FULL PAPER *Virology*

First Finding of Southeast Asia Topotype of Foot-and-Mouth Disease Virus in Kinmen, Taiwan, in the 2012 Outbreak

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ABSTRACT. Foot-and-mouth disease virus, a member of genus *Aphthovirus* within the family *Picornaviridae*, affects cloven-hoofed animals, causing foot-and-mouth disease characterized by vesicle development. The Southeast Asia topotype, one of the topotypes within serotype O of the virus, is prevalent in some Asian countries, but had not previously been found in Taiwan. The topotype was first found in pigs in Kinmen Island, Taiwan, in 2012 and identified by nucleotide sequence comparison and phylogenetic analysis. Outbreaks were reported at 4 farms, resulting in the culling of 628 pigs and 1 cattle. Pigs were the only species infected during the outbreak. The incursion of Southeast Asia topotype into Taiwan implies the expansion of the topotype in East Asia.

KEY WORDS: foot-and-mouth disease, Kinmen, Southeast Asia topotype, Taiwan

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Foot-and-mouth disease (FMD) is one of the world's most important animal diseases. The disease is highly contagious and affects cloven-hoofed animals, notably pigs, cattle, sheep and goats. Clinically, FMD is characterized by vesicles in the mouth and on the muzzle, feet and, sometimes, udders. Severe myocarditis causes high mortality among infected newborns. While the infection in adult animals is rarely lethal, it does results in significant losses due to both a decrease in production and the ban on international trade of animals and animal products [10].

Foot-and-mouth disease virus (FMDV) is a member of the genus *Aphthovirus* within the family *Picornaviridae*. The non-enveloped virus contains a single-stranded, plus-sense RNA genome with a length of approximately 8,500 bases. Seven FMDV serotypes have been identified: O, A, C, Asia 1, SAT1, SAT2 and SAT3 [3]. Each serotype also has a number of subtypes. In Asia, prevalent topotypes of serotype O are Cathay, Southeast Asia (SEA), Middle East-South Asia and PanAsia [1, 2, 9, 15].

Two topotypes of FMDV serotype O, namely Cathay and PanAsia, have been identified in Taiwan. A pig-adapted strain of the Cathay topotype [13], designated O/Taiwan/1997, invades in 1997, and the invasion results in a severe epidemic among the pig farms and a devastating economic loss of 378 million US dollars [22]. The O/Taiwan/1999 and O/Taiwan/2000 strains of the PanAsia topotype [18] were isolated from Chinese yellow cattle, dairy cattle and goats in 1999 and 2000. The 2 strains are phylogenetically close to FMDVs from the Middle East and India [11]. Isolates obtained in 2009 are genetically close to the Cathay topotype and may be a derivative of O/Taiwan/1997 [17].

In the present study, we report the identification of the first SEA topotype of the FMDV in Kinmen Island and infer the incursion of a new topotype of FMDV serotype O into Taiwan's territory.

MATERIALS AND METHODS

Geography and livestock industry of Kinmen: Kinmen County comprises 12 islands, including the 2 largest: Kinmen and Lieyu. Kinmen Island is a 150-km^2 island near the coast of Fujian Province, People's Republic of China. This dumbbell-shaped island spans approximately 20 km east to west and approximately 15 km north to south, although the narrowest region in the center is only 3 km wide (Fig. 1).

Pigs, cattle and meat goats are the major livestock in Kinmen County with populations of approximately 16,700, 5,900 and 8,800 heads, respectively (Agriculture Statistics Yearbook 2012). Most owners are backyard farmers raising a small number of animals, ranging from only a few to hundreds.

Course of the outbreaks: On January 23, 2012, several porkers on Farm A (289 pigs, including 2 boars and 10 sows) showed lameness and decreased appetite. On January 26, a local governmental veterinarian from the Bureau of Animal and Plant Health Inspection, Kinmen County (BK), visited the farm, and the FMD vaccine was administered. On January 29, hoof tissues, nasal swabs and serum samples were collected and submitted to the Animal Health Research Institute (AHRI). Responding to the clinical observation and the positive results of reverse transcription polymerase chain reaction (RT-PCR) and antibody detection, on January 30, 74 affected pigs were destroyed, and the remained pigs on the farm were destroyed on the next day.

