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A visual DNA chip for simultaneous detection, genotyping, and differentiation of wild-type and vaccine-type classical swine fever virus

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Running title：DNA Chip genotyping and differentiation of wild-type and vaccine-type CSFV

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Summary

Routinely, RT-PCR followed by DNA sequencing has been the method used to detect classical swine fever virus (CSFV) and to exclude the interference of vaccine viruses in clinical samples. Here, a DNA chip assay was developed to enable simultaneous detection, genotyping and differentiation between wild-type and vaccine-type CSFV. Specific oligonucleotide primers and probes were designed in the 3' non-translated region of the CSFV genome. One-step RT-PCR amplification was performed with biotin-labeled primers, followed by hybridization to the DNA probe immobilized on the plastic chips. The DNA chips not only can accurately differentiate three major CSFV genotypes, but also can discriminate between wild-type and vaccine-type CSFV. The limit of detection for wild-type virus was 10 $TCID_{50}$/ml for RT-PCR and 1 $TCID_{50}$/ml for the DNA chips. The sensitivity of the visual DNA chip was 10 times higher than that of the RT-PCR confirmed by agarose gel electrophoresis. We conclude that RT-PCR coupled with DNA probe hybridization provides a highly sensitive diagnostic tool for genotyping of CSFV and for discriminating between wild-type and vaccine-type CSFV in clinical samples.

Keywords: *Classical swine fever virus; DNA chip; Probe; RT-PCR*

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Exp.Rep AHRI.,No.43：89~100 (2008)

Introduction

Classical swine fever (CSF), formerly known as hog cholera, is a serious and highly contagious viral disease caused by CSF virus (CSFV). CSF is a highly contagious disease and is difficult to control in areas of high pig or wild boar density. It is for this reason that it is included in the A list of infectious diseases of most importance for international trade [1, 21]. The course of disease can run an acute, subacute, chronic or late onset course, but can also go unnoticed in the infected pigs [25]. Disease control is attempted by either vaccination or eradication. Vaccination with live attenuated LPC vaccine is currently performed in Taiwan. Owing to the Government's compulsory vaccination policy, CSF is well control in Taiwan. However, sporadic outbreaks of the disease still occur every year.

Classical swine fever virus is classified in the genus *Pestivirus* within the family *Flaviviridae* [28]. Pigs are also susceptible to other pestiviruses, including bovine viral diarrhea virus (BVDV) and border disease virus (BDV) [22]. The genome of CSFV consists of a single-strand RNA of about 12.3 kb, which comprises a single large open reading frame (ORF) [15, 16]. This ORF flanked by 5' and 3' non-translated region (NTR), which are highly conserved among CSFV isolates. By sequencing the full-length genome from three vaccine strains (C-strain, HCLV and LPC), notable T-rich insertions of 12 – 13 nucleotides (nts) in length were found in the 3' NTR of the viral genome [17, 29, 30].

CSF is still endemic in many parts of the world. CSFV isolated around the world have been tentatively divided into three major genetic groups, each with three or four subgroups: 1.1, 1.2, 1.3; 2.1, 2.2, 2.3; 3.1, 3.2, 3.3, 3.4 [20]. Genotyping of CSFV field isolates is very important for molecular epidemiology and tracking the transmission pathways of the virus. In Europe, most of the field isolates collected before 1970 belong to group 1, which is similar to Alfort 187 strain. Since 1970, the presence of group 1 viruses in Europe has been very infrequent. Large number of isolates appeared in the 1980s and 1990s belong to group 2 [3, 20]. The 1997-1998 epidemic of CSF in the Netherlands was caused by subgroup 2.1 viruses [24]. In Latin America, only group 1 viruses have so far been reported. In Asia, all of the major genetic groups and subgroups of CSFV have been identified in different parts of Asia at different times [20]. In Taiwan, a genotype switch from subgroup 3.4 to 2.1a was observed. Currently, subgroup 2.1a viruses were prevalent in the field [18, 19].

DNA microarray (also called DNA chips) technology has emerged in recent years as a powerful tool for simultaneous detection of large numbers of DNA sequences in a sample [26]. DNA microarrays have been successfully employed for molecular epidemiological typing of different isolates [9]; typing and subtyping influenza virus [12]; detection and typing of foot-and-mouth disease virus [2]; and genotyping of human hepatitis B virus [23], human group A rotaviruses [5], and human papillomavirus [10]. In this study, we developed a DNA chip for genotyping of CSFV. The novel assay described here provided a rapid and sensitive method for accurate differentiation of three major CSFV genotypes and for discrimination between wild-type viruses and vaccine strains of CSFV.

Materials and methods

Viruses and vaccine strains

A total of 40 field isolates of CSFV representing 4 genotypes were tested in this study (Table 1). These field isolates were collected in Taiwan from 1989 to 2004 and had previously been genotyped as subgroups 2.1a, 2.1b, 2.2 and 3.4 (Pan et al., 2005). Two laboratory reference strains preserved in Animal Health Research Institute (AHRI), namely ALD virulent strain and A76 strain belonging to subgroups 1.1 viruses, were tested. Three lapinized vaccine strains of LPC were also tested: 1) The LPC/AHRI vaccine strain (AHRI, Tamsui, Taiwan) was made from spleens and lymph nodes of inoculated rabbits; 2) The LPC/TS vaccine strain (AHRI, Tamsui, Taiwan) was cell culture-adapted in PK-15 cell line; 3) The LPC/PRK vaccine strain (Formosa Biomedical Inc, Taiwan) was cell culture-adapted in primary rabbit kidney cells. Antigenically related viruses were also tested: BVDV strain 31 (VR-996TM Global Bioresource Center, Manassas, VA, USA); three BVDV strains, including two BVDV type 1 strains (BVDV/NADL and BVDV/Nose) and one BVDV type 2 strain (BVDV/MD). Porcine reproductive and respiratory syndrome virus (PRRSV), porcine circovirus type 2 (PCV2), and swine influenza virus (SIV) subtype H1N2 viruses, isolated previously from pigs in Taiwan in 2006, were also used as negative controls.

