

Molecular Epidemiology of Influenza A(H1N1)pdm09 Virus among Humans and Swine, Sri Lanka

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After multiple discrete introductions of influenza A(H1N1)pdm09 virus into Sri Lanka, the virus was transmitted among humans, then swine. The spread of virus between geographically distant swine farms is consistent with virus dispersal associated with a vehicle used for swine transportation, although this remains unproven.

The first known transmission of influenza A(H1N1)pdm09 virus to humans from swine was in 2009. As the virus spread among humans worldwide, it was transmitted from humans to swine repeatedly (1), changing the global genetic landscape of swine influenza viruses. We previously reported the spillover of H1N1pdm from humans to swine and absence of North American triple reassortant, classical, and European avian-like swine viruses in swine herds in Sri Lanka during August 2009–May 2012 (2). Here, we extend these studies through August 2013 with the analysis of paired nasal and tracheal swab samples collected from 4,683 animals and serum samples from 3,351 animals (online Technical Appendix Table 1, wwwnc.cdc.gov/EID/article/20/12/14-0842-Techapp1.pdf) and comprehensively analyze full genomes of viruses isolated from samples from 26 swine (11 isolated in 2009, 4 in 2010, and 11 in 2011) and 35 humans (6 isolated in 2009, 17 in 2010, 9 in 2011, and 3 in 2012) and 2 publicly available hemagglutinin sequences of human H1N1pdm viruses from Sri Lanka. Sequences generated in this study are available in GenBank (KJ856002–KJ856446).

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The Study

To understand the molecular epidemiology and spatial and temporal dynamics of spillover events, we compared our data with full-genome sequences of H1N1pdm available in public databases as of August 28, 2013. These include all available full genome sequences from swine H1N1pdm viruses ($n = 82$), all human H1N1pdm viruses from outside of the USA ($n = 957$), and 100 randomly selected full genome sequences from 1,500 human H1N1pdm sequences from the United States. Reassorted swine or human viruses containing H1N1pdm virus genes were excluded from this analysis. Our final dataset included the full genomes of 35 human and 26 swine samples from Sri Lanka and a global sample of 1,057 human and 82 swine virus sequences.

The single breakpoint recombination and genetic algorithm for recombination detection methods (3) excluded the presence of reassortants in our dataset; hence, we used concatenated genomes of 8 gene segments for all subsequent analyses. We conducted multiple-sequence alignment using MUSCLE (4) and optimized the sum of all of the pairs of characters manually. Phylogenetic trees and bootstrap supports were estimated by using the GTR+I+ Γ nucleotide substitution model as identified by using JModeltest (5) and the maximum likelihood method in RAxML (6). We inferred dates of introduction of major Sri Lankan human and swine lineages using the relaxed clock method under a Bayesian Markov chain Monte Carlo approach (BEAST v1.7) (7).

Figure 1 illustrates independent introductions of at least 8 H1N1pdm sublineages into Sri Lanka during 2009–2012. Six of these were exclusively detected in humans: hu1 (2009), hu2 (2009), hu3 (2009), hu5 (2010/11), hu6 (2011), and hu7 (2011). One was exclusively from swine (sw1; 2009/10) and 1 sublineage was detected in both humans (hu4; 2011) and swine (sw2; 2011) (Figures 1, 2). Similar multiple discrete introductions of human H1N1pdm viruses have been reported in the United Kingdom and India (8,9).

In Sri Lanka, swine H1N1pdm clusters sw1 (2009/10) and sw2 (2011) were genetically distinct from each other and from other swine viruses isolated globally, indicating 2 separate introductions to local swine that circulated among swine for 11 and 4 months, respectively, for each cluster. The sw1 and sw2 lineages did not appear to establish long-term sustained transmission within pigs. However, the

