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Novel reassortment of Eurasian Avian-like and pandemic/2009 influenza viruses in swine: infectious potential to humans

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ABSTRACT

Pigs are considered to be intermediate hosts and "mixing vessels", facilitating the genesis of pandemic influenza viruses as demonstrated by the emergence of the 2009 H1N1 pandemic (pdm/09) virus. The prevalence and repeated introduction of the pdm/09 virus to pigs raises the possibility to generate novel swine influenza viruses with the potential to infect humans. To address this, an active influenza surveillance program was conducted on slaughtered pigs in abattoirs in Southern China. Over 50% of the pigs tested were found to be seropositive for one or more H1 influenza viruses, most commonly pdm/09-like viruses. Out of 36 virus isolates detected, one group of novel reassortants had Eurasian avian-like swine H1N1 surface genes and pdm/09 internal genes. Animal experiments showed that this virus transmitted effectively from pig to pig and from pig to ferret, and it could also replicate in *ex-vivo* human lung tissue. Immunization against the 2009 pandemic virus gave only partial protection to ferrets. The continuing prevalence of the pdm/09 virus in pigs could lead to the genesis of novel swine reassortant viruses with the potential to infect humans.

Key words: swine influenza, evolution, reassortant, interspecies transmission

INTRODUCTION

Pigs are considered to be "mixing vessels", facilitating reassortment events among avian, swine and human influenza viruses, which might allow the introduction of novel viruses into the human population (19, 20). The occurrence of the 2009 H1N1 influenza pandemic provided renewed evidence that pigs do play such a role in the influenza ecosystem (5, 11). Accumulated findings from epidemiological and genetic studies have revealed that each of the eight gene segments of the 2009 H1N1 pandemic virus (pdm/09) was generated through multiple reassortant events among viruses that had long been prevalent in and adapted to pigs (8, 24). However, due to a lack of systematic surveillance in northern America, the direct precursor of the pdm/09 virus has not yet been recognized.

Another important lesson learned from the 2009 pandemic was that a virus of the same subtype as the prevailing human seasonal influenza virus could become pandemic if there were significant antigenic differences (8, 30). As such, any virus with the capacity to infect humans, and with novel hemagglutinin genes that are antigenically distinct from circulating human strains, should be considered as having pandemic potential.

Currently circulating swine influenza viruses are associated with the classical swine (CS) lineage, the North American triple reassortant (TR) lineage, or the Eurasian avian-like (EA) lineage (3, 13, 16, 27). Two influenza pandemics (those of 1918 and 2009) were caused by viruses with HA genes closely related to, or directly belonging to, the CS lineage (8, 27, 30). Sporadic human infection cases with different swine virus lineages are not rare events (4, 10, 15, 21).

Although the EA virus has been prevalent in Eurasian pig populations for more than 30 years, it is only occasionally detected in humans (1, 6, 9, 17). Currently, the majority of the human population are immunologically naïve to EA-like virus (27). Antibodies against pdm/09 are unlikely to confer substantial protection against EA-like viruses as convalescent human sera post pdm/09 infection or human sera post vaccination did not cross-react with an EA H1N1 virus (26).

After the occurrence of the 2009 pandemic, the pdm/09-like virus was repeatedly introduced back to pigs in many countries (18, 26, 29). Recent influenza surveillance in Hong Kong showed that the CS, TR and EA swine influenza lineages were co-circulating in pigs in southern China (24, 26, 27). Many of the contemporary swine virus isolates were reassortant variants of different swine influenza viruses (24, 26, 27)

and the pdm/09 virus has also reassorted with other circulating swine influenza viruses (14, 25, 26). This raises concerns that pdm/09 reassortant variants within pigs may cause new threats to human health.

As approximately half of the world's population of domestic pigs is farmed in China (7), this represents the largest localized collection of "mixing vessels" for influenza viruses in the world, and therefore, the greatest opportunity to generate reassortant viruses with the potential to infect humans. To understand the further development and impact of the pdm/09-like virus in pigs, active surveillance of influenza in pigs in Southern China has been conducted since December 2009. Over 50% of pigs were seropositive for at least one H1 influenza virus (mostly pdm/09) and several viruses with novel genotypes were isolated. One isolate with EA-like surface genes and pdm/09 internal genes was tested for intra- and inter-species transmissibility in mammalian models. The findings emphasize the need for ongoing influenza surveillance of the pig population.

MATERIALS AND METHODS

Surveillance. On an approximately weekly basis from December 2009 to June 2010, a total of 3,600 tracheal swabs and 1,020 sera samples were collected from slaughtered pigs at abattoirs in Guangdong (swabs, n=2,240; sera, n=625; sampling occasions, n=21) and Guangxi (swabs, n=1,360; sera, n=395; sampling occasions, n=13) provinces of the People's Republic of China. The samples collected in Guangdong represent pigs introduced from many neighboring provinces, while those collected in Guangxi are mainly from local pigs.

Hemagglutination inhibition (HI) assays. Sera were pre-treated with a receptor-destroying enzyme (RDE, Denka Seiken Co. Ltd, Tokyo, Japan) to destroy nonspecific inhibitors, followed by heat inactivation at 56˚C for 30 mins. RDE-treated sera were then absorbed with Turkey red blood cells (TRBC) to remove nonspecific agglutination substances. Antibody titer was determined by testing serial two-fold dilutions (1:10 to 1:2,560) of each serum in duplicate. HI assays were performed in 96-well microtiter plates (Corning Costar Co.) with 0.5% turkey erythrocytes using four hemagglutination units of virus.

Serological survey. HI assays were performed on each of the 1,020 sera samples collected, with a contemporary human H3N2 virus (A/Shantou/1328/2008) and five representative swine H1 influenza virus strains: A/Sw/HK/294/09 (CS H1N2), A/Sw/HK/915/04 (TR H1N2), A/Sw/HK/NS1583/09 (pdm/09 H1N1), A/Sw/HK/2433/09 (EA H1N1) and A/Sw/HK/1532/09 (EA-like H1N1 variant) (24, 26, 27).

