Comparison of embryonated chicken eggs with MDCK cell culture for the isolation of swine influenza virus

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Abstract

Embryonated chicken eggs (ECE) and the Madin-Darby canine kidney (MDCK) cell line were compared for isolation of swine influenza virus (SIV) from nasal swabs and tissue samples. Samples originated from 30 pigs experimentally inoculated with 2×10^6 to 2×10^7 embryo infectious dose 50% (EID_{50})/mL of swine influenza strain A/Swine/Indiana/1726/88 (H1N1). The results were analyzed with McNemar's chi-squared test for symmetry. The results indicated that more samples were SIV-positive with ECE than with tissue culture ($P < 0.001$), suggesting that ECE remains the system of choice for isolation of SIV. It is recommend that routine use of both SIV isolation systems will increase the sensitivity of detection of virus shedding by considering the differences in growth and tropism of diverse SIV strains.

Résumé

Une comparaison entre des embryons de poulet (ECE) et la lignée cellulaire de rein de chien Madin-Darby (MDCK) fut faite quant à leur efficacité pour isoler le virus de l'influenza porcin (SIV) à partir d'écouvillon nasaux et d'échantillons de tissus. Les échantillons provenaient de 30 porcs inoculés expérimentalement avec 2×10^6 à 2×10^7 dose-infectante 50 % d'embryons (EID_{50})/mL de la souche du virus de l'influenza porcin de type A/Swine/Indiana/1726/88 (H1N1). Les résultats analysés à l'aide du test de χ^2 de McNemar pour la symétrie démontrent que plus d'échantillons étaient positifs pour SIV par culture sur ECE qu'en culture cellulaire (P < 0,001). Il est recommandé que les deux systèmes soient utilisés de routine pour l'isolement du SIV afin d'augmenter la sensibilité de la détection de l'excrétion du virus étant donné les différences dans la croissance et le tropisme des divers isolats de SIV.

(Traduit par Docteur Serge Messier)

Influenza virus is an enveloped RNA virus with a helical nucleocapsid with 8 segments of single-stranded negative-sense RNA. The envelope contains hemagglutinin (H) and neuraminidase (N) proteins, of which there are 15 H and 9 N subtypes (1). Influenza A viruses are widespread in nature and are highly infectious respiratory pathogens of pigs, horses, humans, and birds (2,3). Swine influenza virus (SIV) has been detected in swine populations of North America since 1930 (4). Infection of pigs with SIV leads to weight loss or retarded weight gain and, in some cases, to death. Clinical disease is marked by a sudden onset of anorexia and lethargy, with labored breathing, especially when animals are forced to move. Other clinical signs include rhinitis, nasal discharge, sneezing, and coughing. Morbidity is high, but mortality is low, unless there are concurrent infections or the pigs are very young. Reproductive problems have also been associated with outbreaks of swine influenza (5).

The objective of this study was to compare 2 systems for the isolation of SIV. The SIV strain A/Swine/Indiana/1726/88 (H1N1) for this study was kindly provided by Dr. Christopher Olsen of the University of Wisconsin in Madison, Wisconsin, USA. The virus was passed once in 9-day-old chicken embryos and the allantoic fluid was harvested and stored at −70°C until used for the inoculation experiment. Infectivity titer of the virus preparation was determined in 9-day-old chicken embryos according to standard methods (6). A group of 40 apparently healthy, crossbred, 10-week-old pigs, without history of swine influenza or vaccination against the disease, were selected and were acclimatized for 7 d prior to the start of the experiment. All the pigs were confirmed to be serologically negative for SIV antibodies by competitive enzyme-linked immunosorbent assay (c-ELISA) (7), the hemagglutination inhibition (HI) test (8), and the agar gel immunodiffusion (AGID) test. They were fed a commercial, non-medicated, age-appropriate ration, ad libitum, and had full access to water. All laboratory and animal experiments were conducted in facilities operated under biosafety level 3 agriculture containment. A veterinarian observed the pigs daily for any clinical signs of disease. The local animal care committee approved all animal procedures prior to initiation of the study.

