sw	1/20	<	400	12 800	3 200		320	160	640	320	<	160	<	<	<	<	<
Sw/ 5550/88 H1N2 Sw	1/40	<	400	12 800	1 600	800	160	320	*640*	320	<	40	<	<	<	<	<
A/PC/1/73 (MRC 11) H3N2	1/60	25 600	<	400	1 600	800	320	<	<	*2 560*	40	<	<	<	<	<	<
A/SICH/2/87 H3N2	1/15	<	<	<	<	<	<	<	<	160	*640*	160	40	<	<	<	<
A/SHANG/16/89/H3N2	1/15	<	<	<	<	<	<	<	<	<	40	*640*	40	80	<	<	<
A/BEIJ/353/89 H3N2	1/10	<	<	<	<	<	<	<	<	<	<	40	*240*	40	<	<	<
A/BEIJ/32/92 H3N2	1/30	<	<	<	<	<	<	<	<	<	40	480	240	*640*	<	<	<
Sw/4510/87 H1N1 Sw	1/10	<	<	<	<	<	<	<	<	<	<	<	<	<	*640*	<	<
Sw/6578/89 H1N1 Sw	1/20	<	<	<	<	<	<	<	<	<	<	<	<	<	<	*320*	<
A/TAIW/1/86 H1N1	1/10	<	<	<	<	<	<	<	<	<	<	<	<	<	<	30	320

(or only partially interact) with the two viruses Sw/France/5027/87 and Sw/France/5550/88. Ferret antisera to both H1N1 porcine strains and human strains do not react (or only partially) with the neuraminidase of these two viruses.

The antiserum to virus Sw/France/5550/88 inhibits the neuraminidase activity of H3N2 sub-type viruses isolated from swine in 1984 and 1987, but not that of viruses of the same sub-type isolated in 1990. In contrast, this antiserum does not react with the neuraminidase of the swine H1N1 prototype virus.

The results obtained with ferret antisera show that the neuraminidase of viruses Sw/France/5027/87 and Sw/France/5550/88 belong to the N2 sub-type and that it is related with that of the H3N2 viruses isolated from swine between 1984 and 1987.

The results obtained with the six monoclonal antibodies to N2 prepared against Sw/France/3633/84 and A/PC/1/73 confirm the foregoing observations. Besides, it clearly seems that although the neuraminidase of swine H3N2 viruses underwent antigenic drift between 1987 and 1990, the neuraminidase of Sw/France/5027/87 and Sw/France/5550/88 is related to that of the swine H3N2 viruses of 1987.

Genetic studies

The antigenic analysis of the isolated viruses Sw/France/5027/87 and Sw/France/5550/87 showed that their N2 neuraminidase was identical to the N2 from the recent swine strains.

According to the details of the antigen analysis, this N2 swine antigen was probably of human origin. We decided to sequence, in priority, the N gene in order to compare human, swine and swine reassortant genes.

The sequence of the virus Sw/France/5027/87 was compared with the human A/BK/1/79 strain, the swine reference strain Sw/France/3633/84 and a swine virus Sw/France/457/89 (Sw H3N2) isolated in the same geographical area as Sw/France/5027/87 during the subsequent epidemic. We had sequenced the 4 viruses from nucleotide (Nu) 70 to Nu 500, from Nu 855 to Nu 885, from Nu 1158 to Nu 1189 and from Nu 1399 to Nu 1464.

Compared to the human sequence, the swine H1N2 strain Sw/France/5027/87 varies at 30 Nu positions and only 11 of these results in amino acid differences: 20 Ile → Met, 43 Ser → Asp, 46 Pro → Ala, 54 Glu → Lys, 56 Ile → Met, 60 Arg → Lys, 62 Ile → Thr, 77 Ile → Val, 113 Gly → Ala, 143 Lys → Arg and 385 Thr → Lys (Table 5). 25 of the Nu changes are shared with another swine strain and of 11 AA substitutions, 8 are also observed with another swine strain isolated from 1984 to 1989.