Virus isolation and sequencing. Isolation of viruses from tracheal swabs in Madin-Darby canine kidney (MDCK) cells, viral RNA extraction, cDNA synthesis, PCR and sequencing were carried out as previously described (24, 26, 27). All 288 nucleotide sequences of the segments of the 36 influenza isolates detected in this study have been deposited in GenBank under accession numbers JN374994-JN375281. The virus A/Swine/Guangdong/1361/2010 (H1N1) was plaque-purified and re-sequenced to confirm its identity before use in subsequent experiments.

Phylogenetic analysis. For each gene segment identified here and representative influenza virus sequences from GenBank, maximum likelihood (ML) phylogenies were inferred using the heuristic tree search method Garli 1.0 (34). Phylogenetic

support for branch points was estimated by bootstrap analysis with 100 replicates using the same ML method.

Animals. All pigs (n=16, local domestic hybrid pigs, Putian White×Nianbian variants) used were confirmed to be free of influenza virus by virus isolation in MDCK cells and to be seronegative against circulating swine and human influenza viruses (CS, TR, EA, pdm/09, seasonal H1N1 and H3N2), as well as avian H5N1 and H9N2 viruses by HI assays prior to the start of the study. Ferrets (n=9) negative for both influenza virus and antibodies were obtained from the experimental ferret breeding program at Sangosho Pet Park Co., Ltd. Animal experiments were approved by the Shantou University Medical College, in compliance with the University Policies "Animal Ethics and Welfare" and "Use of Animals in Research", and the guidelines of the World Health Organization and the International Council for Laboratory Animal Science.

Viral infectivity studies. Infection and transmission studies were carried out in biosafety level 3 (BSL-3) containment laboratories at 20-21˚C and 76.5±2.1% relative humidity. All animals were moved to the BSL-3 lab at least 4 days prior to the experiment for acclimatization. A microchip (Implantable Programmable

157 Temperature Transponder™ IPTT-300, BioMedic Data Systems) was implanted into
158 the skin of each animal, between the shoulder blades, to measure subcutaneous
159 temperatures.
160
161 **Immunization of ferrets with the CA4 virus.** Twenty-two week old male ferrets
162 (n=3) were inoculated with 10^6 PFU (plaque forming units) of A/California/04/2009
163 (CA4, the prototype pdm/09 virus) and gained high antibody titers (HI>1,280) to this
164 virus on the 14th day post-primary infection (dpi). These immunized ferrets were used
165 for the transmission experiment at 80 dpi.
166
167 **Infection and transmission experiments with Sw/GD1361 virus.** Four-week-old
168 piglets (n=7) were intranasally inoculated with 2×10^6 PFU, i.e. 2.56×10^6 TCID50
169 (median tissue culture infective dose) of Sw/GD1361 virus in 2 ml of MEM, delivered
170 with a mucosal atomization device (MAD® Nasal Drug Delivery Device, Wolfe Tory
171 Medical, Inc.) to mimic aerogenous infection (2). Two naïve pigs and three naïve
172 ferrets inoculated with Phosphate buffered saline (PBS) were used as negative control
173 animals.
174

175 At 24 hours post-inoculation (hpi) of the pigs, immunologically naïve pigs (n=7,
176 4-week old) and ferrets (naïve ferrets, n=3; ferrets with antibodies against CA4, n=3;
177 33.5-week old) were introduced into the animal infection and transmission facility
178 holding the inoculated pigs (n=7) (Fig. S3). Four immunologically naïve pigs were
179 used as physical contact animals, and three were used for aerosol contact. The aerosol
180 contact pigs and ferrets were housed in adjacent double layer steel wired cages with a
181 distance of at least 10 cm from those holding the directly inoculated pigs. Airflow
182 inside and between each pair of cages was <0.1 m/sec. To avoid inadvertent physical
183 contact and artificial transmission, aerosol contact animals were always handled first;
184 drinking water and food stock, gloves and any other items in contact with the animals
185 or their bedding were kept sterile by decontamination or reserved for the exclusive
186 use of each individual animal.
187
188 **Animal Monitoring.** Body weights and temperatures were recorded daily around the
189 same time (9:30-10:30 am) for each animal. Clinical signs were observed twice daily.
190 Nasal swabs from each piglet were collected daily and placed into 0.6 ml of cold
191 sterile phosphate buffered saline (PBS) with antibiotics. Nasal washes from each
192 ferret were collected daily into 1 ml of PBS with antibiotics. The end point infectivity
193 titration ($TCID_{50}$) was determined for all swabs and nasal washes in MDCK cells.

194

195 **Animal serology and histology**. Blood was collected via venipuncture of the anterior
196 vena cava of the pigs or from the ferret tail artery. Seroconversion was monitored by
197 determining the HI titers of pre- and post-exposure sera.

198

199 Four directly inoculated pigs were euthanized for post-mortem examination (2 at 4dpi
200 and 2 at 6 dpi) by intracardiac injection of pentobarbital sodium (100-200mg/kg). One
201 physical contact pig was similarly euthanized at 5 days post contact (dpc). Freshly
202 excised tissues of the trachea, lungs, nasal turbinates and other major organs of
203 euthanized animals were fixed in 10% phosphate-buffered formalin, dehydrated,
204 embedded in paraffin, and cut into 5 μm thick sections. Standard Hematoxylin and
205 Eosin (Sigma) (H&E) staining as well as immunohistochemistry (IHC) assays with a
206 mouse anti-NP (nucleoprotein) monoclonal antibody were performed as previously
207 described (32).