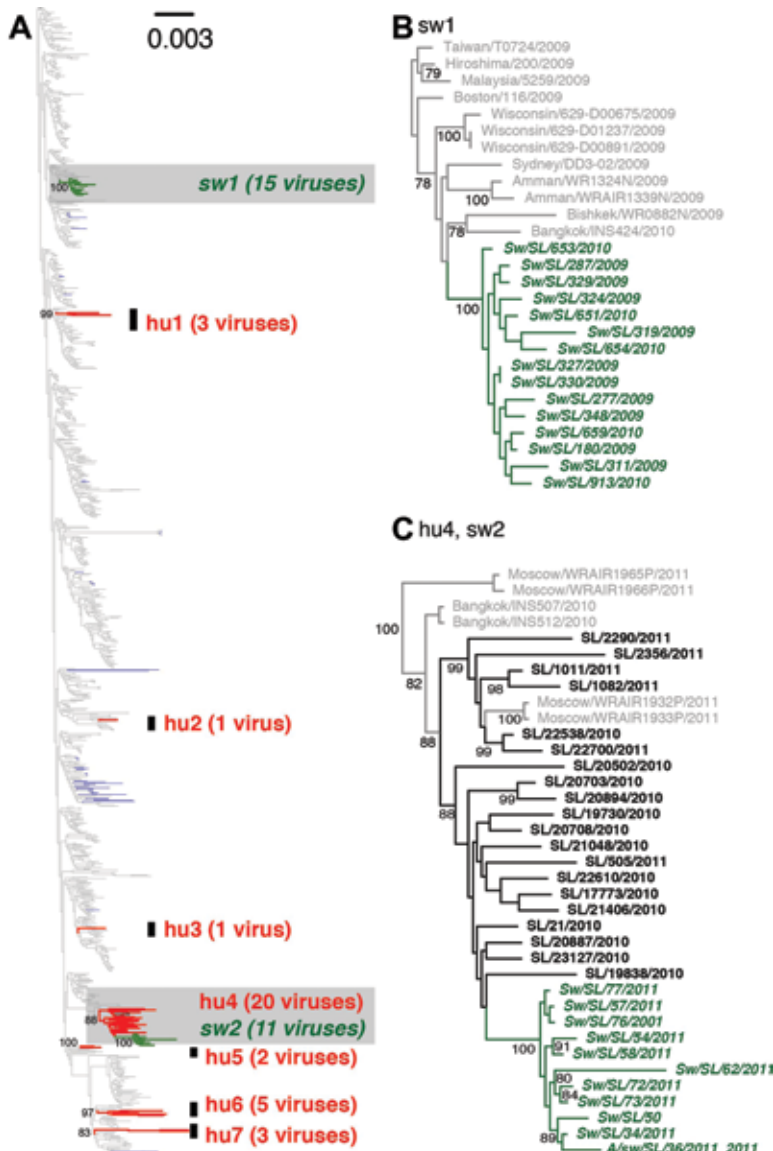


Figure 1. Phylogenetic relationships of influenza A H1N1pdm viruses isolated from human and swine during 2009–2012 in Sri Lanka. (A) Maximum likelihood tree generated from a concatenated dataset of 8 gene segment sequences from 1,057 human and 82 swine H1N1pdm viruses isolated globally during 2009–2012, and 35 human and 26 swine H1N1pdm viruses isolated in Sri Lanka in 2009–2012. Red and green branches represent human (hu) and swine (sw) viruses isolated in Sri Lanka, respectively; gray and blue branches represent globally sampled human and swine viruses, respectively. Highlighted regions (gray) are shown with virus names in (B) and (C), respectively. Support values estimated from 500 maximum likelihood bootstrap replicates are shown along the node for each swine and human clusters identified in Sri Lanka in (A) and for each node with >70% support for (B) and (C).

reduced surveillance of farms during the period 2012–2013 (online Technical Appendix Table 1) means that this conclusion has to be qualified in regard to the sw2 lineage. We did not identify ancestors of sw1 in Sri Lanka, however sw2 appears to have been directly derived from hu4, which included the majority of the human viruses (20/35) sequenced from Sri Lanka. The lack of identification of a human ancestor for sw1 may be related to insufficient human influenza genomic data obtained from Sri Lanka during 2009–2010 (Figures 1, 2). Although the 11 sw2 viruses were isolated from pigs on farms A, C, and G, which were separated by >25 km from each other (Table, Figure 2), they form a monophyletic clade with no human isolates within this cluster. Even though the paucity of human viruses sampled is a limitation in this study, the data suggest a single introduction of human viruses into swine followed

by transmission within and between swine farms for >4 months (Figure 2).

To clarify transmission patterns between affected swine farms in Sri Lanka, we obtained contact patterns by interviewing pig farmers using a structured questionnaire (online Technical Appendix) with approval from the Ethics Review Committee, Faculty of Medicine, Ragama, Sri Lanka. There was no evidence of movement of persons or fomites between farms. However, during the peak demand period (November–December) of each year that surveillance was performed, a common truck owned by farm M (Table), driven by a single driver and an assistant, provided transportation from multiple farms to the abattoir, including from affected farms A, C, and G (Table). On some occasions, animals taken to the abattoir for slaughter were returned to the farm. Pools of water or body fluids were often noted within this