Thirty pigs were challenged with a virus dose equivalent to 2×10^6 to 2×10^7 embryo infectious dose 50% (EID_{50})/mL diluted in 0.01 M phosphate-buffered saline (PBS). Ten negative control pigs

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Table I. Virus isolation in embryonated chicken eggs and MDCK cell culture from nasal swabs

Pig ID	0 ECE	0 CC	1 ECE	1 CC	3 ECE	3 CC	5 ECE	5 CC	7 ECE	7 CC	9 ECE	9 CC	11 ECE	11 CC
2[a]	–	–	–	–	–	–	–	–	–	–	–	–	–	–
3[a]	–	–	–	–	–	–	–	–	–	–	E	E	E	E
7[a]	–	–	–	–	–	–	–	–	–	–	E	E	E	E
10[a]	–	–	–	–	–	–	–	–	–	–	E	E	E	E
12[a]	–	–	–	–	–	–	–	–	–	–	E	E	E	E
15[a]	–	–	–	–	–	–	–	–	–	–	–	–	–	–
18[a]	–	–	–	–	–	–	–	–	–	–	–	–	–	–
23[a]	–	–	–	–	–	–	–	–	–	–	E	E	E	E
32[a]	–	–	–	–	–	–	–	–	–	–	–	–	–	–
33[a]	–	–	–	–	–	–	–	–	–	–	–	–	–	–
1	–	–	+	–	+	+	+	+	–	–	–	–	–	–
4	–	–	+	–	+	+	+	+	+	–	E	E	E	E
5	–	–	–	–	+	–	+	+	+	–	–	–	–	–
6	–	–	+	–	+	+	+	+	–	–	E	E	E	E
8	–	–	–	+	+	+	+	+	–	–	E	E	E	E
9	–	–	–	–	+	+	+	+	–	–	–	–	–	–
11	–	–	+	+	+	+	+	+	–	–	–	–	–	–
13	–	–	+	+	+	+	+	+	–	–	–	–	–	–
14	–	–	–	–	+	+	+	+	+	–	–	–	–	–
16	–	–	+	–	+	+	+	+	+	–	–	–	–	–
17	–	–	+	–	+	+	+	+	–	–	E	E	E	E
19	–	–	+	+	+	+	+	+	–	–	E	E	E	E
20	–	–	+	–	+	+	+	+	–	–	–	–	–	–
21	–	–	–	–	+	+	+	+	+	–	+	+	–	–
22	–	–	+	+	+	+	+	+	+	–	E	E	E	E
24	–	–	+	–	+	+	+	+	–	–	E	E	E	E
25	–	–	–	–	+	+	+	+	–	–	E	E	E	E
26	–	–	–	–	+	+	+	+	+	–	–	–	–	–
27	–	–	–	–	+	+	+	+	–	–	–	–	–	–
28	–	–	+	–	+	+	+	+	–	–	E	E	E	E
29	–	–	+	+	+	+	+	+	–	–	E	E	E	E
30	–	–	+	–	+	+	+	+	+	–	–	–	–	–
31	–	–	+	–	+	+	+	+	+	–	E	E	E	E
34	–	–	–	–	+	+	+	+	+	–	E	E	E	E
35	–	–	+	–	+	+	+	+	–	–	–	–	–	–
36	–	–	–	–	+	+	+	+	+	–	E	E	E	E
37	–	–	–	–	+	+	+	+	–	–	–	–	–	–
38	–	–	+	–	+	+	+	+	–	–	E	E	E	E
39	–	–	–	+	+	+	+	+	–	–	E	E	E	E
40	–	–	–	–	+	+	+	+	+	–	–	–	–	–

ECE = embryonated chicken eggs; CC = MDCK cell culture; E = euthanized; + = positive; – = negative
[a] control pig

were inoculated with sterile allantoic fluid diluted in 0.01 M PBS. Each pig was inoculated with a syringe or by aerosolization with an atomizer. All the pigs were anesthetized with a short-acting injectable anesthetic before administration of the challenge inoculum or the placebo. Nasal swabs were collected prior to inoculation and on days 1, 3, 5, 7, 9, and 11 postinoculation. The swabs were immediately placed into transport medium [1% bovine serum albumin (BSA) in brain heart infusion (BHI) containing 1 mg/mL of streptomycin, 20 μg/mL of vancomycin, 500 μg/mL of gentamicin, and 50 units of nystatin]. On days 7 and 13 postchallenge, a blood sample was collected from the pigs for detection of serum antibodies to SIV, using C-ELISA (7) and the HI test. Antibodies against SIV were detected in 26 out of 30 pigs at 7 d postinoculation with both tests. All the animals had seroconverted by 13 d postchallenge.