208

209 **Infection of human lung tissue in *ex vivo* culture.** Similar to an earlier report (31),
210 fresh lung tissues were surgically removed from patients with lung carcinoma, in
211 accordance with a protocol approved by the Ethical Review Board of Shantou
212 University Medical College. Only normal nonmalignant tissue fragments that were

213 excess to the requirements of clinical diagnosis were used. Tissues were cut into
214 ~3mm×4mm×2mm cubes and placed into F-12K nutrient mixture (Gibco) with
215 L-glutamine and antibiotics. Three viruses were used for inoculation: CA4 (pdm/09),
216 Sw/GD1361 (the novel reassortant, this work) and A/Wuhan/359/1995 (seasonal
217 H3N2). For each virus, infections were performed in triplicate, with 7 lung tissue
218 cubes per replicate inoculated with 0.5×10^6 PFU of virus in 0.5ml inocula in 6-well
219 plates, and allowed to absorb for 1 h at 37˚C, 5% CO_2. The tissue cubes were then
220 washed three times with culture medium and incubated with 0.5ml of F-12K medium
221 supplemented with 0.2% TPCK (N-tosyl-L-phenylalanine chloromethyl
222 ketone)-Trypsin, 1% BSA (bovine serum albumin) and antibiotics. At 18 and 36 hpi,
223 lung tissue cubes (n=5 each per virus) were individually rinsed with medium and
224 homogenized in 0.5 ml of cold PBS, then clarified by centrifugation. Virus titers of
225 the homogenates were determined by $TCID_{50}$ assays in MDCK cells. The remaining
226 cubes (n=11, per virus) were fixed and examined for viral NP expression by
227 immunohistochemical staining (31, 32).
228

229 **RESULTS**

230

231 From December 2009 to June 2010, active surveillance of pigs in the Guangdong (GD)
232 and Guangxi (GX) provinces of southern China was undertaken for evidence of
233 influenza infection and virus isolation.

234

235 **Seroprevalence of influenza virus in pigs of southern China.** In the study period,
236 based on hemagglutination inhibition (HI) assays, about 51% (521/1,020) of the sera
237 were positive (HI titer ≥1: 80) against at least one of five reference H1 strains, while
238 all were negative to the contemporary human H3N2 influenza virus (HI titer ≤ 1: 20)
239 (Fig. S1, Table 1).

240

241 Of the 625 sera collected in GD (over the entire surveillance period), 104 (16.6%)
242 were solely positive to the pdm/09-like virus, while 35 (8.9%) of the sera from GX
243 were solely positive to this virus. Additionally, 295 (28.9%) sera (GD n=166, GX
244 n=129) were positive to pdm/09 and one or more other viruses (Table 1), indicating a
245 high frequency of co-infection or multiple virus exposure in the pigs from southern
246 China, although cross-reaction cannot be completely excluded. Some of the sera had

247 extremely high HI titers (≥ 1: 2560) to different H1 reference viruses (Fig. S1),
248 suggesting a recent infection.
249
250 **Genetic characterization of swine influenza isolates.** Thirty-six H1N1 and H1N2
251 influenza viruses were isolated from tracheal swabs obtained in Guangdong but none
252 from those taken in Guangxi. Full-length sequences were obtained for each of the
253 eight gene segments of all 36 swine virus isolates. Phylogenetic analyses of the H1
254 hemagglutinin (HA) gene revealed that these viruses clustered into three different
255 lineages: the pdm/09, CS and EA virus lineages (Fig. S2).
256
257 For each of the remaining genes, phylogenetic analyses revealed that these viruses
258 belonged to four distinct genotypes: pdm/09-like H1N1 (n=12, four sampling
259 occasions); a previously undescribed EA-like H1N2 variant with its HA gene from the
260 CS lineage and NA (N2) gene from the human lineage (n=5, single sampling
261 occasion), a novel reassortant with EA-like H1N1 surface genes and pdm/09-like
262 internal genes (n=10, single sampling occasion) and an EA-like H1N1 variant with
263 the non-structural (NS) gene from the TR lineage (n=9, single sampling occasion)
264 (Figs. 1 and S2).
265

266 It was noted that viruses isolated from the same sampling occasion were very closely
267 related to each other, and always clustered together in the phylogenetic trees (Fig. S2).
268 All pdm/09-like viruses were detected from late December 2009 to the end of January
269 2010 (from four sampling occasions), while the remaining viruses were isolated in
270 February and May, 2010 (Fig. 1).
271
272 To see if there is any evidence of early adaptation of pdm/09-like viruses in pigs, the
273 sequences of the 22 viruses containing pdm/09-like genes were compared with all the
274 pdm/09 sequences in GenBank (as of 5 Mar 2011). Nineteen of these viruses
275 contained a total of 11 substitutions in 6 internal genes that were absent in all human
276 pdm/09 virus sequences (Table 2). Ten of the novel reassortant viruses, represented
277 by A/swine/Guangdong/1361/2010 (Sw/GD1361), had 6 of these substitutions (in the
278 PB2, PA, M1 and NP genes), while Sw/GD/286/2010 had 2 unique substitutions (in
279 the PB2 and PA genes). The other 8 viruses contained only one substitution, in the
280 PB2 gene (5 viruses, represented by Sw/GD/275/2010) and the NS1/NS2 gene (3
281 viruses, represented by Sw/GD/94/2009). Only the substitution in the NS1/NS2 gene
282 was observed on more than one sampling occasion.
283