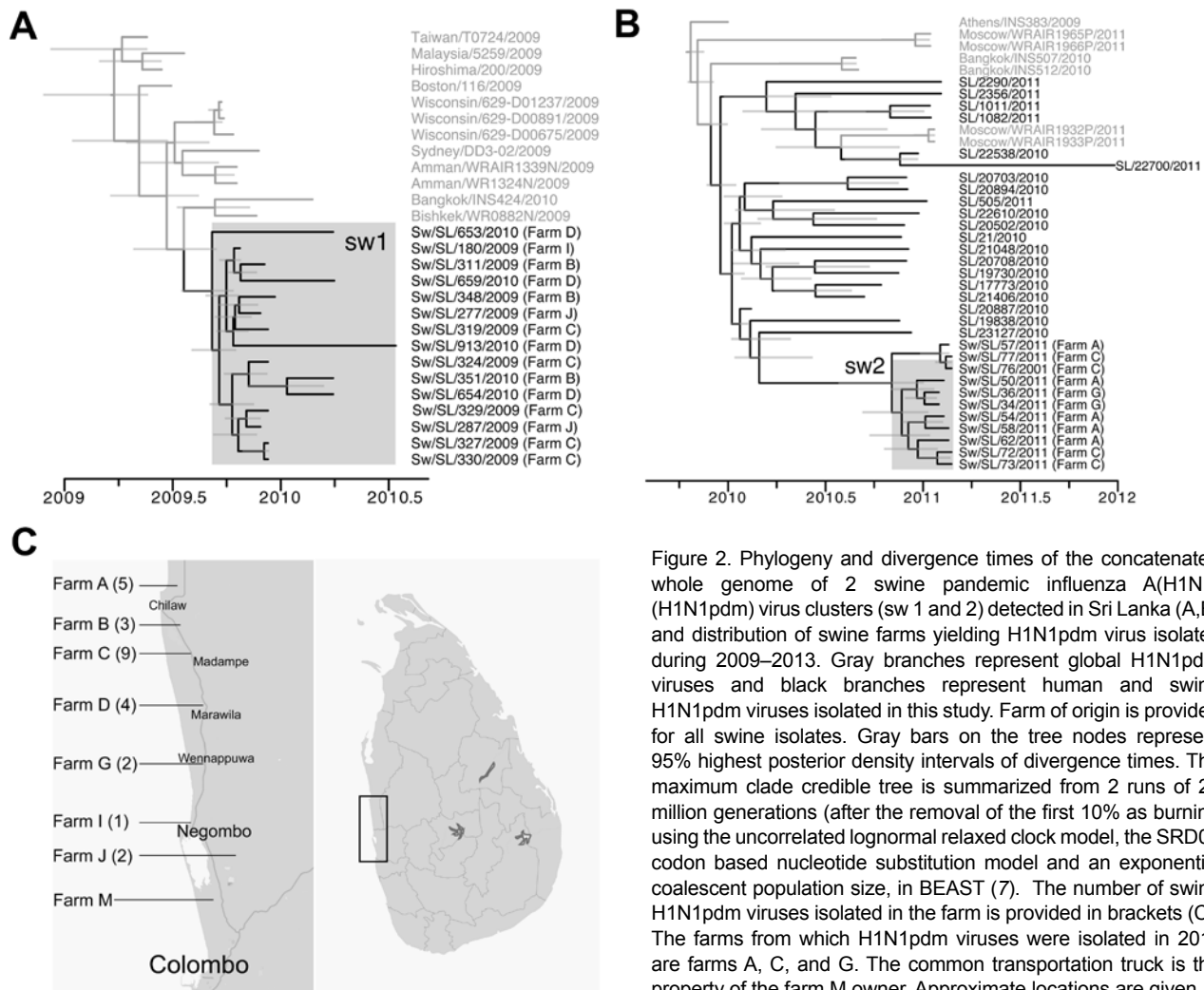


Figure 2. Phylogeny and divergence times of the concatenated whole genome of 2 swine pandemic influenza A(H1N1) (H1N1pdm) virus clusters (sw 1 and 2) detected in Sri Lanka (A,B) and distribution of swine farms yielding H1N1pdm virus isolates during 2009–2013. Gray branches represent global H1N1pdm viruses and black branches represent human and swine H1N1pdm viruses isolated in this study. Farm of origin is provided for all swine isolates. Gray bars on the tree nodes represent 95% highest posterior density intervals of divergence times. The maximum clade credible tree is summarized from 2 runs of 20 million generations (after the removal of the first 10% as burnin), using the uncorrelated lognormal relaxed clock model, the SRD06 codon based nucleotide substitution model and an exponential coalescent population size, in BEAST (7). The number of swine H1N1pdm viruses isolated in the farm is provided in brackets (C). The farms from which H1N1pdm viruses were isolated in 2011 are farms A, C, and G. The common transportation truck is the property of the farm M owner. Approximate locations are given.

truck, and it is possible that viable swine influenza viruses may have survived for varying periods. We did not test these fluids from the common transportation truck for influenza viruses; this is also a limitation of the study.

