

Seroepidemiology Survey and Isolation of Swine Influenza Viruses from Subclinical Infections in Israel During the Years 2009-2011

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ABSTRACT

The exposure of the swine population in Israel to swine influenza viruses (SIV) was actively assessed during the years 2009-2011 by serological and virological assays. Around 90% of 777 sera from 52 herds and 2 wild boars were positive by ELISA. The antibody subtype specificity was determined on 407 sera from 27 herds by haemagglutination inhibition assay with 4 viruses, A/sw/Flandre/1/98(H3N2), A/sw/Scotland/410440/94(H1N2), A/sw/Cotes d'Armor/0388/09(H1N1) and A/ck/Israel/1525(H9N2). All herds had antibodies to SIV H1N2 and H3N2 while only 10 herds had antibodies to SIV H1N1. The highest HI titers against SIV H1N2, SIV H3N2 HI titers were of intermediate values, while SIV H1N1 exposure produced the lowest titers. No antibodies to AIV subtype H9N2 were detected. Sub-clinically infected pigs yielded five positive samples, of which two were identified as H3N2 and the pandemic H1N1, respectively. We suspect that these were acquired by contact of the pigs with infected humans.

Keywords: Swine Influenza Virus, Active Surveillance, Haemagglutination Inhibition, Virus Isolation, Real Time Reverse Transcription RRT-PCR, ESNIP 3.

INTRODUCTION

Influenza A virus infection of pigs is of great interest and concern worldwide, particularly since the emergence of the pandemic virus (A(H1N1)pdm09) in 2009. The circulating swine influenza viruses (SIVs) are extensively surveyed virologically and seroepidemiologically in many parts of the world, including North and South America (1), Europe (2), China (3) and other Far Eastern countries. Influenza viruses have the potential for rapid spread amongst the swine population. Swine are infected through contact with influenza-

infected humans (4), turkeys (5) and other birds, and are in a continuous course of genetic change. Airway epithelia of the upper respiratory tract of pigs express the two types of cellular sialic acid receptors that preferentially bind avian (NeuAc -alpha2,3-Gal) and human (NeuAc -alpha2,6-Gal), influenza viruses facilitating the formation of inter-species reassortments. As a result, the emergence of new SIV variants with potential zoonotic significances is a realistic scenario, and pigs can serve as mixing vessels for the generation of new human and avian viruses.

The main enzootic SIV subtypes are H1N1, H3N2 and H1N2, except pandemic H1N1 virus that evaded the swine population since 2009, as reviewed by Zell *et al.* (6) European SIV lineages differ genetically from classical SIVs and from the triple (avian, human and swine) North American and Asian SIV reassortants. Avian-like H1N1 SIVs emerged in 1979 and spread to Europe. From further reassortment with the human seasonal H3N2, the human-like swine H3N2 SIV emerged in 1984. During 1994, the human-like swine H1N2 also emerged after reassortment of human-like swine H3N2 with human seasonal H1N1. These reassortants, comprising genes of avian and human origin, prevailed in European pigs and replaced the classical swine H1N1 viruses. Among the various SIV reassortant viruses, the most eminent SIV is A(H1N1)pdm09, which emerged in swine and caused the 2009 pandemic, and then re-infected the swine population. Emergence of A(H1N1)pdm09 resulted from the reassortment of two SIVs, the North American triple reassortant, H1N1 or H1N2 and the European avian-like H1N1 (2).

Active monitoring of swine influenza is conducted on apparently healthy, subclinically-infected pigs, side-by-side with passive monitoring, performed on clinically-affected pigs with apparent morbidity. Lately, subclinical infections became a topic of increased awareness as the movement and exhibition of pigs at agricultural fairs in the US presents a risk of SIV dissemination (7).

The present study reports for the first time evidence of subclinical SIV infections in Israel in apparently healthy animals, as in the previous serological survey, no evidence of SIV infection of Israeli pigs was demonstrated (8). We now illustrate an active serosurveillance of the swine population at slaughter and attempts to isolate virus from subclinically infected pigs. In so doing, the SIV subtype that prevailed in the most eastern European border of swine herds before entering the Far East was revealed.