Farm B (241 pigs, including 3 boars and 23 sows) is 200 m

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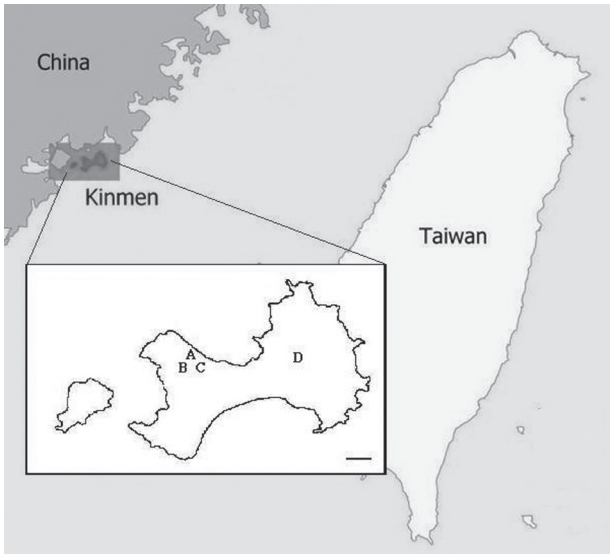

Fig. 1. Map of Kinmen County. The letters represent the locations of the 4 pig farms invaded by the Southeast Asia topotype of foot-and-mouth disease virus during the 2012 outbreak. The bar scale represents 2 km.

southwestern of Farm A. A sow on Farm B showed fever and anorexia on January 21. On February 1, the owner of Farm B observed lameness in the pigs and notified the BK. The BK veterinarians observed vesicles and ulcers on the coronary bands of hooves and detached hooves. On February 3, all the 241 pigs were destroyed.

In response to the outbreaks, restriction zones were imposed on a 3-km-radius movement restriction zones around Farms A and B, and an on-farm investigation was conducted within the zones. On February 7, typical FMD lesions were observed in sows on Farm C (34 pigs, composed of 6 sows, 11 weaning pigs and 17 porkers), located 400 m southeastern of Farm A. The pigs at Farm C were sampled and destroyed on February 7. Clinically FMD-suspected animals were also observed at 5 other farms, designated S1 to S5 (Table 2), within the restriction zones. The FMD-like lesions of the animals were also sampled.

On February 10, veterinarians observed healing lesions and re-growing hooves in pigs of Farm D (64 porkers and 1 Chinese yellow cattle), located on the eastern Kinmen Island. Samples were collected from 15 clinically healthy pigs, 15 pigs with lesions and the cattle on the farm and shipped to the AHRI. All of the pigs and cattle of Farm D were destroyed on the same day. Locations of Farms A, B, C and D are marked in Fig. 1.

Sampling: During the field investigation, diagnostic samples were collected from animals suspected to be ill and submitted to the AHRI. Blood samples were collected from external jugular vessels of pigs, external jugular vein or coccygeal vein of cattle and jugular vein of goats. The sera were collected by centrifugation after sampling and tested for neutralizing antibodies and antibodies to 3ABC non-structural protein. Swab samples were collected from the nostrils or throats of suspected animals. Vesicle fluid, ruptured vesicles and hooves were also sampled from pigs showing visible vesicular lesions, using appropriate restraint.

To compare levels of humoral immunity against FMDV between populations in Kinmen County, 319 pig sera, 529 cattle sera and 240 goat sera were collected during an annual surveillance program from July to December 2011 and tested by virus neutralization test (VNT).

VNT: The VNT was used to measure levels of neutralizing antibodies against FMDV. The test was performed following the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, published by the World Organization for Animal Health. Briefly, serum samples were serially diluted in 96-well microtiter plates, and a virus suspension containing 100 $TCID_{50}$ of FMDV O/Taiwan/1997 strain was added to each well. After a 1-hr incubation, a suspension of BHK-21 baby hamster kidney cells was added, and the plates were incubated at 37°C in a humid incubator with 5% CO_2 for 2 days. The neutralizing antibody titer was expressed as the log2 of the reciprocal serum dilution that protected cells from the cytopathic effect.