Clinical samples

A total of 100 clinical samples collected from different vaccinated pig farms were submitted to AHRI for routine CSF diagnosis by local animal disease control centers (LADCC) between 2005 and 2007. These specimens were prepared as 10% (w/v) emulsions by mixing and homogenizing tonsils and lymph nodes in Eagle's minimum essential medium (Sigma–Aldrich, St. Louis, MO, USA).

Primer and probe design

The 3' NTR sequences for 72 CSFV, 11 BDV, 15 BVDV type 1 and 8 BVDV type 2 strains were obtained from the NCBI GenBank and stored in a database. To generate CSFV-specific primers as well as CSFV-common and genotype-specific probes, the program Clustal V of MegAlign 5.03 (DNASTAR, Inc., Madison, WI, USA) was used. One set of CSFV-specific primers was designed based on the conserved region of 3' NTR of the CSFV genome and encompassed the T-rich insertion region that is unique to the lapinized CSF vaccine strains. For genotyping, two

CSFV-common and six genotype-specific probes were designed based on the conserved and the variable regions within the 3' NTR of CSFV. Two vaccine-type specific probes were designed to anneal to the T-rich insertion site. To ensure the specificity of the probe for its target, the sequences of the probes were compared with the CSFV database using the BLAST program for sequence similarity and uniqueness. A tail composed of 20 T bases was added to the 5' end of each oligonucleotide probe as well as the hybridization control probe-H. Biotin was used to label the 5' end of each oligonucleotide primers. Primer and probe sequences used in the DNA chip assays are listed in Table 2.

RNA extraction and RT-PCR amplification

Viral RNA was extracted directly from 100 μl of the 10% (w/v) emulsion of tissue specimens or cell culture viruses using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Reverse transcription (RT) and subsequent PCR were performed in one tube with a single reaction buffer. One-step RT-PCR amplification was performed as previously described (Pan et al., 2008a). The amplified products were analyzed by electrophoresis through 2% agarose gels containing 1x SYBR® Safe DNA gel stain (Invitrogen, Carlsbad, CA, USA) in 1× Tris-acetate-EDTA (TAE) buffer solution.

DNA chips preparation

Ten microliters of each probe mixed with an equal volume of probe solution were placed on a plastic disc (DR. Chip Biotech, Miao-Li, Taiwan) and then spotted to each specific position on a commercialized polymer plastic chips (DR. Chip Biotech) using a manual spotting machine, DR. Fast Spot™ (DR. Chip Biotech), according to the manufacturer's instructions. Probe immobilization was performed using1.2 joules/cm of UV-irradiation (Spectroline UV Crosslinker, SelectTM Series, USA) at a wavelength of 254 nm.

Hybridization reaction and image analysis

The hybridization reaction between each DNA template and probe was carried out with DR. Chip DIY™ Kit (DR. Chip Biotech). The procedures were performed according to the manufacturer's protocol with little modifications. The PCR product was first denatured at 95℃ for 10 min and then cooled in an ice bath for 2 min. A total of 200 μl of Hybridization Buffer (containing the 5' end-biotinylated oligonucleotide complementary to the sequence of the hybridization control probe) was added to the chip chamber followed by the addition of 10 μl of the denatured PCR product. The chamber was incubated at 50℃ under constant vibration for 1 hr, and then washed three times with the Wash Buffer prewarmed to 50℃. The blocking reaction was then initiated by adding a mixture of 0.2 μl of Strep-AP (Streptavidin conjugate alkaline phosphates) and 200 μl of Blocking Reagent, and then incubated at room temperature for 30 min. After washing three times with Wash Buffer, the colorimetric reaction was performed by adding a mixture of 4 μl of NBT/BCIP and 196 μl of Detection Buffer in the chamber followed by developing in the dark at 37℃ for 5 min, and then washed with plenty of water. The hybridization procedure took about 2 hours. The hybridization results were indicated by the developed pattern on the chips, which was read directly by eye.

Sensitivity and specificity test

To examine the sensitivity of RT-PCR and the DNA chips, viral RNA was extracted from 10-fold serial dilutions of CSFV field isolate strain 93-N1211 (virus titer: 10^5 $TCID_{50}$/ml) and was amplified by one-step RT-PCR. The labeled target sequences for each dilution were hybridized to the CSFV DNA chips to determine sensitivity. To test the specificity, two BVDV type 1 strains, one BVDV type 2 strain, BDV, PRRSV, PCV2 and SIV were used.