284 **Assessment of a novel H1N1 EA-pdm/09 reassortant virus.** The infectivity,
285 transmissibility and pathogenicity of a representative isolate with EA-like HA and NA
286 genes and pdm/09-like internal genes (Sw/GD1361) were tested in experimental
287 animals. Interspecies transmissibility and the potential for cross-protection from prior
288 pdm/09 infection were investigated.
289
290 **Infectivity and transmissibility in pigs.** Seven uninfected pigs were co-housed,
291 either in physical or aerosol contact, with seven pigs experimentally inoculated with
292 the Sw/GD1361 virus (Fig. S3). Virus shedding from each of the inoculated pigs was
293 detected from the first day post-inoculation (dpi) till the 7th day, with a peak at 4 dpi
294 (6.0±0.3 log $TCID_{50}$/ml swab material) (Fig. 2a). In the physical contact pigs, virus
295 replication in the nasal cavity lasted from day 1 to day 7 post-contact (dpc), with peak
296 titers of 6.0±0.7 log $TCID_{50}$/ml (Fig. 2b). In the aerosol contact group, pigs started to
297 shed virus from the nasal route between 3-5 dpc, and virus could be detected for at
298 least 4-6 days, with peak titers of 5.9±1.3 log $TCID_{50}$/ml (Fig. 2c). There were no
299 statistically significant differences in viral shedding among the inoculated, physical
300 contact or aerosol contact pigs (Fig. 2a-c), and the peak virus shedding titers were
301 comparable with those of prototype EA-like (27) and pdm/09 viruses (2, 12, 28, 29,
302 33) as previously reported. Lethargy, lower activity levels and reduced interest in food

303 occurred in the pigs, but no major clinical symptoms of infection were observed. On
304 15 dpi or 14 dpc, all experimental pigs, either inoculated or exposed by physical or
305 aerosol contact, had seroconverted with HI titers ranging from 160 to 1280 (Table 3),
306 which were comparable with those of EA-like viruses (27), and higher than those of
307 pdm/09-like viruses (33). Cross-reaction of swine sera was only observed to the EA
308 viruses. Naïve pigs inoculated with PBS did not show virus shedding or
309 seroconversion (data not shown).
310
311 **Interspecies transmissibility of Sw/GD1361 to ferrets.** Ferrets were used as sentinel
312 animals for an aerosol contact, interspecies transmission test. Two of the three
313 immunologically naïve ferrets (F1 and F2), held in separate cages with a distance of
314 10 cm to the cage of the infected pigs, began to shed virus at 2 dpc, with the third (F3)
315 shedding virus at 5 dpc. Large amounts of virus (peak titer > 5 log $TCID_{50}$/ml) were
316 secreted from the nasal discharges of the ferrets, lasting for 5-6 days (Fig. 2d).
317 Sneezing, nasal discharge, inactivity and slight fever (1.3-1.4˚C increase in body
318 temperature) were observed in the ferrets. On 14 dpc, all ferrets had seroconverted
319 with an HI titer of at least 640 against the homologous virus (Table 3). In general,
320 ferret sera showed broad cross-reactivity to all H1 viruses tested. Naïve ferret controls
321 did not show virus shedding, clinical signs or seroconversion (data not shown).

322

323 Ferrets (F1* to F3*) seroconverted against the prototype pandemic virus
324 (A/California/04/09, CA4), developed signs of infection when co-housed with the
325 other ferrets. Symptoms, similar to its naïve counterpart (F1), developed in ferret F1*
326 at 2 dpc with virus shedding for at least four days (2-5 dpc). The other seroconverted
327 ferrets (F2* and F3*) also shed virus, although the onset was delayed 5-6 days (Fig.
328 2e). Comparison of the pre- and post-exposure HI titers from these ferrets showed that
329 two of them (F1* and F2*) had 4-8 fold increases in antibodies against the
330 Sw/GD1361 virus, whereas the third one (F3*) had a 2 fold increase (Table 3). Even
331 though ferrets seropositive to pdm/09 could be infected by Sw/GD1361, the amount
332 of virus shed and duration of shedding were reduced when compared with the
333 immunologically naïve ferrets (Fig. 2d and e). In the viruses recovered from the nasal
334 washes of all six contact ferrets, no amino acid substitutions were observed that
335 related to the antigenic sites (Ca1, Ca2, Cb, Sa and Sb) of the HA protein (data not
336 shown).

337

338 **Infectivity in the pig respiratory tract and *ex vivo* human lung tissue.** Euthanized
339 pigs (four inoculated and one contact) showed similar lung lesions and clear evidence
340 of virus replication in the nasal turbinate, trachea and lower respiratory tract.

341 Pulmonary consolidation and extensive bronchioalveolitis were also observed,
342 characterized by multiple foci of severe inflammatory infiltrates and necrosis of
343 bronchus epithelia (Fig. 3), which is similar to the effect of pdm/09-like virus
344 infections (2, 12, 28, 29).
345
346 Immuno-staining of viral proteins and $TCID_{50}$ titers showed that Sw/GD1361 and the
347 prototype pdm/09 virus (CA4) could infect human lung tissues, whereas only very
348 limited infection was observed with human seasonal H3N2 influenza virus
349 (A/Wuhan/359/1995) (Fig. 4).
350

DISCUSSION

In the present study, virological surveillance revealed that pdm/09-like, TR-like, CS-like and EA-like viruses were co-circulating in pigs in southern China with relatively high prevalence (Figs. 1, S1, Table 1). Serological data from this surveillance suggested that infections with multiple different H1 viruses occur commonly in pigs (Table 1). Multiple infections, as observed here, highlight the possibility of further reassortment among these swine influenza lineages. Isolation of novel reassortant viruses with three differing genotypes during this surveillance demonstrated that such reassortment events do occur (Fig. 1).

Although the pdm/09-like virus has been repeatedly detected in pigs from different countries (18, 26, 29), whether it can become established in pigs remains unknown. Throughout this study a high seroconversion rate to the pdm/09 virus was observed in pig populations in southern China. Repeated detections of genetic reassortment between pdm/09-like and other swine viruses (14, 25, 26), in this study with EA-like viruses, suggest that the pdm/09-like virus might have been maintained in pigs for a period of time. It appears likely that the pdm/09-like virus will eventually become established in pigs.