Detection of antibodies to 3ABC non-structural protein: A commercially available enzyme-linked immunosorbent assay (ELISA) kit, PrioCHECK FMDV-NS (Prionics Lelystad B.V., Lelystad, The Netherlands), was used to detect serum antibodies to the 3ABC non-structural protein of the FMDV. The kit used a blocking ELISA with baculovirus-expressed 3ABC polypeptide [20].

Virus isolation: Fluid was extracted from the nasal and throat swabs and sterilized with a membrane filter. Ruptured vesicles and hoof tissues were homogenized and centrifuged, and their supernatants were filter-sterilized. The filtrate was inoculated into a baby hamster kidney cell line BHK-21 and an embryonic bovine kidney cell line EBK, and the cultures were observed daily for 3 days. Whenever a cytopathic effect was observed, the supernatant of the cell culture was analyzed for the possible presence of FMDV using 1-step RT-PCR. If cytopathic effect was not exhibited, a blind passage was carried out for an additional 3 days.

FMD antigen-detecting ELISA: When an FMDV was isolated, its serotype was firstly identified by the Indirect Sandwich ELISA for Detection of Antigens of FMDV and SVDV, purchased from the Pirbright Institute, U.K. The ELISA was performed in accordance with the instruction manual.

RNA extraction: Viral RNA was extraction from 200 μl of vesicle fluid or supernatant of the inoculated cell culture by the Quick-RNA™ MiniPrep kit (Zymo Research Corporation, Irvine, CA, U.S.A.). The extracted RNA was dissolved in 100 μl of DEPC-treated water.

RT-PCR: The full-length VP1 gene of the FMDV was amplified by 1-step RT-PCR, using a primer pair based on the nucleotide sequences of 1C and 2A genes of O/Taiwan97/106/112 strain (GenBank Accession No. AY593835, [5]). The sequence of the forward primer FVP1-F was 5′-AGGGATGGGTCTGTTTGTT-3′, corresponding to the O/Taiwan97/106/112 strain nucleotides 3,108 to 3,126; the sequence of the reverse primer FVP1-R was 5′-AAGTC-GGGTCCGTGTTTCGTT-3′, corresponding to nucleotides

Table 1. Isolates of the foot-and-mouth disease virus employed in the phylogenetic analysis

Isolate	Serotype	Place of isolation	Year of isolation	Animal of isolation	Reference
O/KM/6/2012	O	Kinmen, Taiwan	2012	Pig	This article
O/Taiwan97/102/116	O	Taiwan	1997	Pig	[5]
O/TAW/2/99	O	Kinmen, Taiwan	1999	Cattle	[18]
O/TW/256/2001	O	Taiwan	2001	Pig	[17]
O/TW/257/2009	O	Taiwan	2009	Pig	[17]
O/BY/CHA/2010	O	China	2010	Pig	[23]
O/GZ/CHA/2010	O	China	2010	Cattle	[23]
O/NC/CHA/2010	O	China	2010	Pig	
O/HongKong/S17/2002	O	Hong Kong, China	2002	Wild boar (*Sus scrofa*)	
O/HongKong/P404/2010	O	Hong Kong, China	2010	Wild boar (*Sus scrofa*)	
O/JPN/2000	O	Miyazaki, Japan	2000	Cattle	[12]
O/JPN/1/2010	O	Miyazaki, Japan	2010	Cattle	[21]
O/LAO/1/2009	O	Laos	2009	Buffalo	[2]
O/MAY/3/2000	O	Gombak, Selangor, Malaysia	2000	Pig	[1]
O/MAY/7/2001	O	Malaysia	2001	Cattle	[1]
O/MAY/8/2005	O	Kuala Langat, Selangor, Malaysia	2005	Pig	[1]
O/MAY/7/2007	O	Jasin, Melaka, Malaysia	2007	Cattle	[1]
O/MOG/1/2010	O	Mongolia	2010	Cattle	[14]
O/SKR/1/2000	O	Kyunggi, Republic of Korea	2000	Cattle	
O/SKR/5/2010	O	Gyeongbuk, Republic of Korea	2010	Pig	[21]
O/RUS/Aug-2010	O	Russia	2010	Unknown	[21]
O/TAI/22/2009	O	Lamphun, Thailand	2009	Pig	[21]
O/UKG/9327/2001	O	United Kingdom	2001	Sheep	[7]
O/VIT/2/2010	O	Vietnam	2010	Cattle	[14]
A/IND17/82	A	India	1982	Cattle (vaccine strain)	
A/TAI/3/2001	A	Thailand	2001	Cattle	[2]
Asia l/YZ/CHA/06	Asia 1	China	2006	Pig	
Asia 1/BAM/AFG/L-590/2009	Asia 1	Afghanistan	2009	Cattle	[4]