Of the 15 farms on which the common truck was used, swine on 3 (20%) were infected by a sw2 clade virus; on 2 farms on which the common truck was not used, no swine were infected. This association was not statistically significant ($p = 1.0$), however, given the small numbers of farms investigated. Our findings are consistent with dispersal of sw2 clade viruses in association with the truck to infect multiple farms that were geographically distant, but this remains unproven. It was previously documented that influenza viruses can remain viable for prolonged periods of time in water at a temperature of $\approx 28^{\circ}\text{C}$ (comparable ambient temperature in the Western Province, Sri Lanka) (10) and are reported to survive longer periods on nonporous surfaces (11). Influenza virus has been detected in air samples from rooms of experimentally infected pigs (12) and

in the exhaust air samples collected up to 1 mile away from the index farm (13), indicating the possibility of aerosol transmission for some distance. Notably, studies of the swine populations in the United States have demonstrated spatial dissemination of swine influenza viruses of human origin to match long-distance swine movements (14).

Despite widespread inter-farm transmission of sw1 and sw2, our results show that only animals on farm C were infected in both spillover events. Farms A and G, on which swine were infected by sw2 in 2011, appeared not to have had infected swine during 2009–2010, as shown by both virus isolation and serologic testing (online Technical Appendix Table 1). Maternally derived antibodies transferred through colostrum from dams infected during 2009–2011 may have provided passive protection to offspring born in 2010–2011. On swine farms in Sri Lanka, female swine are used for breeding for ≈ 2 –3 years. In experimental challenge, maternally derived antibodies provided some protection against disease, but not complete protection from infection (15).

Table. Demographic data, internal and external biosecurity measures practice by swine farms, Sri Lanka, 2009–2013*

Farm	Pig replacement source	Used specific clothing and footwear	Used disinfectants for wheel and foot baths	Dedicated employees for fattening and nursery units	Type of truck used to transport pigs to the GSHD	Visitor restriction grade	Service provider visits	Cats and/or dogs allowed in the facility	H1N1pdm detection
A	Internal	No	Yes	Yes	Hired/farm M	M	Nil	Yes	Clade 2
B	Internal	Yes	Yes	Yes	Dedicated	A	No	Yes	Clade 1
C	Internal	No	No	No	Hired/farm M	N	Occasionally	Yes	Clades1/2
D	Internal	No	No	Yes	Dedicated	M	Occasionally	Yes	Nil
E	Internal	No	No	No	Hired/farm M	N	Occasionally	Yes	Nil
F	Internal	No	No	No	Hired/farm M	M	Nil	Yes	Nil
G	Internal	No	No	No	Hired/farm M	M	Nil	Yes	Clade 2
H	Internal	No	No	No	Hired/farm M	M	Nil	Yes	Nil
I	Internal	No	No	No	Hired/farm M	M	Occasionally	Yes	Clade 1
J	Internal	No	No	No	Hired/farm M	N	Occasionally	Yes	Clade 1
K	Internal	No	No	No	Hired/farm M	M	Nil	Yes	Nil
L	ND	No	ND	ND	Hired/farm M	ND	ND	ND	Nil
M	External	No	No	No	Self-owned	M	Yes	Yes	Nil
N	Internal	No	No	No	Hired/farm M	N	Nil	Yes	Nil
O†	NA	No	No	No	NA	N	Nil	Yes	Nil
P	Internal	No	No	No	Hired/farm M	N	Nil	Yes	Nil
Q‡	Internal	Yes	No	Yes	NA	A	Yes	Yes	Nil
R	Internal	No	No	No	Hired/farm M	M	Occasionally	Yes	Nil
S	Internal	No	No	No	Hired/farm M	N	Occasionally	Yes	Nil

*None of the swine farms housed domestic poultry in the facility. Farms are listed in alphabetical order, A being in the northernmost location and S the southernmost farm in this study. GSHD, Government Slaughter House Dematagoda. The common transportation truck is the property of the farm M owner. Visitor restriction is scored as M-moderate, and A-absolute restriction, N-nil, on the basis of level of visitor restrictions (absolute/moderate/nil) and selection of veterinary surgeons (designated/freelance); service providers are veterinary surgeons, drug, and vaccine providers. NA, not applicable; ND, no data available.

†Backyard slaughterhouse.

‡Pigs not slaughtered at the GSHD.

Conclusions

This study demonstrates natural independent spill-over events of H1N1pdm influenza viruses from humans to swine. H1N1pdm viruses appear to be spread by multiple, discrete introductions to swine, after which clonal expansion occurs within the swine. The spread of such virus lineages across multiple farms is consistent with virus dispersal by breaches of external biosecurity measures, including the manner of swine transportation, although this remains unproven given the small sample size. Unlike classical swine influenza, North American triple reassortant, and European avian swine viruses that have persistently circulated among swine for several decades in other countries (15), H1N1pdm does not appear to establish long-term lineages in swine in the absence of further reassortment. This observation requires confirmation in other geographic settings.