370

371 Several unique substitutions were recognized in the pdm/09-like swine viruses
372 isolated here that were absent in all human pdm/09-like viruses. Some of these viruses
373 had multiple such substitutions over several genes. These changes implied that a
374 process of adaptation of the current pandemic virus to swine might be in progress.
375 This has parallels with the association of classical swine viruses with the 1918
376 pandemic virus and the emergence of the 1968 pandemic virus in pigs (22, 23) .

377

378 Current H1 influenza viruses circulating in mammals fall into two major clades, the
379 EA-like and the CS/human H1 clades (Fig S2). All human H1 viruses established in
380 the 20th century cluster with the CS lineage, from which the TR viruses and the
381 pdm/09 virus were derived, and are distinct from the EA viruses. Viruses with
382 EA-like HA genes rarely infect humans and the human population would likely be
383 immunologically naïve to such a virus (1, 6, 9). Therefore the ability of the
384 EA-pdm/09-like reassortant detected here to cross the species barrier is relevant to the
385 possibility of novel threats to human health arising from multiple infections of pigs
386 with the pdm/09-like and other swine influenza viruses.

387

388 It was shown that the Sw/GD1361 virus could not only be transmitted efficiently from
389 pig-to-pig, but also could spread by aerosol from pig to ferret. These animal
390 experiments and the replication of the virus in *ex-vivo* human lung tissue suggest that
391 the Sw/GD1361 EA-pdm/09-like reassortant virus could have the potential to cross
392 the species barrier and infect humans.
393
394 Ferrets previously inoculated with and seroconverted to the pdm/09 virus could not
395 avoid symptomatic infection with the Sw/GD1361 virus, indicating there was no
396 substantial cross-protection between this EA-pdm/09 reassortant and the pdm/09 virus.
397 As such, this reassortant, and others like it that will be generated if pdm/09-like
398 viruses become established in pigs, may represent a new threat to contemporary
399 human populations. Prior exposure to currently circulating viruses is unlikely to
400 provide protection from novel viruses of this type. Intensive surveillance of influenza
401 viruses in pigs appears warranted to closely monitor their future evolution, their
402 extent of reassortment and their potential to impact on public health.
403

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542

Figure Legends

FIG. 1. Genotypes of the swine influenza viruses identified. The name of a representative virus and the numbers of each variant isolated are given (left). Dates of the sampling occasions are A: 2009.12.25, B: 2009.12.31, C: 2010.01.08, D: 2010.01.29, E: 2010.02.27, F: 2010.05.07, G: 2010.05.28.

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564 (A) Immunohistochemical detection of viral antigen (nuleoprotein) in *ex vivo* human lung
565 tissue sections (36 hpi). NP-positive cells are shown by brown staining. (B) Virus titers in the
566 *ex vivo* human lung tissue cubes. The values are means (±standard deviation) of five replicate
567 tissue cubes from three independent inoculations.
568

569 **Table 1. Seroprevalence of antibodies against different swine influenza virus lineages**

570

Seropositive to				Guangdong		Guangxi		Subtotal	
				No.	(%)	No.	(%)	No.	(%)
None				306	(49.1)	193	(48.9)	499	(48.9)
Pdm				104	(16.6)	35	(8.9)	139	(13.6)
	TR			2	(0.3)	3	(0.8)	5	(0.5)
		CS		8	(1.3)	6	(1.5)	14	(1.4)
			EA	35	(5.6)	19	(4.8)	54	(5.3)
Pdm	TR			15	(2.4)	11	(2.8)	26	(2.5)
Pdm		CS		25	(4.0)	24	(6.1)	49	(4.8)
Pdm			EA	13	(2.1)	8	(2.0)	21	(2.1)
	TR	CS		0	(0.0)	0	(0.0)	0	(0.0)
	TR		EA	0	(0.0)	0	(0.0)	0	(0.0)
		CS	EA	3	(0.5)	7	(1.8)	10	(1.0)
Pdm	TR	CS		25	(4.0)	23	(5.8)	48	(4.7)
Pdm	TR		EA	4	(0.6)	0	(0.0)	4	(0.4)
Pdm		CS	EA	15	(2.4)	18	(4.6)	33	(3.2)
	TR	CS	EA	1	(0.2)	3	(0.8)	4	(0.4)
Pdm	TR	CS	EA	69	(11.0)	45	(11.4)	114	(11.2)
Total sera positive				319	(51.0)	202	(51.1)	521	(51.1)
Total sera tested				625		395		1020	

571

572 Abbreviation of representative viruses: Pdm: A/Sw/HK/NS1583/09 (H1N1); TR:

573 A/Sw/HK/915/04 (H1N2); CS: A/Sw/HK/294/09 (H1N2); EA: A/Sw/HK/2433/09 (H1N1)

574 and A/Sw/HK/1532/09 (H1N1). Viruses against which the swine sera showed an HI titer ≥80

575 are given in the table.

576

577 **Table 2 Unique positions in the novel isolates *vs* human and swine pdm/09 viruses.**

578

Protein	Substitution	No. of isolates	Sampling occasion	Genotype	Representative strain
PB2	T81A	10	F	EA-pdm/09	Sw/GD/1361/2010
	Q300K	5	D	pdm/09-like	Sw/GD/286/2010
	N348D	1	B	pdm/09-like	Sw/GD/213/2009
	I615V	9	F	EA-pdm/09	Sw/GD/1361/2010
PA	I38T	1	D	pdm/09-like	Sw/GD/286/2010
	S140T	10	F	EA-pdm/09	Sw/GD/1361/2010
	V521I	1	F	EA-pdm/09	Sw/GD/1425/2010
NP	H289Y	10	F	EA-pdm/09	Sw/GD/1361/2010
	S344L	10	F	EA-pdm/09	Sw/GD/1361/2010
NS1/NS2	T5N	3	A, B	pdm/09-like	Sw/GD/94/2009
M1	V31I	10	F	EA-pdm/09	Sw/GD/1361/2010

579

580 Substitutions to the residues in the novel isolates are relative to the human pdm/09 numbering

581 and consensus sequences. For dates of sampling occasions see Fig. 1.

