

Swine Influenza in Sri Lanka

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To study influenza viruses in pigs in Sri Lanka, we examined samples from pigs at slaughterhouses. Influenza (H3N2) and A(H1N1)pdm09 viruses were prevalent during 2004–2005 and 2009–2012, respectively. Genetic and epidemiologic analyses of human and swine influenza viruses indicated 2 events of A(H1N1)pdm09 virus spillover from humans to pigs.

Data on swine influenza in southern Asia are limited (1–3). Sri Lanka is an island in this region with a human population of 21 million and a swine population of $\approx 83,785$ (4,5). Pigs are not routinely imported into Sri Lanka. Most (61%) swine farms are located in the western coastal belt spanning the Puttlam, Gampaha, Colombo, and Kalutara districts. In 2010, for these 4 districts, pig population densities were 7, 15, 12, and 1 animal per km², respectively (4,5). In 2001, for these districts, the human population densities were 246, 1,539, 3,330, and 677 persons per km², respectively (6).

The Study

During 2004–2005 and 2009–2012, tracheal and nasal swab and serum samples were collected from pigs at government slaughterhouses in Sri Lanka (Table 1). Culture tubes with MDCK cells were inoculated with the swab samples, and 2 blind passages were made. Also, embryonated eggs were inoculated by the allantoic route with swab samples collected during 2004–2005. Virus isolates were subtyped by hemagglutination inhibition (HAI) testing and neuraminidase inhibition testing with reference antiserum as described (7,8), and results were confirmed

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by sequencing the hemagglutinin and neuraminidase gene segments.

RNA extraction, cDNA synthesis, PCR, genome sequencing (9), and one-step quantitative real-time reverse transcription (RT-PCR) for rapid genotyping of all 8 gene segments (10) of A(H1N1)pdm09 isolates were performed as described. Methods used for the phylogenetic analysis are described in online Technical Appendix 1 (wwwnc.cdc.gov/EID/article/19/3/12-0945-Techapp1.pdf). GenBank accession numbers assigned to the sequences determined in this study are KC197816–KC197855 and KC190041–KC190078.

Serum samples were tested by HAI as described (8) by using the virus antigens shown in Table 2. The number of human influenza A(H1N1)pdm09 viruses detected by RT-PCR in Sri Lanka during July 2009–March 2012 was obtained from the World Health Organization FluNet and from the Epidemiology Unit, Ministry of Health, Sri Lanka (11,12). Seven A(H1N1)pdm09 viruses isolated from humans during 2009–2011 were obtained from the National Center for Influenza, Medical Research Institute, Sri Lanka, and were genetically sequenced as described above. Genetic sequence data of the hemagglutinin gene of 5 additional human A(H1N1)pdm09 viruses isolated in Sri Lanka were provided by the World Health Organization Influenza Collaborating Centre, Melbourne, Australia.

One influenza A virus, A/swine/Colombo/48/2004(H3N2), was isolated in MDCK cells from a tracheal swab sample collected in 2004–2005. All genes of this virus were closely related to human influenza (H3N2) virus isolate A/Ragama/190/2003 from Sri Lanka and to other subtype H3N2 influenza viruses isolated worldwide at this time (data not shown). During January 2004–March 2005, a total of 185 (61.6%) of 300 serum samples tested were positive for A/swine/Colombo/48/2004(H3N2); HAI titers ranged from 40 to $\geq 1,280$ (Table 2), indicating that this human-like influenza (H3N2) virus was widespread in the swine population. Serum samples collected from swine during 2009–2012 were also mostly seronegative to this and to more contemporary human influenza (H3N2) viruses.

Table 1. Swine influenza viruses isolated from pigs, Sri Lanka*

Collection years, location	No. pigs sampled/no. viruses isolated (source)
2004–2005	
Welisara†	40/0
Dematagoda‡	260/1 (tracheal swab)
2009–2012, Dematagoda‡	2,710/26 (7 tracheal swabs, 19 nasal swabs)

*From each pig, 1 tracheal swab, 1 nasal swab, and 1 serum sample was collected, except during 2009–2012, when only 1,773 serum samples were collected from 2,710 pigs beginning in February 2010.