MATERIALS AND METHODS

Israeli swine population

The yearly swine production, coordinated by the Israeli Veterinary Services, comprises around 200,000 pigs. Most farms are concentrated in four regions, three of them are located in the Northern part of the country and the fourth is located in the Southern part. The countries bordering Israel

do not focus on swine breeding; therefore, the swine population in Israel is relatively isolated and reflects the epidemiological situation in the most easterly European region, before the Far East.

ELISA

The presence of SIV nucleocapsid antibodies was determined in sera collected during the years 2009-2010 by ELISA using the ID Screen Influenza A Antibody Competition kit (IDVet Innovative Diagnostics, Fluaca ver 0509 GB), and on sera collected during 2011 using the LSIVET Suis Influenza (LSI version SIVL 002 VA – 070409). Both kits performed similarly.

Serum samples tested by the haemagglutination inhibition assay

Sera from 27 herds were obtained at slaughter from apparently healthy animals. Each was treated with receptor destroying enzyme (RDE) to eliminate competitive inhibitors of the haemagglutination inhibition (HI) test, according to the method of Hofling *et al.* (9), as recommended by the OIE (10), and then inactivated at 56°C for 30 minutes. Briefly, 50 µl of sera were incubated at 37°C for 1 hr with 100 Units/ml of RDE in calcium saline solution.

Reference sera and viruses

In the context of the ESNIP 3 Consortium, reference sera and viruses were distributed to all consortium members by Dr. Gaëlle Simon (Anses, Laboratoire National de Référence Pestes Porcines, Ploufragan, France). The reference viruses and their respective hyperimmune sera were:

1. A/sw/Flandre/1/98 H3N2
2. A/sw/Cotes d'Armor/0388/09 H1N1
3. A/sw/California/04/09 pH1N1
4. A/sw/Scotland/410440/94 H1N2
5. A/sw/Finistre/2899/82 H1N1

In addition, an avian influenza virus, subtype H9N2, A/ck/Israel/1525/H9N2 (11) was added to the HI panel.

Haemagglutination inhibition assay

The HI assay (10) was used for serological identification of SIV antibodies using the reference viruses and their hyperimmune sera. As the initial serum dilution was 1:10, the HI detection limit was 1:20. The mean HI titer of the herd was calculated on the basis of the titer dilution antilog.

Table 1: Subtype specificity of reference hyper-immune sera and viruses by the HI assay

Hyperimmune serum	H3N2 Flandre	H1N2 Scotland	pH1N1 California	H1N1 Cotes d'Armor
A/sw/Flandre/1/98 (H3N2)	1:5120	1:160	Negative	Negative
A/sw/Cotes d'Armor/0388/09 (H1N1)	1:20	1:80	1:160	1:2560
A/sw/California/04/09 (pH1N1)	1:20	1:80	1:1280	1:40
A/sw/Scotland/410440/94 (H1N2)	1:160	1:5120	1:160	Negative
A/sw/Finistre/2899/82 (H1N1)	1:20	1:80	1:320	1:320
Negative serum	Negative	Negative	Negative	Negative

SIV sampling and virus isolation

Five apparently healthy swine holdings were actively sampled. From each holding five different groups of 7-10 week-old piglets, housed separately, were sampled. From each group five animals were sampled for virus identification and isolation in embryonated specific pathogen free fowls' eggs using both dry and transport medium (Copan, Ltd., Brescia, Italy) soaked nasal swabs; a total of 125 swabs, each. The five animals from each group were pooled, obtaining 25 pools. Separate pools of dry and "wet" swabs in transport media were used to purify RNA directly, and to amplify the viruses in embryonated eggs, respectively. The RNA purification was carried out using the QIAamp Viral RNA Mini KIT (QIAGEN, GmbH, Germany) according to the manufacturer's instructions. The swabs in transport media were inoculated into allantoic cavities of 10-day-old embryonated eggs (SPAFAS, U.S.A.) which were incubated at 37° C for 6 days. The embryos were observed for 5 days after inoculation; those dead within the first day were discarded. Allantoic fluids from eggs in which embryos were dead from the second day post-inoculation, or from eggs with surviving embryos up to 5 days, were used to demonstrate the presence of SIV by the haemagglutination activity (HA) assay (10) and by molecular amplification of RNA purified from the allantoic fluid.