Table 2. Sampled farms, clinical samples and the results of laboratory testing during the foot-and-mouth disease outbreak in Kinmen, Taiwan, in 2012

Date of sampling	Farms involved	Laboratory tests			
		Virus isolation	RT-PCR	Antigen ELISA	NSP Ab[a)] detection (Positive/Tested)
January 29	Farm A (pig)	FMDV	Positive	Serotype O	Positive (4/30)
January 31	Farm A (pig)	FMDV	Positive	Serotype O	Positive (3/8)
February 1	Farm B (pig)	FMDV	Positive	Serotype O	Negative (0/5)
February 7	Farm C (pig)	Negative	Positive	NT	Positive (1/34)
February 10	Farm D (pig)	Negative	Negative	NT	Positive (13/30)
	Farm S1 (pig)	Negative	Negative	NT	NT
	Farm S2 (pig)	Negative	Negative	NT	Negative
	Farm S3 (goat)	Negative	Negative	NT	NT
	Farm S4 (pig)	Negative	Negative	NT	Negative
	Farm S5 (pig)	Negative	Negative	NT	NT

a) NSP Ab: Antibodies against 3ABC non-structural protein of foot-and-mouth disease virus.

3,991 to 4,011. A KAPA2G™ Fast PCR Kit (Kapa Biosystems, Wilmington, MA, U.S.A.) was used for the RT-PCR and 4 U of avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI, U.S.A.), and 8 U of RNase inhibitor (Promega) were added to each reaction. The 1-step RT-PCR was carried out using the GeneAmp PCR System 9700 (Applied Biosystems, Life Technologies, Carlsbad, CA, U.S.A.) with the following procedure: reverse transcription for 40 min at 42°C, denaturation for 3 min at 94°C, followed by 35 cycles of denaturation for 40 sec at 94°C, annealing