†National Livestock Development Board swine farm, Welisara, Sri Lanka (slaughters 2–3 pigs/wk).

‡Government slaughterhouse, Colombo Municipal Council, Dematagoda, Colombo, Sri Lanka (slaughters 10–20 pigs/d).

Table 2. Homologous serological reaction profile to subtypes of influenza viruses among pigs, Sri Lanka, 2004–2005 and 2010–2012*

Virus antigen (lineage)	Seroprevalence, no. (%)				
	Jan 2004–Mar 2005, n = 300	Feb–Aug 2010, n = 149	Sep 2010–Mar 2011, n = 284	Apr–Oct 2011, n = 577	Nov 2011–May 2012, n = 763
A/swine/Colombo/48/2004 (H3N2) (human-like)	185 (61.6%)	06 (4.0%)	0	0	0
A/swine/HK/2422/98 (H3N2) (swine) (human)	0	0	0	0	0
A/Sydney/5/97 (H3N2) (human)	0	0	0	0	0
A/swine/HK/1774/99 (H3N2) (European swinelike)	0	0	0	0	0
A/HK/44062/2011 (H3N2) (human)	Not tested	Not tested	0	0	0
A/swine/Colombo/330/2009 (H1N1) (H1N1pdm09)	0	16 (10.7)	95 (33.5)	14 (25.1)	77 (10.1)
A/swine/HK/29/2009 (H1N1) (Eurasian avian)	0	01 (0.6)	0	0	0
A/swine/HK/1110/2006 (H1N1) (North American–triple reassortant)	0	01 (0.6)	0	0	0
A/swine/HK/915/2004 (H1N2) (North American–TR)	0	0	0	0	0
A/swine/HK/4167/99 (H1N1) (classical swine)	0	0	0	0	0
A/swine/Ghent/G112/2007 (Eurasian avian)	0	0	0	0	0

*Hemagglutination inhibition reciprocal antibody titers ≥ 40 were considered positive. The range of the antibody titers was 40 to $\geq 1,280$. If serum reacted to multiple antigenically related influenza H3 or H1 subtype viruses, we categorized the serum as having a homologous reaction profile to the virus to which titer was ≥ 4 -fold higher than that for other viruses of the same subtype. For example, during 2004–2005, some serum samples were seropositive for influenza A/swine/HK/2422/98 (H3N2) virus; however, because in the same sample, titer to influenza A/swine/Colombo/48/2004 (H3N2) virus was >4 -fold higher, reactivity was attributed to the latter.

Of the nasal and tracheal swab samples collected from 2,710 pigs during 2009–2012, a total of 26 (0.5%) viruses were isolated in MDCK cells; all were identified as A(H1N1)pdm09 viruses. All 8 gene segments of these viruses were similar to those of A(H1N1)pdm09 virus; no evidence of reassortment with other swine or human viruses was found. These isolates were collected on 12 sampling occasions from apparently healthy pigs on 7 farms. The 2 peaks of A(H1N1)pdm09 detection in swine followed peaks of human A(H1N1)pdm09 outbreaks that occurred during June 2009–January 2010 and October 2010–February 2011, which represented the first and second waves of the pandemic in Sri Lanka (Figure 1). Overall, virus yield was

higher from nasal swab samples than from tracheal swab samples (Table 1).