SIV detection by real-time reverse transcription and conventional PCR amplification

RNA samples were amplified by real-time reverse transcription PCR (RRT-PCR) for the SIV Matrix gene using the "perfect match" method described by Slomka et al. (12), as well as for the presence of A(H1N1)pdm09 (14-16), both by the TaqMan chemistry (Ambion Ag-Path, Life Technologies, USA). Conventional amplification of the RNA samples was performed using the multiplex RT-PCR

Table 2: Survey of antibodies to SIV nucleocapsid antigens by ELISA

Year	No. of swine herds	Total sera tested	No. total positive sera (%)	Mean (%) positive sera per herd \pm s.d.
2009	1 (wild boar)	1	1	100
	19	241	151 (63)	91.5 \pm 18.6
2010	1 (wild boar)	1	1	100
	21	346	306 (88)	84.6 \pm 30.4
2011	12	190	164 (86.3)	90.8 \pm 18.3

assay (Qiagen OneStep RT-PCR kit, QIAGEN) for differentiating European SIV subtypes H1N1, H1N2 and H3N2 as described by Chiapponi *et al.*, 2012 (13). The presence of A(H1N1)pdm09 was determined by RRT-PCR using TaqMan chemistry, with the RRT-PCR assay (Ambion Ag-Path master mix (Life Technologies, USA)) (14-16).

RESULTS AND DISCUSSION

Subtype specificity of reference viruses and their homologous and heterologous hyperimmune sera

Table 1 shows the assessment of the homologous and heterologous HI assays using reference prototype SIVs and hyperimmune sera raised against these viruses. It was apparent that the homologous HI reactivity of the four reference viruses, A/sw/Flandre/1/98 (H3N2), A/sw/Cotes d'Armor/0388/09 (H1N1), A/sw/California/04/09 A(H1N1)pdm09 and A/sw/Scotland/410440/94 (H1N2) was very robust, while the fifth virus, A/sw/Finistre/2899/82 (H1N1) was weaker, and the virus titer did not allow it to be included in the HI checkerboard matrix.

Active monitoring of antibodies to SIV in swine sera and determination of their subtype specificity

An initial serological survey was performed by ELISA to demonstrate the presence of antibodies to the SIV nucleocapsid antigens. Table 2 shows that 90% of the 777 swine sera tested, which were sampled between the years 2009-2011, were positive for SIV antibodies, as well as the sera from two wild boars. This therefore strongly suggests that the swine population in Israel was extensively exposed to SIV.

Next, the SIV subtype specificity was determined by HI on a total of 407 sera from 27 herds and on serum from one wild boar. The HI was performed with three SIV prototypes; namely, A/sw/Flandre/1/98 (H3N2),

A/sw/Scotland/410440/94 (H1N2) and A/sw/Cotes d'Armor/0388/09 (H1N1). As pigs are receptive to infection with avian influenza viruses, and as the AIV subtype33 H9N2 is endemic in Israel, an AIV H9N2 virus (A/ck/Israel/1525/H9N2) was included in the subtype specificity examination of the swine sera.

Table 3 presents the mean antilog titers of the HI assay performed with the four viruses. Firstly, it is evident that the swine sera did not possess any antibodies to AIV H9N2, indicating that although AIV H9N2 is endemic in Israel within the poultry population (11), and its biological ability to infect swine (17-19), it has not infected the pig population, at least not the animals sampled in the present study. All swine sera had antibodies to SIV H1N2 and H3N2, while SIV H1N1 HI antibodies were detected only in 10 herds. The highest HI titers were against SIV H1N2, SIV H3N2 HI with titers of intermediate values, while SIV H1N1 ex-

posure initiated antibody with the lowest titers. The wild boar sera contained a high antibody HI titer to SIV H1N1. No antibodies to H9N2 virus were detected.