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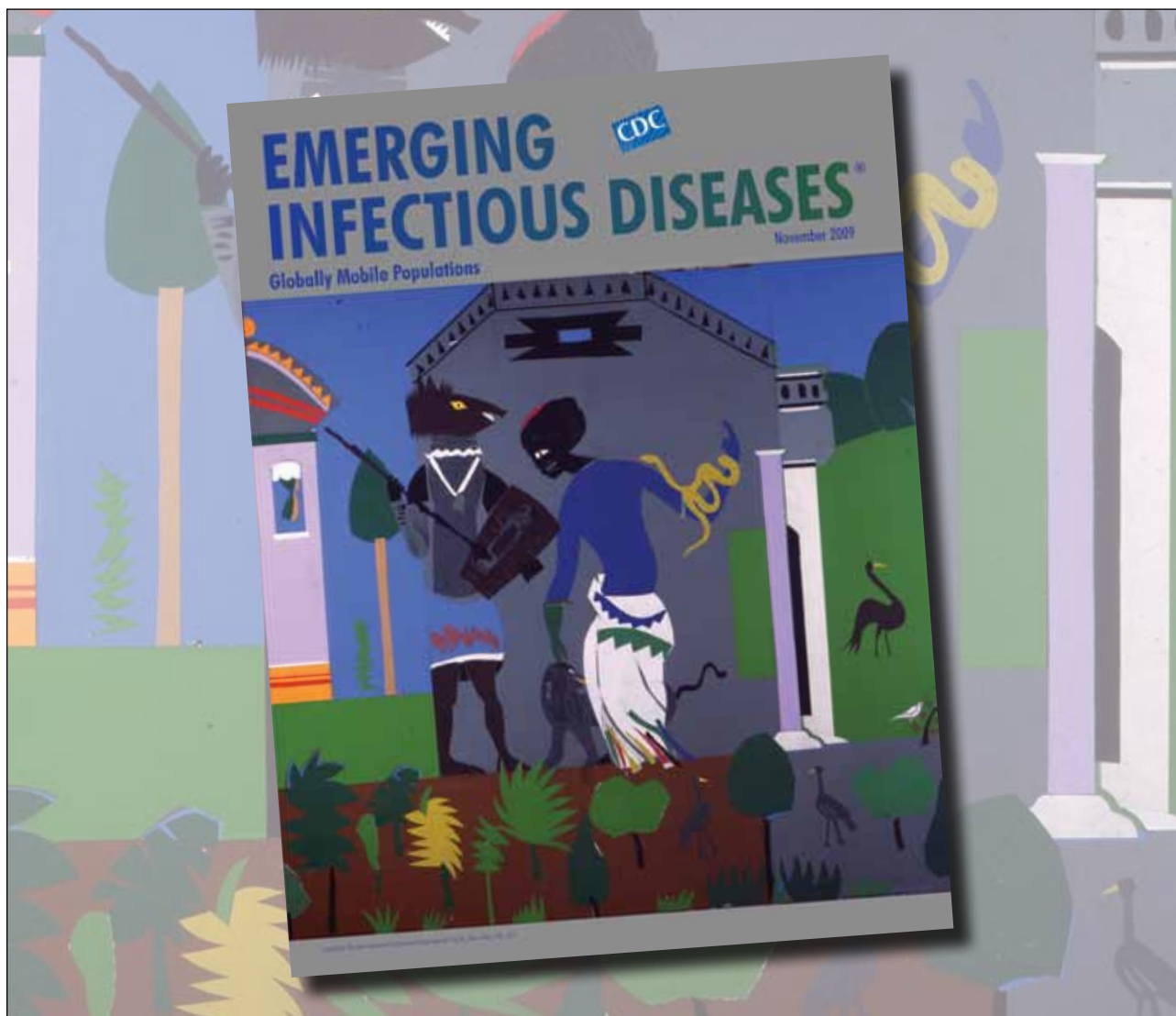
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Molecular Epidemiology of A(H1N1)pdm09 Virus among Humans and Swine, Sri Lanka

Technical Appendix: Overview of the swine industry and related influenza surveillance in Sri Lanka

Swine farms in Sri Lanka are predominantly (~61%) located in the western coastal belt spanning Puttlam, Gampaha, Colombo and Kalutara administrative districts of the country, which is identified as the ‘pig belt’ of Sri Lanka, with a standing population of approx. 80,000 pigs. Pigs are not imported on a regular basis to the country (1).