Phylogenetic analysis showed that the 15 A(H1N1)pdm09 viruses isolated from swine during October 2009–July 2010 clustered together and with other A(H1N1)pdm09 viruses isolated from humans during this period. In contrast, swine A(H1N1)pdm09 viruses isolated in 2011 clustered separately from swine viruses isolated during 2009–2010 and clustered with human A(H1N1)pdm09 viruses isolated in 2010 and 2011 in Sri Lanka and elsewhere (Figure 2). The amino acid signature changes occurring within human A(H1N1)pdm09 viruses in the first and second pandemic waves are reflected in the

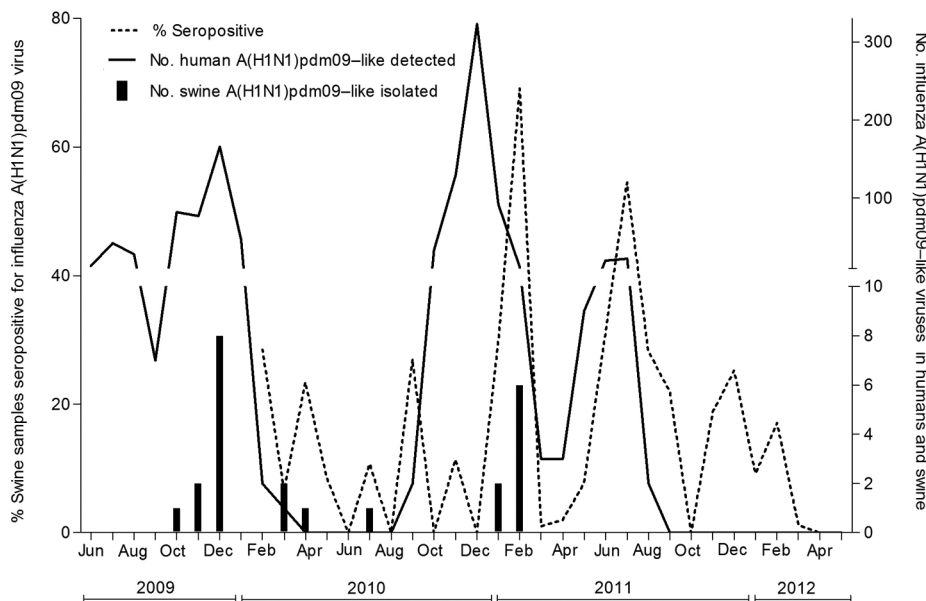


Figure 1. Distribution of percentage of swine serum samples seropositive for influenza A(H1N1)pdm09 viruses, by month, and number of A(H1N1)pdm09 viruses detected in humans and swine. The left y-axis represents the percentage of swine serum samples positive for A(H1N1)pdm09 virus. The right y-axis represents the number of swine A(H1N1)pdm09 isolated in the study and reverse transcription PCR–positive human A(H1N1)pdm09 detected in Sri Lanka.



Figure 2. Phylogenetic relationship of the hemagglutinin 1 gene of the human and swine influenza A(H1N1)pdm09-like viruses isolated during 2009–2012 in Sri Lanka. Underlining indicates swine and human viruses characterized in this study; *indicates swine A(H1N1)pdm09 virus isolates. Nucleotide sequences from selected, related avian, equine, swine, and human virus strains available in GenBank are included for comparison. The phylogenetic tree was generated by the maximum-likelihood method and rooted to A/duck/Miyagi/66/77(H1N1) virus (online Technical Appendix 1, wwwnc.cdc.gov/EID/article/19/3/12-0945-Techapp1.pdf). Scale bar represents number of nucleotide substitutions per site. Vertical dashed line indicates influenza A(H1N1)pdm09-like virus lineage. Branch labels record the stability of the branches >500 bootstrap replicates. Numbers above and below branches indicate neighbor-joining bootstrap values and Bayesian posterior probabilities, respectively. Only bootstrap values >70% and Bayesian posterior probabilities >95% are shown. Three genetic variants with >1 aa difference in hemagglutinin 1 are indicated.

corresponding waves of swine infections, and each lineage that occurred in swine led to extinction (online Technical Appendix 2, wwwnc.cdc.gov/EID/article/19/3/12-0945-Techapp2.pdf). This finding suggests that the A(H1N1)pdm09 infections among swine that occurred during January–February 2011 were separate spillover events from the second wave of human infections during October 2010–February 2011 rather than from continued epizootic transmission among swine from October 2009. The upper 95% CI of the prevalence of each viral genetic variant, given no positive isolates since the last detection, declined to almost zero during the course of observation, indicating probable extinction of these genetic variants (online Technical Appendix 2).