Virus isolation and identification from subclinically infected, apparently healthy pigs

Table 4 shows the results of the PCR assays that were performed on seven samples obtained from the subclinically infected pigs. Five apparently healthy swine holdings were selected for investigation. From each holding, five groups of 7-10 week-old piglets were sampled. Five animals were swabbed from each of the 25 groups. Pools of five swabs, representing the five animals from each group, were examined both directly and after being passaged twice in embryonated fowls' eggs for virus replication.

RNA was purified from dry swabs and from allantoic fluids after virus amplification in embryonated eggs. In five cases, SIV was detected by RRT-PCR, three of them were positive in both RNAs, while the swab RNA from one case had a low C_T value, representing a low virus load, in the other case, the virus could be detected only after the egg inoculation had been performed.

The multiplex conventional PCR was used to identify the SIV subtype. Isolate A/sw/Israel/3/2011 had a high C_T value for the M-gene by RRT-PCR, was positive for N1, but not for H1 or H3, therefore, the isolate was suspected to be A(H1N1)pdm09 (Figure 1). By applying the RRT-PCR

Table 3: The subtype specificity of SIV antibodies by HI assay

Sampling date	Herd	No. sera	AIV H9N2	H3N2	H1N2	H1N1
28.01.2010	1	33	Negative	905	5120	Negative
05.08.2010	2	7	Negative	905	1810	Negative
11.01.2011	3	2	Negative	183	2228	Negative
11.01.2011	4	2	Negative	92	3151	Negative
12.01.2011	5	3	Negative	226	970	Negative
25.01.2011	6	2	Negative	184	1114	Negative
06.02.2011	7	3	Negative	1280	1575	Negative
15.02.2011	8	6	Negative	485	788	Negative
16.03.2011	9	20	Negative	32	2560	49
16.03.2011	10	20	Negative	69	905	12
21.03.2011	Wild boar		Negative	Negative	3151	6303
21.03.2011	11	20	Negative	160	3151	139
22.03.2011	12	20	Negative	519	1351	40
22.03.2011	13	20	Negative	211	1700	17
23.03.2011	14	10	Negative	905	735	14
23.03.2011	15	10	Negative	5120	2743	Negative
23.03.2011	16	10	Negative	452	1470	14
23.03.2011	17	10	Negative	686	3620	Negative
24.03.2011	18	20	Negative	905	5881	Negative
01.08.2011	19	20	Negative	485	2560	Negative
01.08.2011	20	20	Negative	1371	2940	40
01.08.2011	21	20	Negative	459	1940	Negative
04.08.2011	22	20	Negative	1575	2560	Negative
04.08.2011	23	20	Negative	367	1810	Negative
04.08.2011	24	20	Negative	442	2079	Negative
04.08.2011	25	20	Negative	970	3377	40
28.08.2011	26	20	Negative	905	1810	Negative
24.11.2011	27	29	Negative	1280	6303	Negative

Table 4: Virus identification from sub-clinically infected, apparently healthy pigs using RT-PCR

SIV samples	Real-time RT-PCR (C_T value) against M- gene using RNA purified from		SIV subtype specificity identification
	Nose Swab	Allantoic fluid	
A/sw/Israel/1/2011	32-36 ^A	Negative	Negative
A/sw/Israel/2/2011	Negative	Negative	Negative
A/sw/Israel/3/2011	27-30	22.0	pH1N1 ^B
A/sw/Israel/4/2011	27-30	30-34	Negative
A/sw/Israel/5/2011	27-30	34	Negative
A/sw/Israel/6/2011	Negative	Negative	Negative
A/sw/Israel/7/2011	Negative	17-19	H3N2 ^C

A – Range of C_T values obtained from several assay repeats performed at KVI and the AHVLA.