582

583 **Table 3. HI titer of sera from pre-exposure (3 days prior to Sw/GD1361 inoculation)**

584 **and post-exposure (15 dpi or 14 dpc) animals infected with Sw/GD1361.**

Serum		Virus: CS Sw/HK4167 pre	CS Sw/HK4167 post	pdm/09 CA4 pre	pdm/09 CA4 post	pdm/09 Sw/GD106 pre	pdm/09 Sw/GD106 post	reassortant Sw/GD1361 pre	reassortant Sw/GD1361 post	EA Sw/HK29 pre	EA Sw/HK29 post	EA Sw/HK2433 pre	EA Sw/HK2433 post
Sw/GD1361 Direct inoculation pig	1	<10	ND	<20	ND	<20	ND	<10	ND	<10	ND	<10	ND
	2	<10	<10	<10	<10	<10	<10	<10	320	<10	320	<10	320
	3	<10	ND	<10	ND	<10	ND	<10	ND	<10	ND	<10	ND
	4	<10	<10	<10	<10	<10	<10	<10	320	<10	320	<10	80
	5	<10	ND	<10	ND	<10	ND	<10	ND	<10	ND	<10	ND
	6	<10	<10	<10	<10	<10	<10	<10	160	<10	160	<10	80
	7	<10	ND	<10	ND	<10	ND	<10	ND	<10	ND	<10	ND
Sw/GD1361 Physical contact pig	P1	<10	10	<10	<10	<10	<10	<10	640	<10	640	<10	160
	P2	<10	<10	<10	<10	<10	<10	<10	320	<10	320	<10	320
	P3	<10	<10	<10	<10	<10	<10	<10	320	<10	320	<10	160
	P4	<10	ND	<10	ND	<10	ND	<10	ND	<10	ND	<10	ND
Sw/GD1361 Aerosol contact pig	A1	<10	<10	<10	<10	<10	<10	<10	1280	<10	1280	<10	320
	A2	<10	<10	<10	<10	<10	<10	<10	320	<10	320	<10	80
	A3	<10	<10	<10	<10	<10	<10	<10	640	<10	640	<10	160
Sw/GD1361 Aerosol contact ferret	F1	<10	1280	<10	1280	<10	>1280	<10	>1280	<10	>1280	<10	>1280
	F2	<10	1280	<10	1280	<10	>1280	<10	640	<10	640	<10	>1280
	F3	<10	>1280	<10	1280	<10	>1280	<10	>1280	<10	>1280	<10	>1280
	F1*	160	>1280	160	1280	160	>1280	160	>1280	80	>1280	80	>1280
	F2*	40	>1280	80	>1280	80	>1280	80	>1280	40	>1280	40	>1280
	F3*	640	320	640	320	640	640	320	640	160	640	160	320
Sw/HK4167 infected	Ferret	ND	**>1280**	ND	>1280	ND	ND	ND	ND	ND	>1280	ND	ND
	Pig	<10	**80**	<10	10	<10	20	<10	<10	<10	<10	<10	<10
Sw/GD106 infected	Ferret	<10	1280	<10	>1280	<10	**>1280**	<10	>1280	<10	640	<10	1280
	Pig	<10	<10	<10	80	<10	**80**	<10	<10	<10	<10	<10	<10
Sw/HK29 infected	Ferret	ND	640	ND	80	ND	ND	ND	ND	ND	**>1280**	ND	ND
	Pig	<10	<10	<10	<10	<10	<10	<10	160	<10	**320**	<10	320

585 Note:

586 (1)Virus abbreviations:

587 Sw/HK4167: A/Swine/Hong Kong/4167/1999 (Classical swine H1N1 virus, CS)

588 CA4: A/California/04/2009 (prototype pandemic H1N1 2009, pdm/09);

Sw/GD106: A/Swine/Guangdong/106/2009 (pandemic H1N1 2009-like swine isolate, pdm/09)

Sw/GD1361: A/Swine/Guangdong/1361/2010 (novel H1N1 reassortant);

Sw/HK29: A/Swine/Hong Kong/29/2009 (Eurasian avian-like swine H1N1 virus, EA)

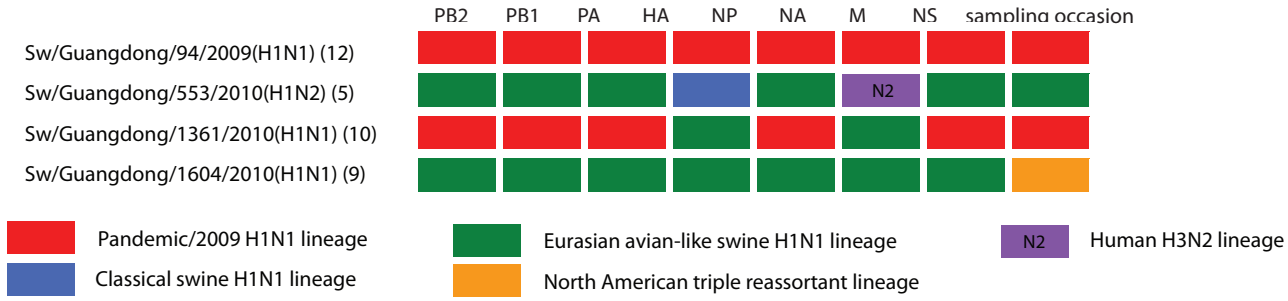
Sw/HK2433: A/Swine/Hong Kong/2433/2009 (Eurasian avian-like swine H1N1 virus, EA).