Results

RT-PCR amplification

A total of 45 CSFV, including 40 field isolates, two reference virus strains (ALD and A76), and three lapinized vaccine strains of CSFV, were all successfully amplified by RT-PCR. As expected, the 40 field isolates of CSFV, ALD and A76 strains had PCR products of 367 base pairs (bp), whereas the three vaccine viruses (LPC/AHRI, LPC/PRK, LPC/TS) had slightly larger products than the wild-type CSFV, as detected by agarose gel electrophoresis. This slight difference is due to the T-rich insertion presented in the 3' NTR of the viral genome of lapinized CSFV vaccine strains. Figure 1 shows the amplification of different genotypes of CSFV as well as of the three lapinized vaccine strains. Other *Pestiviruses* including BVDV/NADL, BVDV/Nose, BVDV/MD and BDV, and other common swine viruses such as PRRSV, PCV2 and SIV, were not amplified (data not shown).

DNA chip assays

A total of 45 CSFV, including 40 field isolates, two reference virus strains (ALD and A76) and three vaccine strains were tested using the DNA chips following the one-step RT-PCR. All viruses were unambiguously detected and divided into three genotypes. Forty field isolates of CSFV belonged to group 2 and group 3. The ALD and A76 strain belonged to group 1. The three vaccine strains belonged to group 1 and were easily differentiated from ALD and A76 by vaccine specific probes. No cross-reactions were detected between unrelated probes. Figure 2 shows the hybridization results for the different genotypes of CSFV and the three lapinized vaccine strains. The colormetric detection of hybridization on the DNA chips used in this study produced results that were clearly detectable with the

Exp.Rep AHRI.,No.43：89~100 (2008)

naked eye, requiring no additional imaging equipment.

Testing of clinical samples with DNA chips

To assess the diagnostic accuracy with clinical samples, we tested 100 clinical samples using the DNA chip assay. In parallel, all of the RT-PCR positive samples were analyzed by DNA sequencing. Virus isolation (VI) followed by antigenic testing using an indirect fluorescent antibody (IFA) staining was also performed. Seventeen of these samples were positive for CSFV by the RT-PCR. Of these 17 samples, four were identified as the wild-type CSFV and 13 were identified as vaccine strains by DNA sequencing. The DNA chip assays identified four as wild-type CSFV, belonging to group 2 genotype, and 16 as vaccine strains. In VI, only nine of the 100 clinical samples tested positive for CSFV with IFA staining, but wild-type and vaccine-type CSFV cannot be differentiate in these positive samples (Table 3). Figure 3 shows the hybridization results from 10 representative clinical samples.

Comparison of the sensitivities of RT-PCR and DNA chip assays

Using RT-PCR, the detection limit for wild-type CSFV was 10 $TCID_{50}$/ml. However, the detection limit of the wild-type viruses for the DNA chip was 1 $TCID_{50}$/ml. The visual DNA chip was 10 times more sensitive than that of the RT-PCR method (Fig. 4).

Discussion

Vaccination is one of the most successful methods for preventing CSFV infection. However, since live attenuated vaccine viruses can persist in a vaccinated pig for a long period of time after immunization designe [7, 11, 14]. Vaccine viruses, which exhibit high degree of genetic similarity with wild-type viruses, could interfere with the detection of wild-type CSFV using current laboratory diagnostic tools [13]. Previously, RT-PCR amplification followed by DNA sequencing has been the primary method for differentiating between the wild-type viruses and vaccine strains of CSFV in Taiwan [18]. CSFV isolated around the world have been clustered into three major genetic groups [20]. Based on the phylogenetic analysis of CSFV isolated from Taiwan, group 2 (including subgroups 2.1a, 2.1b and 2.2) viruses were considered to be of the exotic strains, whereas group 3 (subgroup 3.4) viruses were considered to be of the historical virus strain. No group 1 wild-type virus was found in the field during the past few years [8, 18]. Genotyping of CSFV isolates is important for molecular epidemiological surveillance and tracking the transmission pathways of the virus. Therefore, the viruses that caused each CSF outbreak in farm need to be confirmed for their genotypes by RT-PCR followed by DNA sequencing. To simplify the diagnosis of CSF, a visual DNA chip assay was developed for simultaneous detection, genotyping and differentiation of wild-type and vaccine-type of CSFV.

Since the differential identification of wild-type and vaccine-type CSFV is an important issue, it has been described in some recent studies [6, 13, 19, 31]. All live CSF vaccine viruses were attenuated from group 1 wild-type viruses, and thus high genetic homology exists between the vaccine viruses and the group 1 viruses. Therefore, techniques developed in previous studies to differentiate wild-type from vaccine-type CSFV cannot broadly used in the field [6, 13, 31] and cannot be used for simultaneous genotyping and differentiation between wild-type and vaccine-type [6, 13, 19, 31]. There are seven vaccine strains (LPC, C-strain, HCLV, Porcivac, Rovac, Russian LK and the original Chinese vaccine) containing the 12-14 nts T-rich insertions in the 3' NTR that have been reported [4, 17, 29, 30]. None of the wild-type CSFV has ever been reported to contain these insertions. In this study, the vaccine-type specific probes were designed based on the difference in T-rich insertions that exist uniquely in the 3' nontranslated regions (3' NTR) of the genome of lapinized CSFV vaccine strains. It also separated the vaccine strains from CSFV group 1. Therefore, the DNA chip assay could be applied broadly to most of wild-type CSFV.