Table II. Comparative virus isolation results at different days postinoculation for the detection of swine influenza virus in 240 nasal swab samples

MDCK	0–1 dpi ECE		3–5 dpi ECE		7–11 dpi ECE		Total	Summary performance 0–11 dpi ECE		Total
	Positive	Negative	Positive	Negative	Positive	Negative		Positive	Negative	
Positive	5	2	59	0	1	0	67	65	2	67
Negative	12	61	1	20	12	67	173	25	148	173
Total	17	63	60	20	13	67	240	90	150	240
McNemar's χ^2	5.78		0		10.0			17.926		
P value	0.016		1.00		0.001			< 0.001		

ECE = embryonated chicken eggs; dpi = days postinfection

The antibody titers between 7 and 13 d postinoculation were increased approximately 30% in the C-ELISA and 4-fold in the HI test.

On days 7 and 14 postchallenge, 20 pigs were euthanized, and tracheobronchial lymph nodes, lung, tonsil, and other affected tissues were sampled for virus isolation (lung was the only tissue with macroscopic lesions). Samples were collected aseptically, and a new set of instruments was used for collecting each organ sample. Tissues were placed in sterile plastic bags. Samples and swabs were processed immediately after collection or were stored at −70°C until processed for virus isolation. All carcasses were disposed of according to protocols of the Canadian Food Inspection Agency.

Tissue samples were weighed in a sterile glass vial and were emulsified with heart infusion broth to make a 10% w/v suspension using a Tenbroek tissue grinder. Larger pieces of tissue were removed by centrifugation at 2000 × g at 4°C for 20 min, and the supernatant was harvested. Antibiotic solution was added (1 mg/mL of streptomycin, 20 µg/mL of vancomycin, 500 µg/mL of gentamicin, and 50 units of nystatin), the mixture was incubated at room temperature for 1 h, and the suspension was centrifuged at 2000 × g at 4°C for 20 min. The supernatant was harvested and considered as the undiluted sample. A portion of the original tissue was retained and kept frozen at −70°C. Swabs were transferred to tubes with transport medium, were vortexed vigorously, and were then removed from the tube with sterile forceps. The absorbed medium was squeezed out of the swab, either by gentle pressure of the swab against the tube wall or with sterile forceps. The sample was processed as described for the tissue samples.

Five 9-day-old specific pathogen-free (SPF) embryonated chicken eggs (ECE) were inoculated via the allantoic sac with 0.2 mL of 10% tissue suspension or processed swab. Eggs were incubated in a stationary incubator at 37°C with 55% relative humidity and were candled daily for 7 d for embryo viability. Embryos that died within 24 h were discarded. Allantoic fluid from embryos that died after 24 h was collected aseptically and tested for hemagglutinating virus by the hemagglutination (HA) test. If no deaths occurred in the eggs after 6 d, all of the eggs were opened aseptically. The allantoic fluids were pooled and inoculated into 5 more embryonated eggs. If no deaths occurred after 7 d in the eggs of the second passage, all the eggs were opened and the allantoic fluid of each egg was tested for hemagglutinating activity.

If deaths occurred in the eggs and the material had a positive HA test, samples were considered positive, and this result was confirmed with the HI test. Allantoic fluids from embryos showing early mortality after inoculation were tested for bacterial contamination according to routine procedures.