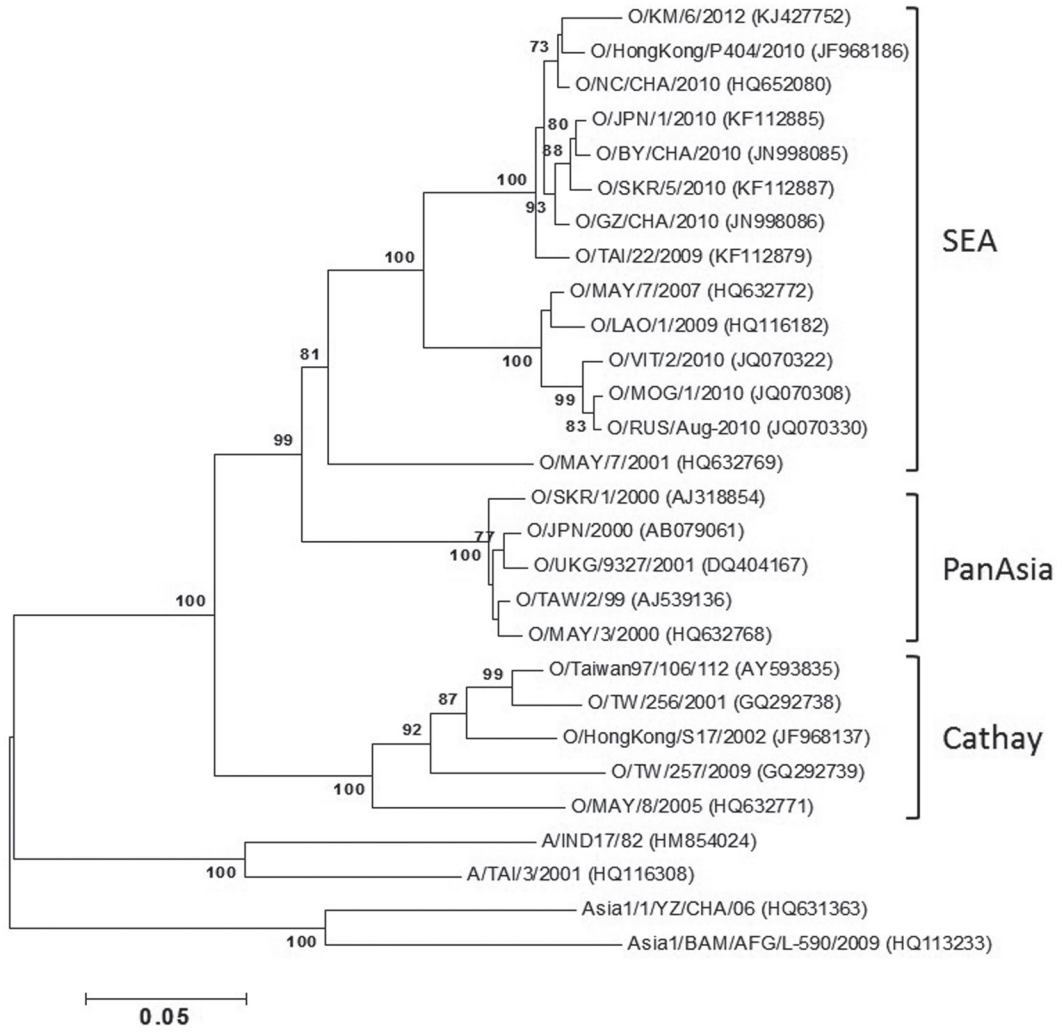

Fig. 2. Neighbor-joining phylogenetic relationships between VP1 genes of foot-and-mouth disease viruses. The tree was generated by Molecular Evolutionary Genetics Analysis version 5 with default parameters. Numbers at the nodes indicate bootstrap confidence values (1,000 replicates) for the groups composed of virus strains to the right of the node. Only the values higher than 70 are shown.

for 40 sec at 50°C and extension for 20 sec at 72°C. A final extension step was performed at 72°C for 5 min. Products of the 1-step RT-PCR were analyzed by agarose gel electrophoresis. The predicted length of the 1-step RT-PCR amplicon was 904 base pairs.

Nucleotide sequencing: Nucleotide sequences of the RT-PCR products were determined by commercial DNA sequencing service (Mission Biotech, Taipei, Taiwan).

Phylogenetic analysis: Phylogenetic relationships between the nucleotide sequences were analyzed by neighbor-joining method with the software Molecular Evolutionary Genetics Analysis version 5 or MEGA 5. Nucleotide sequences of FMDV VP1 genes used for the analysis are listed in Table 1. Sequences of A/IND17/82 (Accession No. HM854024), A/TAI/3/2001 (Accession No. HQ116308, [2]), Asia l/YZ/CHA/06 (Accession No. HQ631363) and Asia 1/BAM/AFG/L/590/2009 (Accession No. HQ113233, [4]) strains were included in the phylogenetic tree as the outgroup.

RESULTS

The sera, tissues and swabs sampled from animals at Farms A, B, C and D and the results of laboratory testing are summarized in Table 2. Notably, FMDVs were isolated from Farms A and B, and the serotype of the isolates was identified as serotype O by determining VP1 sequences of the isolates and by detecting serotype-specific antigen with the indirect sandwich ELISA.