Table 5. Nucleotide and amino acid changes in the N2 of the human H3N2 A/Bangkok/1/79 (*A*) strain compared to the swine viruses Sw H3N2 Sw/France/3633/84 (*B*), Sw H1N2 Sw/France/5027/87 (*C*) and Sw H3N2 Sw/France/457/89 (*D*)

Viruses	Nucleotide no. and change	Amino acid no. and change
A → C and D	79 A → G	20 Ile → Met
A → B-C and D	146 A → G	43 Ser → Asp
	147 G → A	
A → B-C and D	155 G → C	46 Pro → Ala
A → C	179 G → A	54 Glu → Lys
A → C	187 A → G	56 Ile → Met
A → C and D	198 G → A	60 Arg → Lys
	199 A → G	
A → C	204 T → C	62 Ile → Thr
A → C and D	248 A → G	77 Ile → Val
A → C and D	257 G → C	113 Gly → Ala
A → C and D	447 A → G	143 Lys → Arg
	448 G → A	
A → C and D	1173 C → A	385 Thr → Lys

Cloning of virus Sw/France/5027/87 and antigenic analysis of the resulting clones

Virus cloning

Under our experimental conditions, virus Sw/France/5027/87 induces the formation of plaques heterogeneous in size (2–3 mm, 5–6 mm) after four days of incubation at 37 °C. Six plaques of the initial experiment, three of large size (A, B and C) and three of smaller size (D, E and F), were selected for later subcultures.

After five successive subcultures on MDCK cells, the six initial clones generated plaques of homogeneous size, large for series A, B and C, small for series D, E and F.

Antigenic analysis of the resulting clones

Antigenic analysis of all the clones obtained after each of the five subcultures was performed. The results were similar and independent of the size of plaques. Tables 1 and 3 show the characterization of four of these clones, representative of all the clones obtained. Tests carried out with polyclonal as well as monoclonal antibodies did not allow to differentiate them from the initial virus, with respect to both hemagglutinin and neuraminidase. However, it should be emphasized that the neuraminidase of the clones did not react with the specific ferret antisera to viruses H1N1 Sw/France/2899/82 and H1N1 Sw/France/3614/84.

Table 6. Immune response of infected pigs with Sw/France/5027/87 anti HA antibodies

Pig sera	Antigens							
	Sw/2899/82 H1N1	Sw/4510/86 H1N1	Sw/3633/84 H3N2	Sw/4955/87 H3N2	A/BK/2/79 H3N2	A/PHIL/2/82 H3N2	Sw/5027/87 H1N2	Sw/5550/88 H1N2
Natural infection								
– Acute phase (6)	<[a]	<	<	<	<	<	<	<
– Convalescent + 1 month (6)								
No 1	160	160	<	<	<	<	160	160
No 2	160	160	<	<	<	<	160	160
No 3	80	80	<	<	<	<	80	80
No 4	80	160	<	<	<	<	160	80
No 5	40	80	<	<	<	<	40	80
No 6	80	80	<	<	<	<	80	80
Experimental infection								
– Pre inoculation (3)	<	<	<	<	<	<	<	<
– Post inoculation + 1 month (3)								
No 1	80	80	<	<	<	<	160	160
No 2	80	80	<	<	<	<	160	160
No 3	80	80	<	<	<	<	80	80
Chicken anti Sw/5027/87	1 280	1 280	20	20	<	<	*1 280*	*1 280*

Number of tested animals in brackets

[a] < = <20

Table 7. Immune response of infected pigs with Sw/France/5027/87 anti NA neuraminidase