B - Multiplex cPCR for H1N1, H1N2 and H3N2 and real-time RT-PCR for pandemic H1N1

C - Multiplex cPCR for H1N1, H1N2 and H3N2

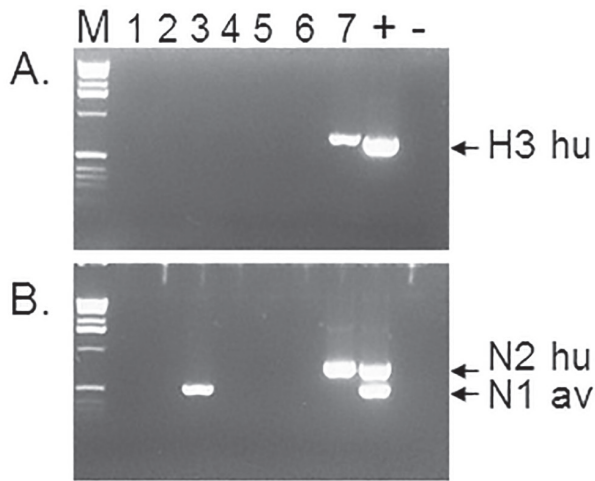


Figure 1: SIV subtype identification of the seven samples by conventional multiplex PCR based on Chiapponi *et al.*, 2012 (13). M: molecular size marker, 1 kb lambda ladder (Invitrogen, Ltd.); 1-7 suspected SIV samples, (+) SIV positive control, (-) Negative control. Panel A.: Identification of H3 human-like SIV subtype. The positive control was RNA purified from the prototype SIV A/sw/Flandre/1/98 H3N2; Panel B.: Multiplex identification of N1 avian-like and N2 human-like SIV subtypes. The positive control was a mix of RNA purified from prototypes A/sw/Scotland/410440/94 (H1N2) and A/sw/Finistre/2899/82 (H1N1). The same positive control, as in panel B was used for the multiplex identification of H1 avian-like and H1-human-like SIV subtypes with negative results (not shown).

against A(H1N1)pdm09, A/Sw/Israel/3/2011 was indeed identified as the pandemic virus (14-16).

Samples A/sw/Israel/4/2011 and A/sw/Israel/5/2011 reacted weakly with the RRT-PCR for the M-gene and by HA, and could not be subtyped. Sample A/Sw/Israel/7/2011 was positive by the M-gene RRT-PCR and was identified as SIV isolate subtype H3N2 by conventional PCR (Figure 1).

CONCLUSION

The present study provides evidence for the exposure of the Israeli swine population to all European SIV subtypes H1N2, H3N2 and H1N1 and also to A(H1N1)pdm09, based on our results from seroprevalence, on the one hand, and virus isolation, on the other. The virological evidence described here confirms the presence of SIV in subclinical infections, because of the asymptomatic clinical appearance and the low virus loads. Wild boars are also known to disseminate SIV, as evidenced now by the presence of antibodies to H1N1 in one wild boar. This study demonstrates for the first time the exposure to, and isolation of SIV from subclinically-infected

pigs in Israel, emphasizing the veterinary and public health importance of SIV infections in domestic and in wild pigs. As implicated previously (4), the two SIV isolates might be acquired by contact of the pigs with infected humans.

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REFERENCES

1. Nelson, M.I., Detmer, S.E., Wentworth, D.E., Tan, Y., Schwartzbard, A., Halpin, R.A., Stockwell, T.B., Lin, X., Vincent, A.L., Gramer, M.R. and Holmes, E.C.: Genomic reassortment of influenza A virus in North American swine, 1998-2011. *J. Gen. Virol.*: 93:2584-2589, 2012.
2. Brown, I.H.: History and Epidemiology of Swine Influenza in Europe. *Curr. Top. Microbiol. Immunol.* 370:133-46, 2013.
3. Su, S., Qi, W., Chen, J., Zhu, W., Huang, Z., Xie, J. and Zhang G.: Seroepidemiological evidence of avian influenza A virus transmission to pigs in southern china. *Clin. Microbiol.* 51:601-602, 2013.
4. Nelson, M.I., Gramer, M.R., Vincent, A.L. and Holmes, E.C.: Global transmission of influenza viruses from humans to swine. *J. Gen. Virol.* 93:2195-2203, 2012.
5. Yassine, H.M., Lee, C.W. and Saif, Y.M.: Interspecies transmission of Influenza A viruses between swine and poultry. *Curr. Top. Microbiol. Immunol.* 370:227-40, 2013.
6. Zell, R., Scholtissek, C. and Ludwig, S.: Genetics, Evolution, and the Zoonotic Capacity of European Swine Influenza Viruses. *Curr Top Microbiol Immunol.* 370:29-55, 2013.
7. Bowman, A.S., Nolting, J.M., Nelson, S.W. and Slemons, R.D.: Subclinical influenza virus A infections in pigs exhibited at agricultural fairs, Ohio, USA, 2009-2011. *Emerg. Infect. Dis.* 18:1945-1950, 2012.
8. Pozzi, S.P., Aborali, G., Cordioli, P. and Rosner, A.: Investigation of swine influenza subtypes H1N1, H3N2, H1N2 in pig population in Israel (2002-2009). *Isr. J. Vet. Med.* 65:11-14, 2010.
9. Hoffing, K., Klenk, H-D. and Herrler, G.: Inactivation of inhibitors by the receptor-destroying enzyme of influenza C virus. *J. Gen. Virol.*; 78:567-570, 1997.
10. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. World Organization for Animal Health. NB: Version adopted by the World Assembly of Delegates of the OIE in May 2010. Chapter 2. 8. 8. Swine Influenza.