On some sampling occasions, A(H1N1)pdm09 viruses were isolated from multiple pigs from the same farm, and on 1 sampling occasion, isolates came from multiple pigs (from the same farm) slaughtered 9 days apart. In such instances, with 1 exception, we found viruses from the same farm to be genetically identical, suggesting continued circulation of the virus in swine herds.

Swine serum samples collected in 2004–2005 showed no seroprevalence to A(H1N1)pdm09, A/California/4/2009, and A/swine/Colombo/330/2009 viruses (Table 2). In 2010, seroprevalence to A(H1N1)pdm09 virus was detected (Figure 1). After peaking in February 2011, seroprevalence declined to undetectable levels in April–May 2012, suggesting that the A(H1N1)pdm09 virus was not sustaining transmission among pigs in the absence of continued human infection. The maximum cross-correlation between incidence of human and swine virus isolates was found after an 8-week lag, indicating that the rise in incidence of human virus preceded that in swine by 7–8 weeks (online Technical Appendix 2).

Conclusions

Isolation of human-like influenza A (H3N2) and A(H1N1)pdm09-like viruses from pigs in Sri Lanka probably represents spillover infection from humans, with self-limited transmission and extinction within pig herds. This finding might reflect characteristics of swine husbandry in Sri Lanka, where swine population density in the study area is relatively low (7.7 pigs/km²), or other factors (5,13). Genetic characterization of individual gene segments of all influenza (H3N2) and A(H1N1)pdm09 viruses from swine showed no evidence of genetic reassortment. This finding contrasts with those from Hong Kong, Thailand, Argentina, and the United States, where reassortment of A(H1N1)pdm09 with other swine influenza viruses has reportedly occurred (14,15). This contrast might reflect the low prevalence of other swine influenza virus lineages (e.g., classical swine, Eurasian avian-like and triple-reassortant

swine) endemic to Sri Lanka. With the exception of subtype H3N2 viruses (Table 2), no evidence of other endemic swine influenza viruses circulating in swine in the country before the emergence of the A(H1N1)pdm09 in 2009 was found, and influenza (H3N2) virus in swine became extinct around the time of the spillover of A(H1N1)pdm09 to swine. These observations might explain the lack of emergence of A(H1N1)pdm09 reassortants among swine. It might also indicate that A(H1N1)pdm09, although able to spill over from humans to swine, is not ideally adapted to establish sustained transmission among swine in the absence of further reassortment with other swine influenza virus lineages.

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Swine Influenza in Sri Lanka

Technical Appendix 1

Methods

The HA sequences were aligned with other sequences from animal and human influenza A viruses available in the GenBank. Maximum-likelihood (ML) tree was estimated using the general time-reversible with invariant sites and 4 gamma distributed heterogeneous substitution rates) in PhyML version 3.0 (1). To evaluate the robustness of the tree topology, a set of 500 pseudo-replicates of the sequences was generated and used in bootstrap analysis. The ML tree topology was also confirmed by using Bayesian analysis in MrBayes version 3.2 (2).