(2) Ferrets F1*, F2* and F3* were pre-immunised with CA4 virus 80 days prior to this study. The codes for each animal are given in Fig. S3.

(3) ND: Not determined. Pigs 1 and 5 were sacrificed at 4 dpi, Pigs 3 and 7 at 6 dpi, Pig P4 at 5 dpc.

(4) Pre: prior to infection (inoculation or contact); post: post-infection (inoculation or contact exposure).

(5) Reference ferret and pig antisera against Sw/HK4167, Sw/GD106 and Sw/HK29 were obtained from previous studies (27, 33).



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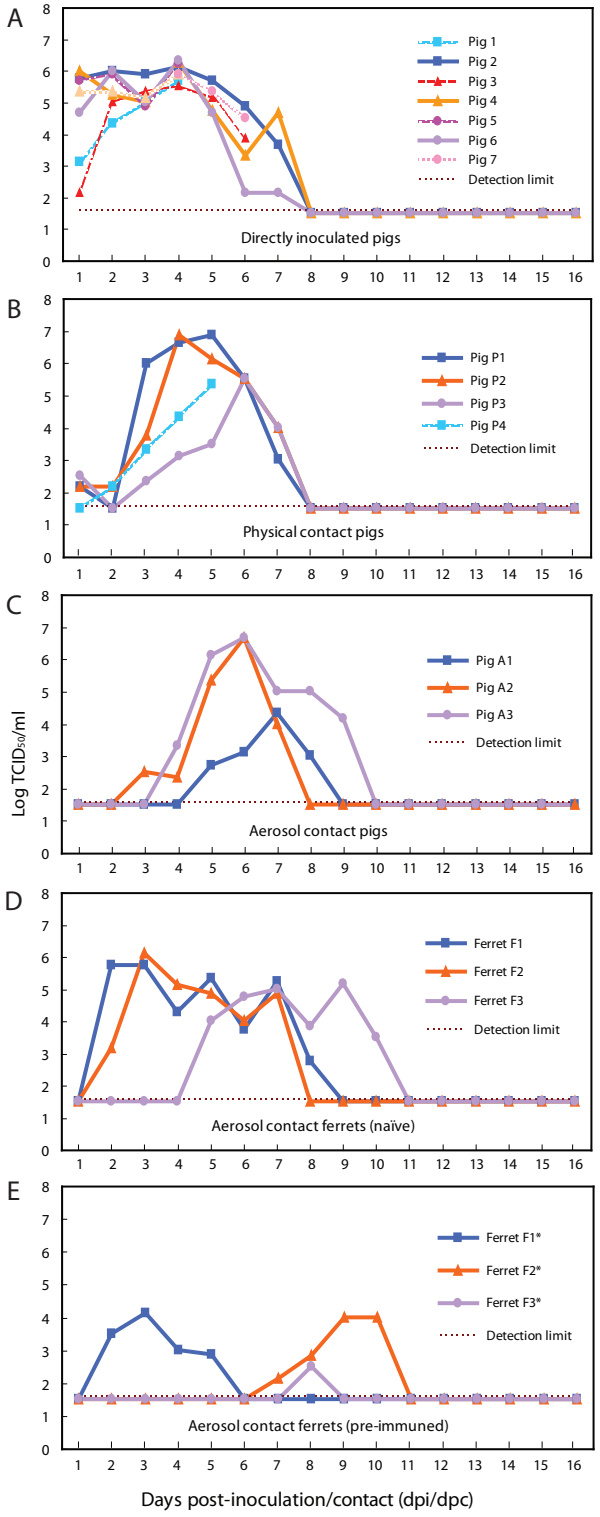

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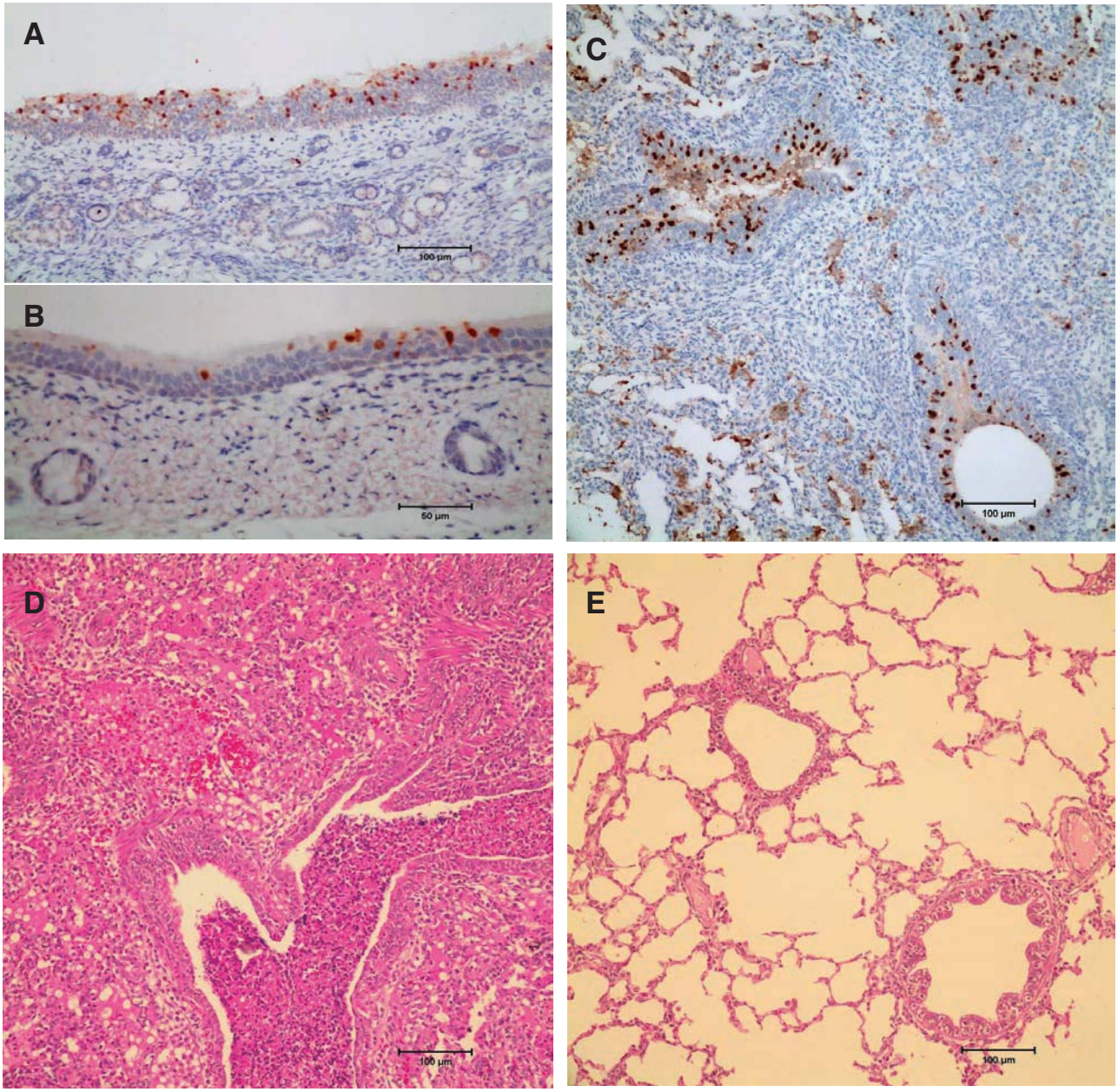