The sensitivity and specificity of these assays are satisfactory. The assay showed that five different genotypes of CSFV, including 40 field isolates of CSFV (subgroups 2.1a, 2.1b, 2,2 and 3.4), ALD and A76 strains (subgroup 1.1) and three lapinized vaccine strains (subgroup 1.1), could be amplified by RT-PCR (Fig. 1) and divided into three groups by the DNA chip assays (Fig. 2). Different target probes combined cooperatively or complementarily to make the obtained results clear and definite. These properties make the DNA chip a good tool for manipulation of multiple genetic variants. In contrast, no cross-reaction was observed in other antigenically related *Pestiviruses* such as BVDV type 1, type 2, and BDV or from other common swine viruses such as PRRSV, PCV2 and SIV (Fig. 2). The limit of detection for wild-type virus was 10 $TCID_{50}$/ml for RT-PCR and 1 $TCID_{50}$/ml for the DNA chips. Results showed that the DNA chip assay was 10 times more sensitive than that of the RT-PCR as confirmed by agarose gel electrophoresis (Fig. 4). Similar results were also been observed for an oligonucleotide microarray designed for detection and differentiation of Newcastle disease and avian influenza viruses, where the array was ten to 100 times more sensitive than the agarose gel [27]. These results demonstrate that the combination of RT-PCR with DNA-DNA hybridization can increase the sensitivity and specificity of the assay. The whole procedure, including the RT-PCR and the 2 hours of hybridization takes just 6 hours, which is significantly faster than the VI or RT-PCR followed by DNA sequencing.

Rapid differentiation between wild-type and vaccine-type CSFV has been of essential importance for laboratory diagnosis and prevention of CSF in Taiwan, where extensive vaccination for CSF has been enforced for decades. Vaccine viruses in clinical sample may

interfere with the detection of CSFV. As described in the result section, four clinical samples were identified as the wild-type CSFV by RT-PCR followed by either sequencing or DNA chip assays. RT-PCR followed by sequencing identified 13 clinical samples as vaccine strains whereas DNA chips were able to identify 16 clinical samples as vaccine strains (Table 3). Due to the maternal antibody existing in the clinical sample influencing the VI, IFA staining was only able to identify nine clinical samples as positive for CSFV and was unable to discriminate between wild-type and vaccine-type CSFV. These results demonstrated that the DNA chip assay was more sensitive than the RT-PCR, and the RT-PCR was more sensitive than the VI.

In conclusion, in countries or areas where CSF live vaccines are implemented, the detection of the vaccine viruses should be considered during diagnosis of CSF. The DNA chip assays presented here will provide a rapid and sensitive diagnostic tool for simultaneous detection, genotyping and differentiation of wild-type from vaccine-type CSFV in the field.

Exp.Rep AHRI.,No.43：89~100 (2008)

Table 1. Forty field isolates of CSFV used in this study.

Isolates	Region of isolation (prefecture)	Isolation years	Genotypes
78-KS	Kaohsiung	1989	3.4
79-60	Pindon	1990	3.4
82-40	Kaohsiung	1993	3.4
82-182	Pindon	1993	3.4
83-19	Tainan	1994	3.4
83-56	Tainan	1994	3.4
83-58	Tainan	1994	3.4
94.4	Ilan	1994	3.4
83-114	Hualien	1994	3.4
83-118	Hualien	1994	2.1a
84-YL1	Yunlin	1995	2.1a
84-FL1	Hualien	1995	2.1a
84-KS1	Kaohsiung	1995	2.2
84-C	Kaohsiung	1995	2.2
84-D	Kaohsiung	1995	2.2
84-108	Taichung	1995	2.1a
85-12A	Pengfu	1996	3.4
85-TD2	Taidon	1996	2.1a
86-HL1	Ilan	1997	2.1a
Q87-278	Pindon	1998	2.1a
88-PT	Pindon	1999	2.1a
89-YL1	Yunlin	2000	2.1a
Q89-240	Pindon	2000	2.1a
90-YL1	Yunlin	2001	2.1b
90-CH1	Chanhua	2001	2.1b
90-CH14	Chanhua	2001	2.1a
90-TN2	Tainan	2001	2.1b
90-TN3	Tainan	2001	2.1b
90-TD2	Taidon	2001	2.1a
Q90-48	Pindon	2001	2.1a
Q90-152	Pindon	2001	2.1a
90-SC2	Sinchu	2001	2.1a
90-CY	Chiayi	2001	2.1a
91-NT1	Nantou	2002	2.1a
Q91-84	Pindon	2002	2.1b
Q92-39	Pindon	2003	2.1a
Q92-43	Pindon	2003	2.1a
92-TN1	Tainan	2003	2.1a
N956	Taidon	2004	2.1a
N1211	Miaoli	2004	2.1a

Table 2. Oligonucleotide sequences of primers and probes designed in this study

Oligonucleotide	Sequence (5'→3')	Virus type
Primer		
CP5	GTAGCAAGACTGGRAAYAGGTA	All genotypes
CP6	AAAGTGCTGTTAAAAATGAGTG	All genotypes
Probe		
CSFV-U1	ATTTATTTATTGAATGAGYAAGAACTGGTACAAACTACCTCA	All genotypes
CSFV-U2	GGTACAAACTACCTCAWGTTACCACACTAC	All genotypes
LPC-1	TAAAAAAGAAAAAAGAAAATTAGTGTTATCTAC	Vaccine-type
LPC-2	GTAGATAACACTAATTTTCTTTTTTCTTTTTTA	Vaccine-type
G1-1	CCCGCCAGTAGGACCCTATT	Wild-type group 1
G1-2	GGAGAGGGGTATGAGCGCG	Wild-type group 1
G2-1	CCGGCCCTTGACCGGGCCCTATCAGT	Wild-type group 2
G2-2	GGGTGTAAGAACGGCCGGCC	Wild-type group 2
G3-1	GAAGGGGCACGTGAGTGCGG	Wild-type group 3
G3-2	CCGCACTCACGTGCCCCTTC	Wild-type group 3
H	ATGAAGCAYGTCAGGGCRTGGATACCTCG	Enterovirus 71

R = A and G; Y= C and T; W = A and T

Table 3. Test results of virus isolation, reverse transcription-polymerase chain reaction (RT-PCR), and DNA chips from 100 clinical samples.