The mean slaughter age and average live weight of a local pig is approx. 9.8 months (4.4-10.1 months) and about 84 kg, respectively (2). However, the mean age of the pigs included in the current study was 6.9 months (and ranged from 6.0–9.0 months).

Pigs were transported in completely covered box-type trucks, whose upper half of rear door is kept open during transportation. The average travel time that animals experience was 3–4 hours, with the exception of farm H that took 8–10 hours (Table, Technical Appendix Table 1, 2).

Animals are not pooled within the same truck during a single transportation session.

The Government Slaughterhouse located in Dematagoda, Colombo, which operates for 6 days per week and slaughters around 20 pigs per day or depending on the available number of pigs on a given day. More than 90% of the animals that are slaughtered at the Dematagoda Government Swine Slaughterhouse receive pigs from the swine farms located in the Puttlam, Gampaha, Colombo and Kalutara administrative districts. Routine vaccination against influenza on both human and swine is not carried in the country. However, a single vaccination program against H1N1pdm was conducted in late 2010 on humans (3). Nevertheless, none of the farmers or employees of the swine farms had received vaccination.

Swine Surveillance

Swine swab samples were obtained from freshly slaughtered pigs at the Government Slaughterhouse Dematagoda Colombo, from August 2009 to March 2013. Swab (tracheal and nasal) and serum samples were collected from these pigs on weekly basis from otherwise healthy animals (Technical Appendix Figure). The swine swabs samples were inoculated into Madin-Darby canine kidney (MDCK) monolayers and 9-11days old embryonated chicken eggs through allantoic route. One blind passage was performed on each negative swine swab sample. Hemagglutination inhibition assay was performed using panel of viral antigens stated in our previous publication (4).

Molecular Detection of Human H1N1pdm Viruses

Influenza A/B typing panel and CDC real time qRT-PCR (A/H1/H3/H1N1pdm09) subtyping panel were used for screening and detection. In summary, viral RNA were extracted using QIAamp™ Viral RNA Mini kits, and 5.5 µL nuclease free water, 0.5 µL of each forward primer and reverse primer, 0.5 µL probe, 0.5 µL superscript TM III/RT-Platinum, 12.5 µL 2 x PCR buffer were run in each qRT- PCR in accordance with following conditions; reverse Transcript 50°C x 30 min, Taq inhibitor activation 95°C x 2min, PCR amplification (40 cycles); 95°C x15sec min and 55°C x 30 sec and fluorescence data was obtained as described in the protocol (5). Human samples tested positive for H1N1pdm viruses were inoculated into MDCK cells as described elsewhere (4) and two blind passages were performed on each culture negative sample.

Temporal Phylogenetic Analysis

Bayesian Markov chain Monte Carlo analyses were performed using the SRD06 codon based nucleotide substitution model (6), a flexible coalescent based demographic model, and the Gaussian Markov Random Field model (7). These models were selected as these have been consistently been shown to be the best-fit models for influenza viruses (8–10).

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Technical Appendix Table 1. Swine swab and serum samples tested for pandemic influenza A(H1N1), Sri Lanka*

Farm	2009	2010		2011		2012		2013	
	Virus isolation (%)	Virus isolation (%)	Sero positive (%)	Virus isolation (%)	Sero positive (%)	Virus isolation (%)	Sero positive (%)	Virus isolation (%)	Sero positive (%)
A	0/22	0/47	0/10 (0)	5/49 (10.2)	13/49 (26.5)	0/60	0/60 (0)	0/11	1/11 (9.1)
B	3/81 (3.7)	0/256	5/30 (16.6)	0/343	72/343 (20.9)	0/423	12/423 (2.8)	0/318	87/318 (27.3)
C	5/81 (6.1)	0/25	7/16 (43.7)	4/76 (5.2)	22/76 (28.9)	NC	NC	NC	NC
D	0/22	4/180 (2.2)	4/49 (8.1)	0/156	46/156 (29.4)	0/178	2/178 (1.1)	0/105	12/105 (11.4)
E	NC	0/40	0/20 (0)	NC	NC	NC	NC	NC	NC
F	NC	NC	NC	0/3	0/32 (0)	NC	NC	NC	NC
G	NC	0/38	NC	2/47 (4.2)	19/47 (40.4)	NC	NC	NC	NC
H	NC	0/29	NC	NC	0/5(0)	0/37	0/37 (0)	NC	NC
I	1/68 (1.4)	0/166	4/28 (14.2)	0/164	53/164 (32.3)	0/172	1/172 (0.6)	0/145	24/145 (16.5)
J	2/24	0/44	NC	NC	NC	NC	NC	NC	NC
K	0/37	0/67	5/28 (17.8)	NC	NC	NC	NC	NC	NC
L	NC	0/28	0/10 (0)	NC	NC	NC	NC	NC	NC
M	NC	0/95	1/13 (17.8)	0/52	16/52 (30.7)	0/241	16/241 (6.6)	0/269	51/269 (18.9)
N	NC	NC	NC	NC	NC	0/5	0/5(0)	NC	NC
O	NC	NC	NC	0/21	0/21 (0)	NC	NC	NC	NC
P	NC	0/41	0/18 (0)	NC	NC	NC	NC	NC	NC
Q	NC	NC	NC	0/17	2/17 (11.7)	NC	NC	NC	NC
R	NC	0/94	5/29 (7.6)	NC	NC	NC	NC	NC	NC
S	0/21	0/43	0/5 (0)	0/61	31/61 (50.8)	0/129	3/129 (2.3)	NC	NC