Pig sera	Antigens							
	Sw/2899/82 H1N1	A/SING/6/86 H1N1	Sw/3633/84 H3N2	Sw/4955/87 H3N2	A/PC/1/73 H3N2	A/SICH/2/87 H3N2	Sw/5027/87 H1N2	Sw/5550/88 H1N2
Natural infection								
– Acute phase (6)	<[a]	<	<	<	<	<	<	<
– Convalescent + 1 month (4)								
No 1	<	<	240	160	<	20	640	240
No 4	<	<	240	320	40 p	<	960	640
No 5	<	<	120[b]	240	<	<	640	240
No 6	<	<	80 p	160	10	<	240	320
Experimental infection								
– Pre inoculation (3)	<	<	<	<	<	<	<	<
– Post inoculation + 1 month (3)								
No 1	<	<	60	160	<	<	960	2 560
No 2	<	<	60	160 p	<	<	640	480
No 3	<	<	40	40	40 p	<	640	640
Chicken anti Sw/5027/87	<	<	1 280	960	40	10	2 560	2 560

[a] < = < 10
[b] Fig. = NI antibody titres
p = Partial inhibition

Inoculation of virus Sw/France/5027/87 to the swine

Clinical observations

The two inoculated swine and the swine placed in contact with them presented the characteristic clinical signs of influenza within 24 h following inoculation: running nose, cough, prostration, hyperthermia. Besides, digestive signs (loss of appetite, diarrhea) developed on the fourth day and persisted one week in the inoculated swine and two weeks in the contact swine. In the three animals, a hemagglutinating virus was isolated from nasal and rectal swab collections until the 5th day and seroconversion with significant titres in response to strain H1N2 Sw/France/5027/87 was observed.

Antigenic analysis of the isolated influenza virus strains

Antigenic analysis of the various strains isolated during the five days of excretion was performed using polyclonal and monoclonal antibodies. All isolates proved identical for each animal and independent of the site of virus development or time of collection. Tables 1 and 3 show the results obtained with 6 of these isolates. The hemagglutinins of the excreted viruses belonged to sub-type H1: they could not be differentiated from those of the inoculated virus Sw/France/5027/87.

The neuraminidase from excreted viruses, sub-type N2, was nearly identical to that of the inoculated strain. However, it did not react with the ferret antisera to H1N1 viruses Sw/France/2899/82, Sw/France/3614/84 and A/Sing/6/86.

Humoral response to experimental infection

The results reported in Tables 6 and 7 show that the animals, which initially lacked antibodies to influenza, seroconverted in response to strain H1N2 one month after inoculation, in accordance with the antigenic characterization of the inoculated virus. No trace of antibodies to H3 sub-type HA or N1 sub-type NA was found.

Thus, inoculation of virus H1N2 Sw/France/5027/87 to the swine made it possible to experimentally transmit the disease, reisolate a virus identical to the initial one and confirm the antigenic characterization of this virus.

Humoral response of swine to natural infection by virus Sw/France/5027/87

Two blood samples were obtained from six animals from which strain Sw/France/5027/87 was derived: one during the acute phase of disease, the second one month later. Tests for antibodies to hemagglutinin and neuraminidase were carried out by inhibition of hemagglutination and neuraminidase activity. The results, reported in Tables 6 and 7, show that all animals seroconverted to H1 and N2 glycoproteins, in accordance with the antigenic characterization of the isolated virus. These results and those of experimental infection are identical. No trace of antibodies to H3 or N1 was found.

Discussion

The present study reports the isolation of an influenza virus with the unusual association of type H1 hemagglutinin and type N2 neuraminidase from the swine. This H1N2 virus was the only virus isolated in this limited outbreak which occured in unvaccinated pigs. We consider the virus to be a recombinant since the immune response of experimentally-infected animals was solely directed against H1 and N2 antigens with cloned virus.

The possibility of accidental production of a recombinant virus in the laboratory can be ruled out since humoral response of swines to infection by virus Sw/France/5027/87 was limited to one Côtes d'Armor farm and these swine exhibited exclusive seroconversion to H1 and N2 antigens of the isolated virus.

Isolation of recombinant viruses from the swine is very rare. Only two cases are reported in the literature, both of them in Japan, one in 1978 [29, 43], the other in 1980 [30, 47]. In both cases, an H1N2 combination was observed: hemagglutinin was similar to that of swine H1N1 viruses in Japan: neuraminidase was related to that of older strains of H3N2 viruses: A/Aichi/2/68.