For isolation of SIV in tissue culture, Madin-Darby canine kidney (MDCK) continuous cell line was used in 24 well plates. Cells were seeded at a concentration of 8 × 10⁴ cells/cm² in MEM-α, 5% v/v fetal bovine serum (FBS), 100 IU/mL of penicillin and 100 µg/mL of streptomycin. Prior to inoculation, the medium from the seeded wells was removed by gentle inversion and washed 3 times with MEM-α. MEM-α containing trypsin (0.3 mL), to a final concentration of 4 µg/mL, was added to each well in all 24 well plates. For each sample, 0.2 mL of the swab preparation or 10% w/v tissue emulsion was inoculated onto 3 wells of MDCK cells. The inoculum was allowed to absorb at 37°C for 60 min in a 5% CO_2 humidified incubator and then 0.5 mL of MEM-α containing 4 µg/mL of trypsin was added to all wells. The cells were incubated at 37°C and 5% CO_2 for 3 to 6 d. The plates were observed daily for cytopathic effect (CPE). After 3 to 6 d, media were removed with a pipette and pools of the sample were tested for HA activity. If the HA test was negative, the samples were passed in MDCK cells and after 6 d tested for the presence of SIV with the indirect immunoperoxidase assay (SIV-IPA). Briefly, cell culture plates were gently washed with 1 mL of warm (37°C) 0.01 M PBS (pH 7.2) and were incubated for 5 min at room temperature. The plate was blot dried and the cell monolayer fully dried with the aid of a hair dryer set on high heat and speed. The monolayer was fixed by adding 500 µL of cold (4°C) fixation fluid [20% acetone, 0.02% bovine serum albumin in PBS (0.01 M, pH 7.6)] to all wells; the cells were allowed to fix for 10 min at room temperature. The fixation fluid was removed by inverting the plate and blotting it dry. The microplate was further dried with a hair dryer as described above. After washing each well with 1 mL per well of PBS at 37°C, the plates were stained with monoclonal antibody (Mab) HB65 (IgG$_{2a}$/ATCC H16-L10-4R5) (9), which is specific for nucleoprotein of influenza A. A 1/10 dilution of Mab (0.5 M NaCl, 0.05%; Tween 20 pH 7.6) was added to the fixed monolayer and then the plate was incubated at 37°C for 30 min and washed 3 times in 0.05% PBS-Tween-20 (PBS-T). Rabbit anti-mouse IgG horseradish peroxidase conjugate was added to all wells of the plate, which was incubated for 30 min at 37°C. The plate was washed as above and then 500 µL of the

Table III. Virus isolation in embryonated chicken eggs and MDCK cell culture from selected tissues from pigs euthanized on day 7

Pig	Lymph node[a]		Lung		Affected tissue[b]		Tonsil	
	ECE	CC	ECE	CC	ECE	CC	ECE	CC
3[c]	NA	NA	–	–	NA	NA	–	–
7[c]	–	–	–	–	NA	NA	–	–
10[c]	–	–	–	–	NA	NA	–	–
12[c]	–	–	–	–	NA	NA	–	–
23[c]	–	–	–	–	NA	NA	–	–
4	–	–	–	–	+	–	–	–
6	–	–	+	–	–	–	NA	NA
8	–	–	+	–	–	–	–	–
17	–	–	+[d]	NA	–	–	–	–
19	–	–	–	–	+	–	–	–
22	–	–	+	–	–	–	–	–
24	–	–	+	–	–	–	–	–
25	–	–	–	–	+[d]	NA	–	–
28	–	–	–	–	+	–	–	–
29	–	–	–	–	–	–	–	–
31	–	–	–	–	+	–	–	–
34	–	–	+	–	+	+	–	–
36	–	–	+	+	+	–	+	–
38	–	–	–	–	–	–	–	–
39	–	–	–	–	–	–	–	–

ECE = embryonated chicken eggs; CC = MDCK cell culture; NA = not available; + = positive; – = negative

[a] Tracheal and bronchial lymph nodes

[b] Affected tissue was lung in all cases

[c] Control pig

[d] Not considered for comparison purposes because no tissue culture results were available with this sample

substrate working solution (3-amino-9-ethylcarbazole substrate; N,N-dimethylformamide; 3% v/v H_2O_2) was added to all wells. The reaction was allowed to occur for 15 min at room temperature. The substrate solution was discarded and the plate was dried. The reaction was read with an inverted light microscope. A positive reaction exhibited a red/brown cytoplasmic staining in response to the precipitating substrate.