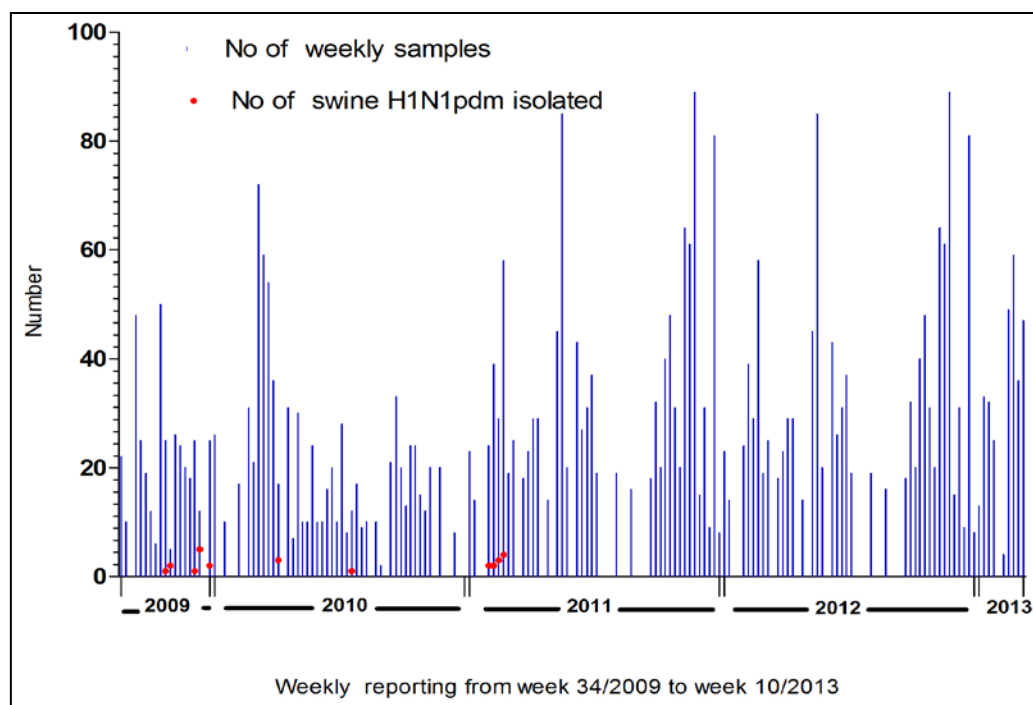
*Farms are listed in alphabetical order, A being in the northernmost location and S the southernmost farm in this study.

Technical Appendix Table 2. Herd size, replacement sources and replacement frequency of swine farms, Sri Lanka

Farm	Approximate herd size	Swine replacement source	Replacement frequency of animals per month
A	200	Internal	30
B	1,500	Internal	30
C	400	Internal	20
D	750	Internal	30
E	250	Internal	10
F	100	Internal	10
G	200	Internal	10
H	100	Internal	10
I	500	Internal	40
J	400	Internal	15

Farm	Approximate herd size	Swine replacement source	Replacement frequency of animals per month
K	300	Internal	15
L	Data not available	Data not available	Data not available
M	300	External	30
N	50	Internal	5
O	Inapplicable	Inapplicable	Inapplicable
P	100	Internal	10
Q	750	Internal	75
R	500	Internal	10
S	160	Internal	10

*Farms are listed in alphabetical order, A being in the northernmost location and S the southernmost farm in this study.



Technical Appendix Figure. Weekly sample distribution. Weekly numbers of pigs sampled from week 34/2009 to week 10/2013. Blue bars indicate the number of pigs sampled each week. Red dots indicate number of swine influenza viruses isolated.

Swine Farm Surveillance Questionnaire:

a) **General characteristics**

1) Identity of the farm.....

2) Address of the farm.....

3) Contact Tel No

4) Herd size.....

5) No of fattening units.....