Several hypotheses may account for such rare isolations: Although the phenomenon of recombination may affect the eight genome segments and give rise to multiple combinations, conventional analysis of the isolated strains only allows identification of recombinants affecting surface antigens.

Scholtissek [37] demonstrated the importance of the swine reservoir in the development of influenza A viruses. It would be interesting to determine the origin of the viral nucleoprotein (NP) for the swine reassortant H1N2 because its pathogenicity and epidemiological potency are different from those of the H3N2 and H1N1 swine viruses.

Virological examinations are not carried out systematically when influenza is suspected on a farm. Besides, when the veterinarian is consulted, it has generally been several days since the onset of disease and it is then difficult to isolate the virus.

For a recombination of this type to occur, a number of particular conditions are required, among which the simultaneous presence in time and space of two viruses capable to infect the same animal. Since 1984, these conditions have been favorable in Brittany, as H1N1 and H3N2 viruses circulate jointly on the pig-rearing farms of this French region [26] where the density of swine-rearing farms is particularly high.

Regarding the origin of the two recombinants, the results of antigenic and molecular analyses allow the assumption that the recombination event occurred in swine. The hemagglutinin is identical to that of H1N1 viruses circulating in swine since 1981 in France, and the neuraminidase is related to that of H3N2 viruses, isolated from swine in 1987, and distinct from that of viruses isolated during the 1989/1992 epidemics in humans. The sequencing of N2 genes from human (1979) and swine 1984–87-89 confirmed the swine origin of the H1N2 reassortant.

It is unlikely that recombinant Sw/France/5027/87 was formed on the rearing farm where it was isolated, as demonstrated by the absence of antibody to H3 or N1 in the six tested animals.

It is possible that recombination may have occurred in another animal or avian species, since the pig is not the only species infected by H1N1 and H3N2 sub-types influenza viruses. These viruses have also been observed in some fowls [1, 3, 16, 18, 21, 36, 41], especially ducks and turkeys.

In France (Brittany), H1N1 viruses were isolated from the turkey on three occasions (1983, 1985 and 1987) [2, 6] and once from pigeons (1985). These viruses proved antigenically identical to the porcine H1N1 virus circulating at the time [6, 15].

As to H3N2 virus, related with human virus A/PC/1/73, we have no data regarding its circulation in France both in domestic fowls and wild birds, but several H3N2 viruses of this sub-type were isolated in other parts of the world from certain wild birds, in particular the common murre [35].

Given the proximity of turkey and swine farms in Brittany, possible formation of these recombinant viruses in birds cannot be ruled out and this hypothesis is supported by the preliminary results of an on-going experiment on the Leghorn hen showing the ability of this virus to multiply in the chick.

Finally, H1N2 recombinants have also been reported in ducks, in Hong Kong [40], and in turkeys, in Israel [24], and an influenza virus of type H1N2 has also been isolated from a man in Japan [31] who had been simultaneously infected by H1N1 and H3N2 viruses.

However, it is not likely that man may have been the origin of the two recombinants isolated from the swine, since porcine H1N1 virus exhibits low infectivity for humans. This is confirmed by a continuous serological survey performed in children and adults with swine H1N1 and H3N2 viruses as antigens. A few sporadic cases were reported in different countries. The H3N2 virus circulating in swine is related to a human strain of this sub-type, isolated in 1973-1975 since then, this strain has been superseded by other variants.

Intratracheal inoculation of H1N2 Sw/France/5027/87 virus to susceptible swine reproduced standard clinical influenza similar to that induced under the same conditions by porcine H1N1 and H3N2 viruses. Unusual digestive signs (in particular diarrhea) were observed in all animals, inoculated or contact, and the virus was isolated several times from deep rectum swab samples, indicating that the virus multiplies in the intestine.