Test	Positive	Negative
Virus isolation followed by IFA	9*	91
RT-PCR followed by sequencing	17†	83
DNA chips	20‡	80

*Nine positive samples cannot be differentiated for vaccine and wild-type CSFV
†The 17 positive samples were differentiated as 4 wild-type and 13 vaccine-type CSFV by DNA sequencing.
‡ The 20 positive samples were differentiated as 4 wild-type and 16 vaccine-type CSFV by the DNA chips.

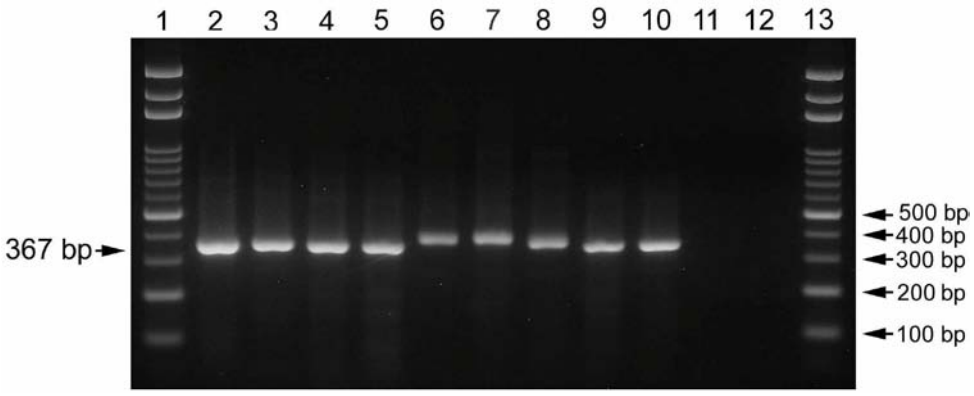

Figure 1. Amplification of different genotypes of CSFV and three lapinized vaccine strains. The reaction products were analyzed by agarose gel electrophoresis. Lanes 1 and 13: 100 bp DNA ladder marker. Lane 2: 83-114. Lane 3: 92-TN1. Lane 4: 90-YL1. Lane 5: 84-KS1. Lane 6: LPC/TS. Lane 7: LPC/PRK. Lane 8: LPC/AHRI. Lane 9: ALD strain. Lane 10: A76 strain. Lane 11: BVDV/NADL. Lane 12: negative control. The detected amplified 367 bp fragments from the wild-type CSFV are indicated with an arrow on the left of gel. Two vaccine viruses (6, 7) had slightly larger products than wild-type CSFV.

Exp.Rep AHRI.,No.43：89~100 (2008)