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Swine Influenza in Sri Lanka

Technical Appendix 2

Statistical Model

Monthly counts of RT-PCR testing of human samples were cumulated from January 2009 to December 2011, and then the data were smoothed by using cubic spline function assuming that each observation took place in the mid-point of the month. Subsequently, weekly human incidence was calculated by dividing weekly number of RT-PCR positive specimens from humans by the interpolated spline function. To assess possible correlation with different time-lags between swine and human epidemics, cross-correlations were estimated between estimated weekly human incidence and swine datasets. Two pieces of swine data, i.e., the proportion seropositive (seroprevalence) and the proportion of virus isolates among test samples (virus prevalence), were examined. Since the time series between human and swine may potentially have shared the trend, we also prewhitened the data. For this process, we fitted polynomial functions to the input series, got residuals from both series and computed cross-correlations from residual series.

To explore the extinction of specific virus type, we grouped the swine H1N1pdm isolates into different genetic variants based on difference in amino acids of the HA molecule. With the close homology of the isolates, a single amino acid variation was considered in grouping. When there has been no observation of a virus that belongs to a specific genetic variant for a long time, it would be useful to understand the likelihood of extinction for interpreting the absence of virus isolation in a conservative way. Because sampling a finite number of test samples during the course of an outbreak cannot prove that swine were never infected with the corresponding virus, a more useful result would be the maximum prevalence with a certain level of confidence if no positive isolates are observed among a total of n samples. To obtain this result, we use the following equation (1a):

$$P_{max} = 1 - \alpha^{\frac{1}{n}} \quad (1a)$$

where p_{\max} is the maximum virus prevalence given n samples and no positive isolates, at a confidence level of $1-\alpha$, e.g. 95% if $\alpha = 0.05$ (A1).

Appendix Results

Comparing human incidence with swine data, maximum cross-correlation between spline-interpolated weekly human incidence versus swine seroprevalence was found at 7-week lag (the cross correlation coefficient, $\rho=0.545$). Consistently, the maximum cross-correlation between human incidence and swine virus isolates was found at 8-week lag ($\rho=0.412$), indicating that the rise in swine prevalence has been seen approximately 7 to 8 weeks later than that in humans. Even after prewhitening, the maximum cross-correlations were observed at 8-week lags for both swine seroprevalence and virus isolates ($\rho=0.383$ and 0.348). The last isolate of virus genetic variant I, variant II and variant III took place on 14 July 2010, 8 February 2011 and 23 February 2011, respectively. Subsequently, these have not been observed even with a large number of test samples from the farms (Appendix Figure 1A), indicating that these viruses may have declined to extinction. Appendix Figure 1B shows the maximum prevalence of the corresponding genetic variants, given no observation of these viruses after the last isolation dates. The maximum prevalence lowered 0.5% in 23rd, 42nd and 46th week in 2011 for genetic variants I, II and III respectively.

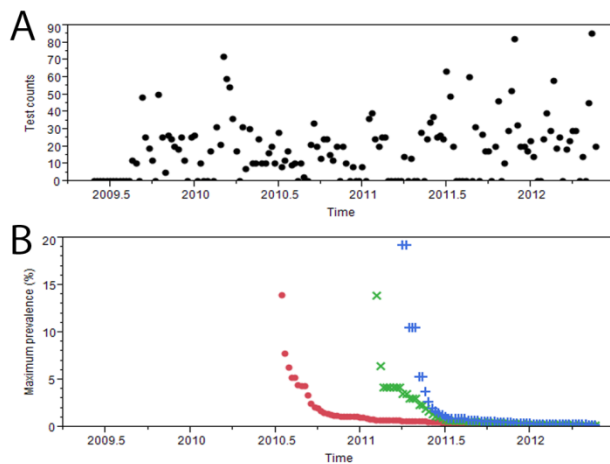


Figure. Decreasing upper bound of influenza prevalence in swine. A) Weekly number of test samples as a function of time. Despite a large number of negative test results, the negative results are informative to ensure the absence of specific viruses isolated in the past. B) Maximum influenza prevalence in swine. The estimate was calculated as the 95% upper bound of the binomial distribution give cumulative counts

of negative test results (B). The maximum prevalence for genetic variant I (filled circles), II (x marks) and III (+ mark) are shown separately.

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