The observed virulence may have a relationship with the combination of an H1 hemagglutinin and an N2 neuraminidase, as suggested by Lipkind et al. [24], after isolating this combination from turkeys in 1984. However, as the virulence of influenza viruses is polygenic [22, 33, 38, 44], that of the recombinant will in fact be dependent on the whole gene pattern, even though the role played by the hemagglutinin and neuraminidase genes may be dominant.

Despite its pathogenicity and marked epidemiological potency, this recombinant virus has not, to date, caused any new outbreak in the same raising farm or in the neighbouring farms. This is probably due to the fact that

vaccination is widely performed in this area of the country, using a bivalent vaccine containing both strains Sw/H1N1 and Sw/H3N2. The vaccination coverage is estimated to be between 20 to 50% of the animals [19].

Should recombination between a strain adapted to man and a strain established in the porcine reservoir occur, a new virus might be produced. Having kept its adaptation to man, this virus might acquire a new antigenic constitution that would cause an influenza outbreak in a non-immunized human population. To date, however, no trace of these H1N2 viruses has ever been detected in humans: neither in Brittany swine farmers nor in the laboratory personnel exposed to many contamination hazards.

Acknowledgements

We are grateful to Mme J. Jolly-Million and Mlle S. Lambert for their technical assistance.

References

1. Alexander DD (1980) Isolation of influenza viruses from avian species in Great Britain. Comp Immunol Microbiol Infect Dis 3: 165–170
2. Andral B, Toquin D, Madec F, Aymard M, Gourreau JM, Kaiser C, Fontaine M, Metz MH (1985) Diseases in turkeys associated with H1N1 Influenza virus following an outbreak of the diseases in pigs. Vet Rec 116: 617–618
3. Arikawa J, Yamane N, Totsukawa K (1983) Antigenic and genetic characterization of H1 influenza viruses isolated from feral ducks and swine in Japan. Arch Virol 78: 19–27
4. Aymard M, Chomel JJ, Thouvenot D, Gourreau JM, Kaiser C (1986) Le diagnostic rapide de la grippe porcine grâce aux anticorps monoclonaux. VIème Colloque Franco-Soviétique sur la Grippe, Lyon, 19–20 juin 1986, pp 1–13
5. Aymard Henry M, Coleman MT, Dowdle WR, Laver WG, Schild GC, Webster RG (1973) Influenza neuraminidase and neuraminidase inhibition test procedures. Bull World Health Organ 48: 199–202
6. Aymard M, Douglas AR, Fontaine M, Gourreau JM, Kaiser C, Million J, Skehel JJ (1985) Antigenic characterization of influenza A (H1N1) viruses recently isolated from pigs and turkeys in France. Bull World Health Organ 63: 537–542
7. Aymard M, Gourreau JM, Kaiser C, Fontaine M, Madec F, Tillon JP (1985) Les marqueurs immunovirologiques du risque d'Influenza A H3N2 chez les porcs. Rev Epidemiol Santé Publique 33: 283–291
8. Bean WJ Jr, Cox NJ, Kendal AP (1980) Recombination of human influenza A viruses in nature. Nature 284: 638-640
9. Daniels RS, Douglas AR, Skehel JJ, Wiley DC (1983) Analysis of the antigenicity of influenza haemagglutinin at the pH optimum for virus-mediator membrane fusion. J Gen Virol 64: 1657–1662
10. Fiszon B (1988) Contribution à l'étude de la circulation du virus de la grippe dans les populations animales. Thèse de Doctorat Vétérinaire, Nantes 1988
11. Gourreau JM, Aymard M, Kaiser C, Fontaine M, Vigouroux A, Salingardes F, Madec F, Labie J, Tillon JP (1983) Utilisation de la technique de l'hémolyse radiale dans une enquête épidémiologique sur la grippe porcine en France. Proc. of the 3rd Intern. Symposium of the World Ass. of Vet. Laboratory Diagnosticians, 13–15 juin 1983, Ames, (Iowa), pp 429–437