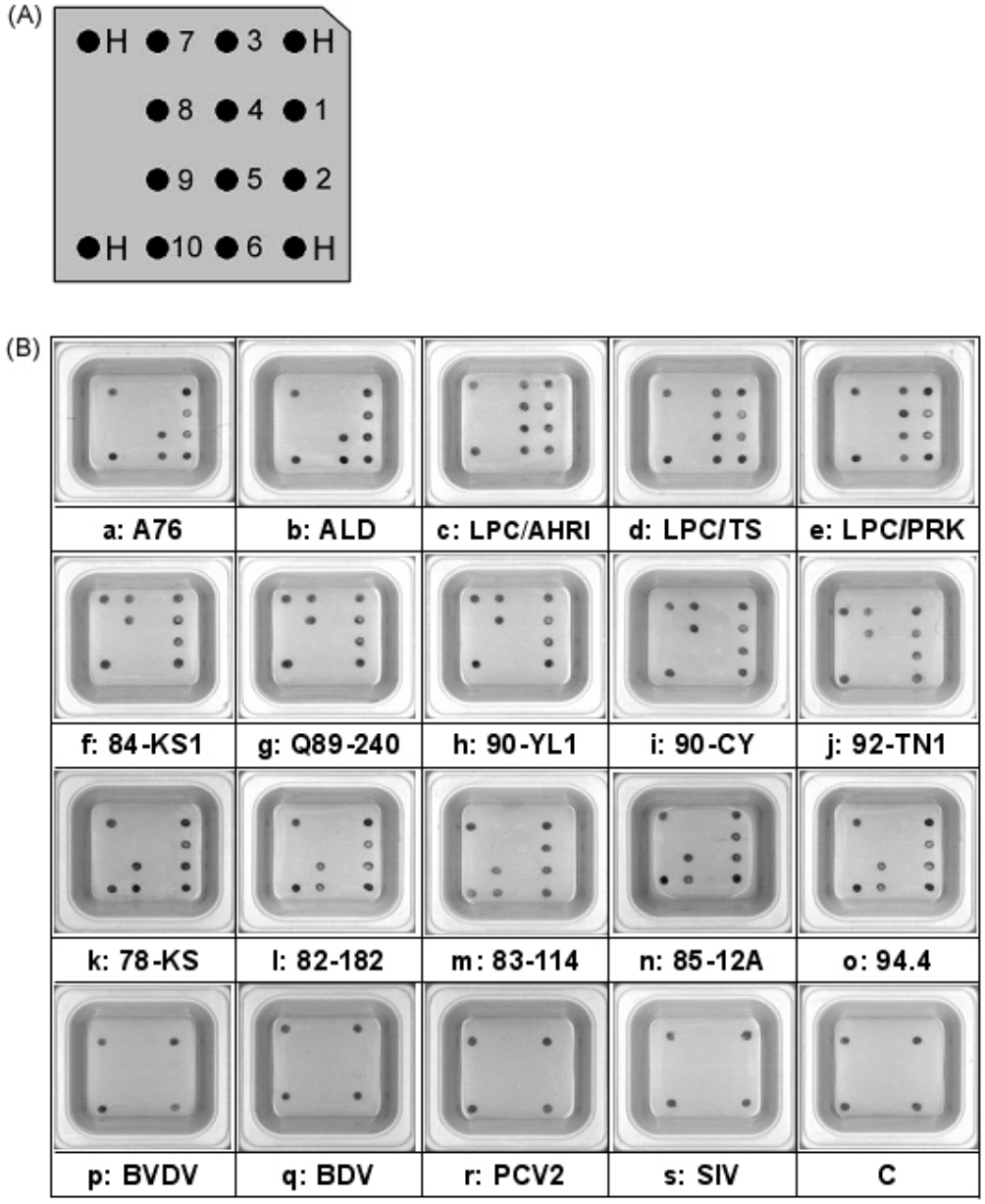

Figure 2. Detection and genotyping of wild-type CSFV and three lapinized vaccine strains using DNA chips. (A) Map pattern. Each dot indicates the spotted position of each probe. 1: CSFV-U1; 2: CSFV-U2; 3: LPC-1; 4: LPC-2; 5: G1-1; 6: G1-2; 7: G2-1; 8: G2-2; 9: G3-1; 10: G3-2. H: Hybridization control. (B) The detection and typing results shown on the chips. a-b: Wild-type CSFV (group 1); c-e: LPC vaccine strain (group 1); f-j: Wild-type CSFV (group 2); k-o: Wild-type CSFV (group 3); p: BVDV; q: BDV; r: PCV2; s: SIV; C: Negative control.

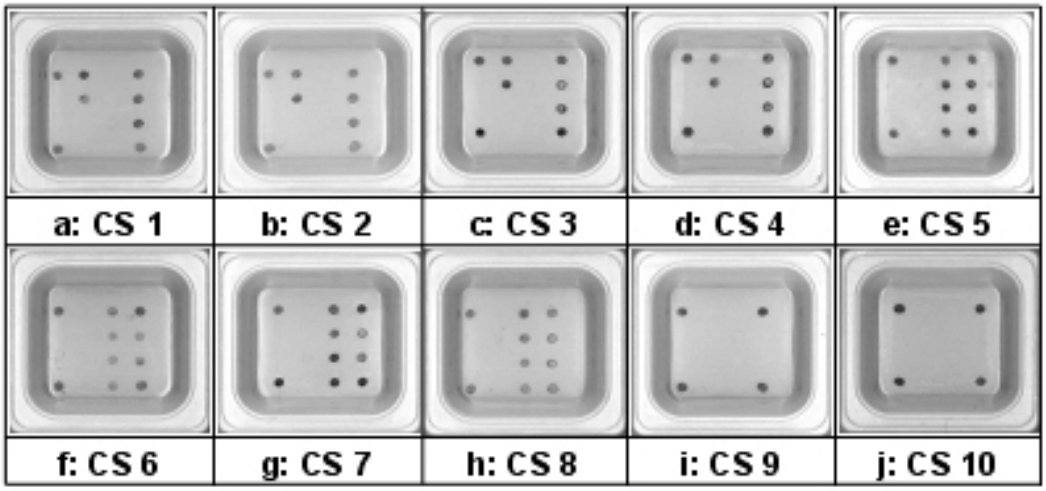

Figure 3. The hybridization results of 10 representative clinical samples (CS) shown on the chips. a-d: wild-type CSFV (group 2); e-h: vaccine-type CSFV (group 1); i-j: Negative samples.