6) Replacements source/s

a) Internal

b) External

c) Both

d)

e) If answered to b or c, the source/s

1.....

2.....

3.....

4.....

7) Replacement frequency

a)

8) Does the farm sell surplus piglets to other farms

a) Yes

b) No

c) If answered yes, name and the address of the farm/s

1.....

2.....

3.....

9) Type of separation between pens:

a) Solid walls

b) Bars

c) other

10) Pen stocking density (m²).....

11) Length of the fattening period.....

12) No of workers in the farm:

a) Full-time

b) Casual.....

13) Educational level of the farmer:

a) No Formal education

b) Primary

c) Secondary

d) University degree

14) Obtained further training on pig framing:

a) Yes

b) No

15) Aware that human diseases could transmit to pigs and *vice versa*:

a) Yes

b) No

b) **Farm biosecurity**

1) Availability of outside fence:

a) Yes

b) No

2) Pigs are allowed to move out the pens:

a) Yes

b) Occasionally

c) Never

3) Use of sanitising wheel baths:

a) Always

b) Occasionally

c) Never

4) Generic name/s of the chemical used in wheel bath

1.....2.....

5) Use of sanitising boot baths:

a) Always

b) Occasionally

c) Never

6) Generic name/s of the chemical used in boot bath

1.....2.....

7) Replacement frequency of wheel/boot bath solution:

a) 12hr

b) 24hr

c) 36hr

- e) 48hr
- f) 1/52
- 8) Separate foot bath for each pen:
 - a) Yes
 - b) No
- 9) Wearing of dedicated clothes before entering the facility:
 - a) Always
 - b) Occasionally
 - c) Never
- 10) Presence of changing rooms and shower in the farm:
 - a) Available
 - b) Not available
- 11) Having a shower before entering the facility:
 - a) Always
 - b) Occasionally
 - c) Never
- 12) Restrictions on entering workers suffering from flu like symptoms in to the farm
 - a) Always
 - b) Occasionally
 - c) Never
- 13) Sharing workers with other swine farms:
 - a) Always
 - b) Occasionally
 - c) Never

If answered to a or b, name of the farm/s

- 1.....
- 2.....
- 3.....

14) Do workers employed in the fattening unit work in the nursery unit:

- a) Yes
- b) No

15) How are the farm animals transported to the slaughterhouse

- a) Dedicated vehicle
- b) Common vehicle in the farm
- c) Hired vehicle

If answered to c, does this vehicle provide service to other swine farms?

- a) Yes
- b) No

If answered yes, name the farm/s

- 1.....
- 2.....
- 3.....

16) Vehicle use to transport nursery pigs to fattening pens

- a) Dedicated vehicle belongs to the farm
- b) Common vehicle in the farm
- c) Hired vehicle

c) If answered to c, the source/s of the vehicle

- 1.....2.....

17) Are animals sourced from different farms “pooled “ during transportation to the slaughterhouse:

- a) Always
- b) Occasionally
- c) Never

c) If answered to b or c, name and the address of the farm/s

- 1.....
- 2.....
- 3.....
- 4.....

19). Presence of other domesticated animals in the farm

- a) Always
- b) Occasionally
- c) Never

d) Type of animal/s (if answered yes to a or b)...../...../.....

c) Feeding management

1) Animals are feed:

- a) Manually
- b) Automatically

2) Type of feed:

- a) Swill
- b) Rice barn
- c) Kitchen refuse
- d) Non-human edible chicken refuses

3) Source of food:

- a) Self made

b) Brought from one source

c) Different sources

d) If answered to c and d, address/es of the feeding source

- 1.....
- 2.....
- 3.....
- 4.....

d) **Visitor restrictions**

1. Restrictions on people visiting the farm:

a) Always

b) Occasionally

c) Never

2. Do service providers * visit the farm

a) Always

b) Occasionally

c) Never

d) If answered to b or c, indicate the purpose/es and identity of the service provider

1 nameservice 1.....2.....3.....

2 nameservice 1.....2.....3.....

3 nameservice 1.....2.....3.....

4 nameservice 1.....2.....3.....

5 nameservice 1.....2.....3.....

3. How often people stated above visit the farm

a) Weekly

b) Monthly

c) > one month

4. Does the farm obtain vet surgeon's consultations

a) Yes

b) No

if yes name of the vet surgeon/s

1.....

2.

5. Does the farm obtain consultations from one designated vet surgeon, when animal/s fall sick:

a) The farm has selected dedicated vet surgeon

b) Not confined to one vet surgeon

6. How often designated "farm" vet visit the premises

a) Weekly

b) Monthly

c) > one month

**Service providers: Sales representatives, drug suppliers, vaccine suppliers, feed suppliers, artificial insemination providers...etc.*