12. Gourreau JM, Hannoun C, Kaiser C (1981) Diffusion du virus de la grippe du porc (Hsw1 N1) en France. Ann Virol Inst Pasteur 132E: 287–294
13. Gourreau JM, Kaiser C, Hannoun C, Vaissaire J, Gayot G (1980) Premier isolement en France du virus de l'influenza du porc (Hsw1 N1) dans un environnement pathologique plurimicrobien. Bull Acad Vet France 53: 181–188
14. Gourreau JM, Kaiser C, Labie J, Duée JP, Gilles G, Letroteur R (1981) La grippe porcine en France. Point Vet 11: 75–82
15. Hinshaw VS, Alexander M, Aymard M, Bachmann PA, Easterday BC, Hannoun C, Kida H, Lipkind M, Mackenzie JJ, Nerome K, Shild GC, Scholtissek C, Senne DA, Shortridge KF, Skehel JJ, Webster RG (1984) Antigenic comparisons of swine-influenza like H1N1 isolates from pigs, birds and humans: an international collaborative study. Bull World Health Organ 62: 871–878
16. Hinshaw WS, Webster RG, Bean WJ, Downie J, Senne DA (1983) Swine influenza-like viruses in turkeys: potential source of viruses for humans? Science 220: 206–208
17. Hinshaw VS, Webster RG, Rodriguez RJ (1979) Influenza A viruses: combinations of hemagglutinin and neuraminidase subtypes isolated from animals and other sources. Arch Virol 62: 281–329
18. Hinshaw VS, Webster RG, Turner B (1980) The perpetuation of orthomyxovirus and paramyxovirus in Canadian waterfowl. Can Microbiol 26: 622–629
19. Kaiser C, Valette M, Million-Jolly J, Lambert S, Labie J, Madec F, Gourreau JM, Aymard M (1991) Mise en évidence de variations des antigènes de surface du virus grippal H3N2 chez le porc en France à l'aide d'anticorps monoclonaux. Bull Epidémiol Sante Anim 19: 75–84
20. Kawaoka Y, Chambers TM, Sladen WL, Webster RG (1988) Is the gene pool of influenza viruses in shorebirds and gulls different from that in wild ducks? Virology 163: 247–250
21. Kida H, Kawaoka Y, Naeve CW, Webster RG (1987) Antigenic and genetic conservation of H3 influenza virus in wild ducks. Virology 159: 109–119
22. Kilbourne ED (1987) Virulence and attenuation as genetic phenomena. In: Kilbourne ED (ed) Influenza. Plenum Press, New York, pp 129–136
23. Kohler G, Milstein C (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256: 495–497
24. Lipkind M, Weisman Y, Shihmanter E (1984) Isolation of viruses antigenically related to the swine influenza virus from an outbreak of respiratory disease in turkeys farms in Israel. Vet Rec 114: 426–428
25. Madec F, Gourreau JM, Kaiser C, Aymard M (1984) Apparition de manifestations grippales chez les porcs en association avec un virus A H3N2. Bull Acad Vet France 57: 513–522
26. Madec F, Kaiser C, Jestin A, Kobisch M, Onno M, Vannier P, Aymard M, Gourreau JM (1978) Les syndromes grippaux en porcherie d'engraissement. Résultats d'une enquête "flash" réalisée en Bretagne. Point Vet 19: 654–662
27. Maes L, Haesebrouck F, Pensaert M (1984) Experimental reproduction of clinical disease by intratracheal inoculation of fattening pigs with swine influenza virus isolates. Proc 8th Congr Int Pig Vet Soc Ghent, p 60
28. Martinez C, Del Rio L, Portella A, Domingo E, Artin J (1983) Evolution of the Influenza virus neuraminidase gene during drift of the N2 subtype. Virology 130: 539–545
29. Nerome K, Sakamoto S, Yano N, Yamamoto T, Kobayashi S, Webster RG, Oya A (1983) Antigenic characteristics and genome composition of a naturally occurring recombinant influenza virus isolated from a pig in Japan. J Gen Virol 64: 2611–2620