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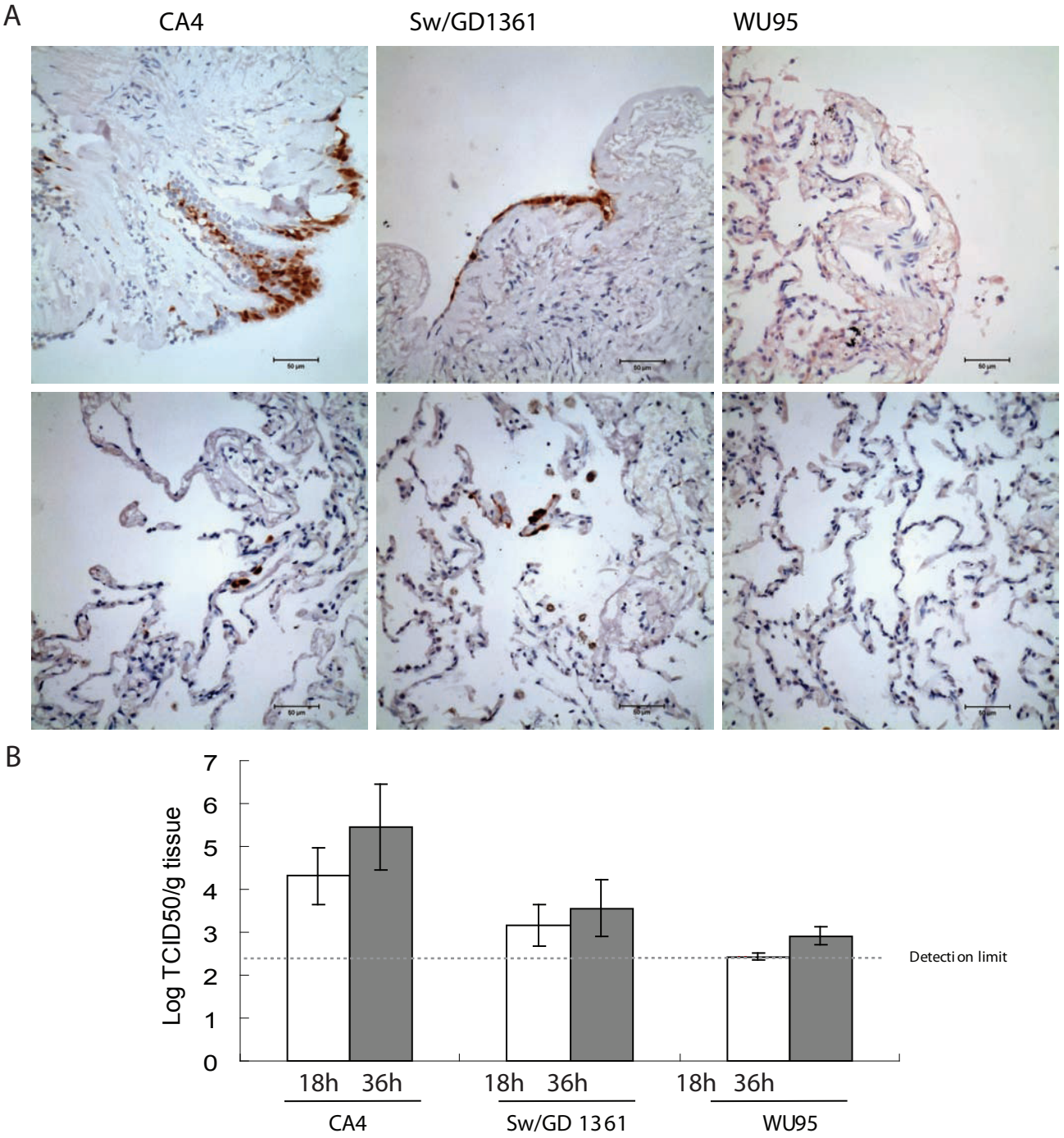

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