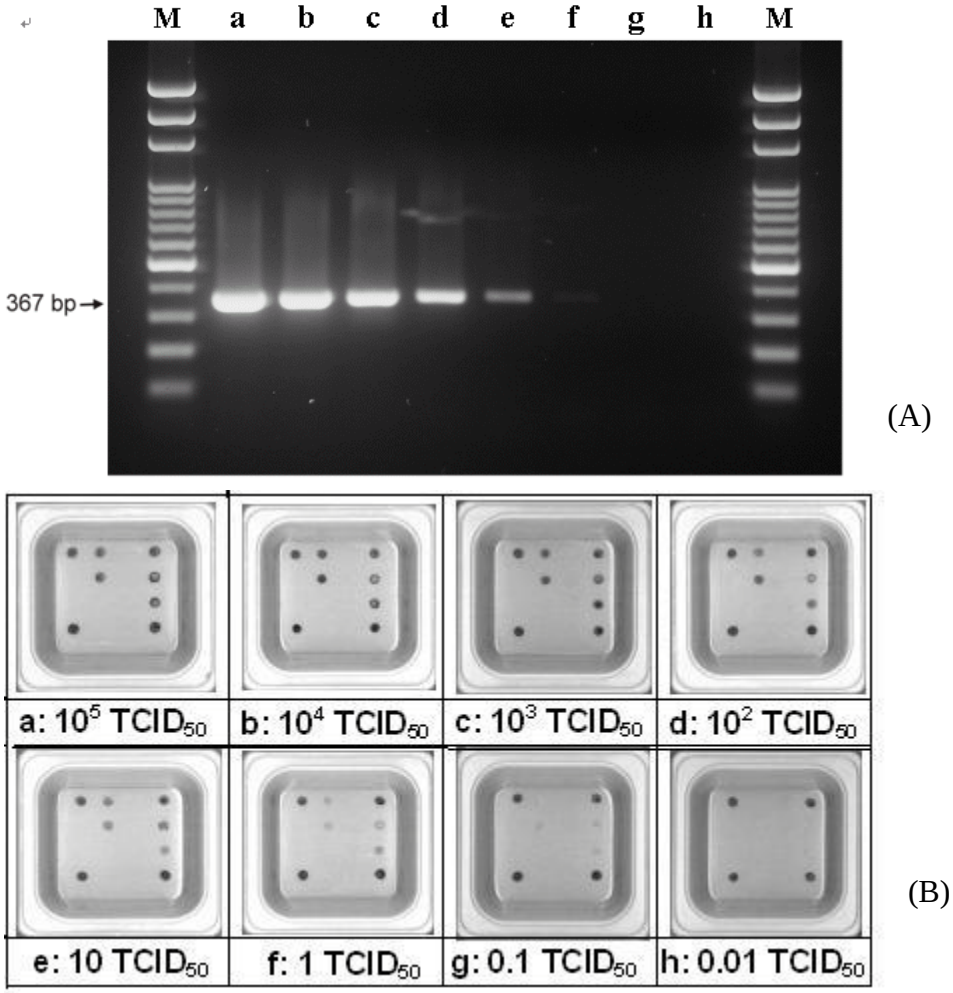

Figure 4. Comparison of the sensitivity between the RT-PCR and the DNA chip assays. (A) Sensitivity of the RT-PCR assay. Lane M: 100 bp (Promega, Madison, USA); lanes 1-8: RNA isolated from 10^5, 10^4, 10^3, 10^2, 10, 1, 0.1, 0.01 $TCID_{50}$/ml of CSFV (2.1a subgroup). RT-PCR products were analysed by electrophoresis on a 2 % agarose gel containing 1x SYBR® Safe DNA gel stain (Invitrogen, Carlsbad, CA, USA). (B) Sensitivity of the DNA chip assays. a-h: Hybridization results from 10^5, 10^4, 10^3, 10^2, 10, 1, 0.1, 0.01 $TCID_{50}$/ml of CSFV.

Exp.Rep AHRI.,No.43：89~100 (2008)