30. Nerome K, Yoshioka Y, Sakamoto S, Yasuhara M, Oya A (1985) Characterization of a 1980 – swine recombinant influenza virus possessing H1 hemagglutinin and N2 neuraminidase similar to that of the earliest Hong-Kong (H3N2) virus. Arch Virol 86: 197–211
31. Nishikawa F, Sugiyama T (1983) Direct isolation of H1N2 recombinant virus from a throat swab of a patient simultaneously infected with H1N1 and H3N2 influenza A viruses. J Clin Microbiol 18: 425–427
32. Palmer DF, Coleman MT, Dowdle WR, Schild GC (1975) Advanced laboratory techniques for influenza diagnosis. US Department of Health, Education and Welfare, Public Health Service, Centre for Disease Control, Atlanta. Immunology Series, vol 6. US Department of Health Atlanta, pp 25–62, 107–129
33. Rott R, Orlich M, Scholtissek C (1979) Correlation of pathogenicity and gene constellation of influenza A viruses. III. Nonpathogenic recombinants derived from highly pathogenic parent strains. J Gen Virol 44: 471–477
34. Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74: 5463–5467
35. Sazonov AA, Lvov DK, Webster RG (1977) Isolation of an influenza virus similar to A/Port Chalmers/1/73 (H3N2) from a common murre at Sakhalin Island. Arch Virol 53: 1–7
36. Scholtissek C, Burger H, Bachmann PA, Hannoun C (1983) Genetic relatedness of hemagglutinins of the H1 subtype of influenza A viruses isolated from swine and birds. Virology 129: 521–523
37. Scholtissek C, Schultz V, Ludwig S, Fitch WM (1992) The role of swine in the origin of pandemic influenza. In: Congress "Option for the control of influenza II", Sept. 26–Oct. 2, 1992, Courchevel, France
38. Schulman JL (1983) Virus-determined differences in the pathogenesis of influenza virus infections. In: Palese P, Kingsbury W (eds) Genetics of influenza viruses. Springer, Wien New York, pp 305–320
39. Schulman M, Wilde CD, Kohler G (1978) A better cell line for making hybridomas secreting specific antibodies. Nature 276: 269–270
40. Shortridge KF (1980) Isolation of ortho- and paramyxoviruses from domestic poultry in Hong Kong between November 1977 and October 1978 and comparison with isolations made in the preceding two years. Res Vet Sci 28: 296
41. Shortridge KF (1983) Avian influenza virus of Southern China and Hong-Kong Ecological aspects and implications for man. Bull OMS 60: 129–135
42. Stallknecht DE, Shane SM (1988) Host range of avian influenza virus in free-living birds. Vet Res Commun 12: 125–141
43. Sugimura T, Yonemochi H, Ogawa T, Tanaka Y, Kumagai T (1980) Isolation of a recombinant influenza virus (Hsw1 N2) from swine in Japan. Arch Virol 66: 271–274
44. Sweet C, Smith H (1980) Pathogenicity of influenza virus. Microbiol Rev 44: 303–330
45. Webster RG, Campbell CH (1972) The in vivo production of "new" influenza viruses. II. In vivo isolation of "new" viruses. Virology 48: 528–536
46. Webster RG, Campbell CH, Granoff A (1973) The "in vivo" production of "New" influenza virus III. Isolation of recombinant influenza virus under simulated conditions of natural transmission. Virology 51: 149–162
47. Yasuhara H, Hirahara T, Nakai M, Sasaki N, Kata J, Watanabe T, Morikawa M (1983) Further isolation of a recombinant virus (H1N2), formerly (Hsw1 N2) from a pig in Japan in 1980. Microbiol Immunol 27: 43–50

Authors' address: Dr. M. Aymard, Laboratoire de Virologie, Hospices Civils de Lyon, Domaine Rockefeller, 8 Avenue Rockefeller, F-69373 Lyon Cedex 08, France.

Received June 24, 1993