11. Davidson, I., Shkoda, I., Golender, N., Perk, S., Lapin, K., Khinich, Y. and Panshin, A.: Genetic characterization of HA gene of low pathogenic H9N2 influenza viruses isolated in Israel during 2006-2012 periods. *Virus Genes*. 46:255-263, 2013
12. Slomka, M.J., Densham, A.L., Coward, V.J., Essen, S., Brookes, S.M., Irvine, R.M., Spackman, E., Ridgeon, J., Gardner, R., Hanna, A., Suarez, D.L. and Brown, I.H.: Real time reverse transcription (RRT)-polymerase chain reaction (PCR) methods for detection of pandemic (H1N1) 2009 influenza virus and European swine influenza A virus infections in pigs. *Influenza and other respiratory viruses*. 4:277-293, 2010.
13. Chiapponi, C., Moreno, A., Barbieri, I., Merenda, M. and Foni, E.: Multiplex RT-PCR assay for differentiating European swine influenza virus subtypes H1N1, H1N2 and H3N2. *J. Virol. Meth.* 2012; 184: 117-120.
14. CDC. 2009. CDC protocol of realtime RTPCR for influenza A (H1N1). http://www.who.int/csr/resources/publications/swine-flu/CDCRealtimeRTPCR_SwineH1Assay-2009_20090430.pdf.
15. Panning, M., Eickmann, M., Landt, O., Monazahian, M., Olschläger, S., Baumgarte, S., Reischl, U., Wenzel, J.J., Niller, H.H., Günther, S., Hollmann, B., Huzly, D., Drexler, J.F., Helmer, A., Becker, S., Matz, B., Eis-Hübinger, A. and Drosten, C.: Detection of influenza A(H1N1)v virus by real-time RT-PCR. *Euro Surveill*. 14; pii 19329, 2009.
16. Hindiye, M.: Evaluation of a multiplex real-time reverse transcriptase PCR assay for detection and differentiation of influenza viruses A and B during the 2001-2002 influenza season in Israel. *J. Clin. Microbiol.*, 43:589-95, 2005.
17. Cong, Y.L., Pu, J., Liu, Q.F., Wang, S., Zhang, G.Z., Zhang, X.L., Fan, W.X., Brown, E.G. and Liu, J.H.: Antigenic and genetic characterization of H9N2 swine influenza viruses in China. *J. Gen. Virol.* 88:2035-2041, 2007.
18. Yu, H., Hua, R.H., Wei, T.C., Zhou, Y.J., Tian, Z.J., Li, G.X., Liu, T.Q. and Tong G.Z.: Isolation and genetic characterization of avian origin H9N2 influenza viruses from pigs in China. *Vet. Microbiol.*, 131: 82-92, 2008.
19. Yu, H., Zhou, Y.J., Li, G.X., Ma, J.H., Yan, L.P., Wang, B., Yang, F.R., Huang, M. and Tong, G.Z.: Genetic diversity of H9N2 influenza viruses from pigs in China: a potential threat to human health. *Vet. Microbiol.* 149: 254-261, 2011.