The FMDV VP1-specific RNA was detected in clinical samples of Farms A, B and C by the RT-PCR. The RT-PCR amplified VP1 sequences from Farms A, B and C demonstrated >99.8% similarity to each other. These sequences

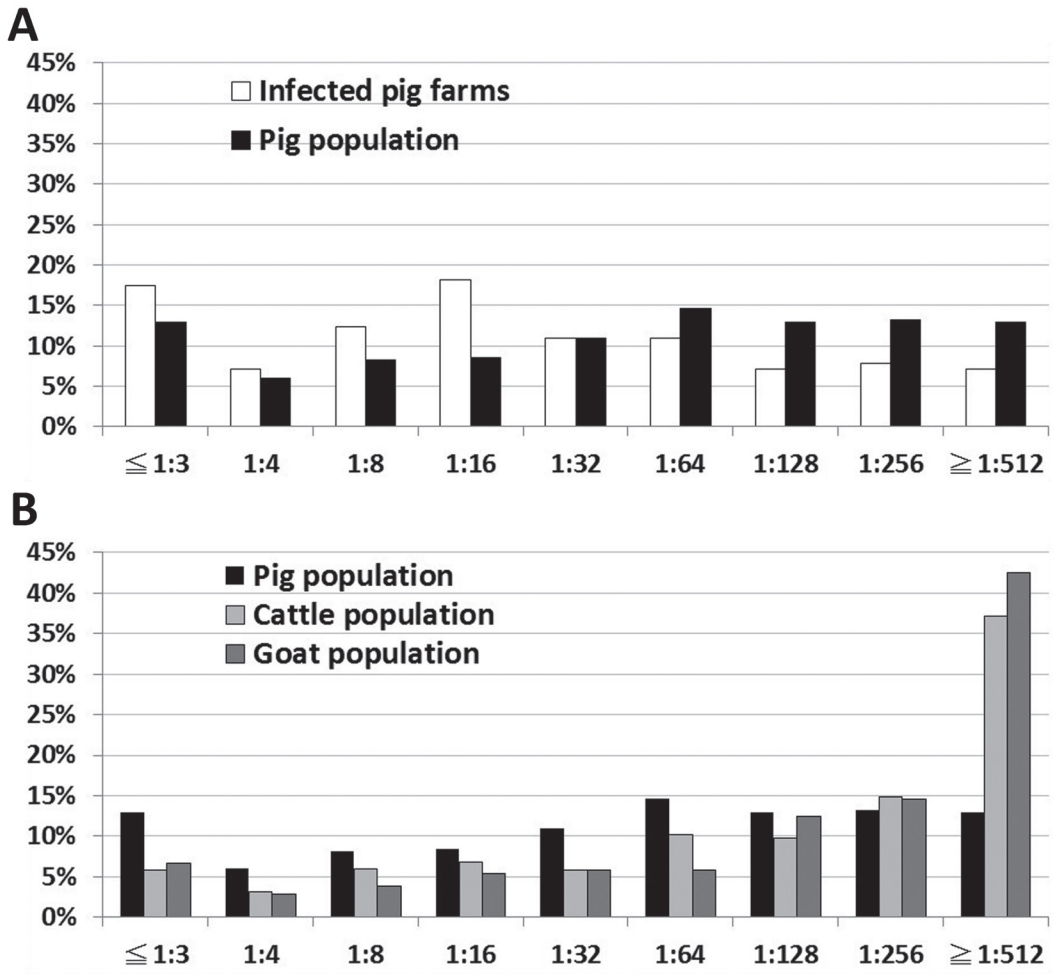

Fig. 3. Frequency distribution of titers of neutralizing antibody to the foot-and-mouth disease virus serotype O in different species in Kinmen, Taiwan. (A) Distribution of titers in the pigs of the 4 infected pig farms of the 2012 foot-and-mouth disease outbreak and in the pigs (n=319) sampled in Kinmen between July and December 2011, a period a half year before the 2012 outbreak; (B) Distribution of titers in 319 pig, 529 cattle and 240 goats sampled in Kinmen between July and December 2011.

showed 77.5%, 76.4% and 96.9% similarities to those of O/Taiwan/1997, O/Taiwan/2009 and FMDV serotype O isolated in Hong Kong in 2010, respectively.

Phylogenetically, the 2012 virus (GenBank Accession No. KJ427752), shown as the isolate O/KM/6/2012 in Fig. 2, was clustered with isolates from Hong Kong, China, Japan, Laos, Malaysia, Mongolia, Korea, Russia, Thailand and Vietnam, which were all identified as SEA topotype.