The results were analyzed with the McNemar chi-squared test, and $P < 0.05$ was considered statistically significant (10). During the study period, 240 nasal swabs were collected in total on different days postinfection and analyzed by virus isolation using ECE and MDCK tissue culture (Table I). A total of 92 (38%) samples were positive by either method. However, the isolation ratio at different days postinfection indicated that 19/80 samples (24%) collected between 0 and 1 d postinfection were positive and that 60/80 samples (75%) collected between 3 and 5 d postinfection were positive. In contrast, only 12/80 samples (15%) were positive on day 7 postinfection. In comparing the performance of the 2 SIV isolation methods, 90 (98%) and 67 (73%) of the 92 positive samples were positive by ECE and MDCK cell culture, respectively (Table II). Overall, more samples were positive by ECE than by MDCK cell culture ($P \leq 0.001$). The isolation ratios for the 2 methods were significantly different only for samples collected from 0 to 1 d postinfection ($P = 0.016$) and from 7 to 11 d postinfection ($P = 0.001$) (Table II).

The proportions of virus positive samples were 67/240 (28%) and 90/240 (38%) by MDCK tissue culture and ECE, respectively, and the confidence interval for the difference was 10% ± 4. On day 7 postinfection, 13 of 69 tissues samples (18%) were positive by ECE and the MDCK method (Table III), whereas on day 14 postinfection no virus was isolated from 73 tissue samples. As expected, lung was the tissue of choice over tonsil for isolation of the virus. A total of 13 samples (19%) were positive by ECE, whereas only 2 (3%) were positive by tissue culture ($P \leq 0.001$; confidence interval 16% ± 8, greater than found for nasal swabs).

Isolation of SIV is routinely performed in 10- to 11-day-old ECE from swab material or the supernatant of 10% lung suspension (11). However, some recent reports in which only a small number of samples (10) was used suggest that isolation of SIV can be improved by using MDCK cell culture (12).

Overall, it was found that nasal swabs remain the preferred sample over lung for virus isolation and that virus isolation is more effective with ECE than with cell culture. The choice of the MDCK cell line for isolation of SIV was based on a previous study (13), in which 3 different cell lines were compared for the isolation

of influenza A virus. The sensitivity of the MDCK cell line was greater than the sensitivity of the Vero and MRC-5 cell lines and, as a result, the MDCK cell line was recommended for the isolation of influenza viruses. MDCK cells were first used in influenza A diagnosis in 1968 (14). In a recent study (15), primary fetal porcine kidney cells were evaluated for the isolation of SIV. Similar results were obtained when compared with isolation by inoculation of ECE.

From 1975 on, MDCK cells were more frequently used for isolation of influenza A viruses after it was found that adding trypsin to the culture stimulates the growth of influenza A viruses and enables many influenza virus strains to form plaques with high efficiency (16). In addition to embryonated eggs, MDCK cells in culture medium containing trypsin are now considered a valuable system for isolation of these viruses from clinical specimens (17,18).

Influenza A viruses have significantly different host properties (Webster, 1997) and possibly different in vitro growth properties, depending on the strain (19). These differences may be associated with the preferential cell receptor, which some influenza A virus strains need to induce infection (2). Some reports describe that some human and swine influenza isolates do not grow well in ECE and that cell culture provides better results for primary isolation (12,20). The H1N1 virus in this study was an egg adapted strain, which could explain the increased sensitivity of isolation in ECE over MDCK. However, the virus was passaged through pigs for the purpose of this study.

Although results indicate that ECE are more suitable for isolation of SIV, the high variability of these viruses and the evidence presented by other authors suggest that both ECE and cell culture need to be used for the primary isolation of SIV. Replacing ECE by cell culture in routine isolation of SIV will reduce the sensitivity of the isolation, as suggested previously (12).

Acknowledgments

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