Serologically, antibodies against 3ABC non-structural protein were detected in porcine sera sampled at each of the 4 infected farms. The mean neutralizing antibody titer, measured by the VNT, of the sampled pigs of the Farms A, B, C and D was 1:18. The 319 pig sera, 529 cattle sera and 240 goat sera sampled from July to December 2011 gave mean neutralizing antibody titers of 1:37, 1:104 and 1:119, respectively.

DISCUSSION

It was the first identification of the SEA topotype of an FMDV in Taiwan. The nucleotide comparison demonstrated that the FMDV VP1 nucleotide sequences from Kinmen were 96.9% similar to the SEA topotype of the FMDV from Hong Kong (Accession No. JF968186). The phylogenetic tree (Fig. 2) further revealed that the Kinmen virus was clustered together with other Asian FMDVs of the SEA topotype, including those from China, Japan, Korea, Mongolia and Russia. Foot-and-mouth disease viruses of this topotype were not only endemic in southeastern Asia, but also invaded East Asian countries recently, as seen in China, Japan, Korea, Mongolia and Russia in 2010–2011 [14, 21]. The found FMDV in Kinmen, based on the phylogenetic closeness to the East Asian isolates, was likely a new member of this genetic pool. The outbreaks in Kinmen highlighted the ter-

ritorial expansion and the continuous threat of this topotype in East Asian countries.

Farms A, B and C may share the same FMDV strain. Farm A, the index case of the outbreak, was first reported on January 23 and was identified as an FMD affected farm on January 31, 2012. Farms B and C demonstrated clinical symptoms in early February. The 99.8% similarity between VP1 nucleotide sequences from these 3 farms further supports that the viruses shared a common ancestor. Additionally, the positive rates of non-structural protein antibodies, 13.3% (4/30) and 37.5% (3/8), implied that Farm A was the first infected farm, because the non-structural protein antibodies usually appear 1 week after infection [6, 16]. With the geographical proximity, chronological sequence of the disease occurrence and close genetic relationship, it was considered that the virus first attacked Farm A and later spread to Farms B and C.

In contrast, Farm D was considered to be affected earlier than Farm A. The healing lesions observed in pigs of Farm D on February 10 indicated that the infection may have occurred about 1 month prior, around early January. A high percentage (43.3%; 13/30) of pigs possessing non-structural protein antibodies also implied that the invading FMDV had been actively circulating among the pigs. Accordingly, we considered that Farm D was the first infected farm, although it was the last identified. Unfortunately, it has not been possible to establish the relationship between the infection at Farm D and those of Farms A, B and C, because neither the FMDV was isolated nor its viral RNA was detected from Farm D.

Insufficient immunity may allow for the entrance of the FMDV. The mean neutralizing antibody titer of the 4 infected farms was significantly lower than that of the pig population in Kinmen ($P<0.001$; *t*-test). Furthermore, the mean titer of the pig population (1:37) in Kinmen was lower than those of the cattle and goat populations (1:104 and 1:119, respectively; $P<0.001$) in the same county. Frequent distribution of neutralizing antibody titers, as demonstrated in Fig. 3, also revealed that the titers in pigs, unlike those in cattle and goats, distributed evenly, and 46.4% of the pigs possessed a titer of 1:32 or lower before the outbreak. The finding could explain why the pig was the only suffering species during the outbreak. The animals with low humoral immunity were not completely protected, possibly giving FMDV more opportunities of infection to them.

The way of FMDV transmission between the farms has been unknown. Field investigation suggested that farm-to-farm visiting and transportation of feeds were not likely to be the cause of transmission. The prevalent north wind in winter might be a means of introduction, and the likelihood of long-distance airborne transmission of FMDV has been considered [8, 19]. Smuggling of animal products including live livestock could be another mode of transmission, considering the geographical proximity to China. However, there is minimal evidence to confirm any route of transmission, as in most of the FMDV incursions.

In conclusion, the FMDV serotype O that invaded Kinmen in 2012 was identified as the first SEA topotype in Taiwan's territory. Pig was the only infected species during the outbreak.

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