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References

1. Anonymous, 1999. Chapter on classical swine fever. In: Animal Health Code of the Office International des Epizooties, eighth ed. Rue de Prony, Paris, pp. 123-127.
2. Baxi MK, Baxi S, Clavijo A, Burton KM, Deregt D, 2006. Microarray-based detection and typing of foot-and-mouth disease virus. Vet J 172:473-481.
3. Biagetti M, Greiser-Wilke I, Rutili D, 2001. Molecular epidemiology of classical swine fever in Italy. Vet Microbiol 83:205-215.
4. Bjorklund HV, Stadejek T, Vilcek S, Belak S, 1998. Molecular characterization of the 3' noncoding region of classical swine fever virus vaccine strains. Virus Genes 16:307-312.
5. Chizhikov V, Wagner M, Ivshina A, Hoshino Y, Kapikian AZ, Chumakov K, 2002. Detection and genotyping of human group A rotaviruses by oligonucleotide microarray hybridization. J Clin Microbiol 40:2398-2407.
6. Cho HS, Park SJ, Park NY, 2006. Development of a reverse transcription-polymerase chain reaction assay with fluorogenic probes to discriminate Korean wild-type and vaccine isolates of classical swine fever virus. Can J Vet Res 70:226-229.
7. Correa GP, Coba-Ayala MA, Salinas LZ, Guadalupe SE, Amalia MA, 2006. Persistence of hog cholera vaccinal virus PAV-250 in tonsils of vaccinated pigs. IPVS Proceed, pp. 117.
8. Deng MC, Huang CC, Huang TS, Chang CY, Lin YJ, Chien MS, Jong MH, 2005. Phylogenetic analysis of classical swine fever virus isolated from Taiwan. Vet Microbiol 106:187-193.
9. Gordon PF, Engstrand L, 2001. Comparison of genetic divergence and fitness between two subclones of Helicobacter pylori. Infect Immun 69:7823-7838.
10. Klaassen CHW, Prinsen CFM, de Valk HA, Horrevorts AM, Jeunink MAF, Thunnissen FBJM, 2004. DNA microarray format for detection and subtyping of human papillomavirus. J Clin Microbiol 42:2152-2160.
11. Koenig P, Hoffmann B, Depner KR, Reimann I, Teifke JP, Beer M, 2007. Detection of classical swine fever vaccine virus in blood and tissue samples of pigs vaccinated either with a conventional C-strain vaccine or a modified live marker vaccine. Vet Microbiol 120:343-351.
12. Li J, Chen S, Evans DH, 2001. Typing and subtyping influenza virus using DNA microarrays and multiplex reverse transcriptase PCR. J Clin Microbiol 39:696-704.
13. Li Y, Zhao JJ, Li N, Shi Z, Cheng D, Zhu QH, Tu C, Tong GZ, Qiu HJA, 2007. Multiplex nested RT-PCR for the detection and differentiation of wild-type viruses from C-strain vaccine of classical swine fever virus. J Virol Methods 143:16-22.
14. Lorena J, Barlic-Maganja D, Lojkic M, Madic J, Grom J, Cac Z, Roic B, Terzic S Lojkic I, Polancec D, Cajavec S, 2001. Classical swine fever virus (C strain) distribution in organ samples of inoculated piglets. Vet Microbiol 81:1-8.
15. Meyers G, Rumenapf T, Thiel HJ, 1989. Molecular cloning and nucleotide sequence of the genome of classical swine fever virus. Virology 171:555-567.
16. Meyers G, Thiel HJ, 1996. Molecular characterization of pestiviruses. Adv Virus Res 47:53-118.
17. Moormann RJM, van Gennip HGP, Miedema GKW, Hulst MM, van Rijn PA, 1996. Infectious RNA transcribed from an engineered full-length cDNA template of the genome of a pestivirus. J Virol 70:763-770.
18. Pan CH, Jong MH, Huang TS, Liu HF, Lin SY, Lai SS, 2005. Phylogenetic analysis of classical swine fever virus in Taiwan. Arch Virol 150:1101-1119.
19. Pan CH, Jong MH, Huang YL, Huang TS, Chao PH, Lai SS, 2008. Rapid detection and differentiation of wild-type and three attenuated lapinized vaccine strains of classical swine fever virus by reverse transcription-polymerase chain reaction. J Vet Diagn Invest 20:448-456.
20. Paton DJ, McGoldrick A, Greiser-Wilke I, Parchariyanon S, Song JY, Liou PP, Stadejek T, Lowings JP, Bjorklund H, Belak S, 2000. Genetic typing of classical swine fever virus. Vet Microbiol 73:137-157.
21. Paton DJ, Greiser-Wilke I, 2003. Classical swine fever-an update. Res Vet Sci 75:169-178.
22. Pringle CR, 1999. The universal system of virus taxonomy, updated to include the new proposal ratified by the International Committee on Taxonomy of Virus during 1998. Arch Virol 144:421-429.
23. Song Y, Dai E, Wang J, Liu H, Zhai J, Chen C, Du Z, Guo Z, Yang R, 2006. Genotyping of hepatitis B virus (HBV) by oligonucleotides microarray. Mol Cell Probes 20:121-127.
24. Stegeman A, Elbers A, de Smit H, Moser H, Smak J, Pluimers F, 2000. The 1997-1998 epidemic of classical swine fever in the Netherlands. Vet Microbiol 73:183-96.
25. Van Oirschot JT, 2003. Vaccinology of classical swine fever: from lab to field. Vet Microbiol 96:367-384.
26. Wang D, Coscoy L, Zylberberg M, Avila PC, Boushey HA, Ganem D, DeRisi JL, 2002. Microarray-based detection and genotyping of viral

pathogens. Proc Natl Acad Sci 99:15687-15692.

27. Wang LC, Pan CH, Severinghaus LL, Liu LY, Chen CT, Pu CE, Huang D, Lir JT, Chin SC, Cheng MC, Lee SH, Wang CH, 2008. Simultaneous detection and differentiation of Newcastle disease and avian influenza viruses using oligonucleotide microarrays. Vet Microbiol 127:217-226.
28. Wengler G, Bradley DW, Colett MS, Heinz FX, Schlesinger RW, Strauss JH, 1995. Flaviviridae. In Virus Taxonomy. Sixth report of the International Committee on Taxonomy of Viruses. Arch Virol Suppl 10:415-427.
29. Wong ML, Peng BY, Liu JJ, Chang TJ, 2001. Cloning and sequencing of full-length cDNA of classical swine fever virus LPC strain. Virus Genes 23:187-192.
30. Wu HX, Wang JF, Zhang CY, Fu LZ, Pan ZS, Wang N, Zhang PW, Zhao WG, 2001. Attenuated lapinized Chinese strain of classical swine fever virus: complete nucleotide sequence and character of 3'-noncoding region. Virus Genes 23:69-76.
31. Zhao JJ, Cheng D, Li N, Sun Y, Shi Z, Zhu QH, Tu C, Tong GZ, Qiu HJ, 2008. Evaluation of a multiplex real-time RT-PCR for quantitative and differential detection of wild-type viruses and C-strain vaccine of classical swine fever virus. Vet Microbiol 126:1-10.

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以目視 DNA 晶片同時檢測，分型及區別豬瘟野外毒及疫苗毒

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摘要　應用反轉錄聚合酶鏈反應 (Reverse transcription-polymerase chain reaction; RT-PCR) 增幅後再進行核酸定序是目前檢測野外豬瘟並排除疫苗毒干擾實驗室診斷之主要方法。本論文為了同時檢測，分型及區別豬瘟野外毒及疫苗毒因而發展目視 DNA 晶片檢測法。豬瘟病毒特異性引子及探針係依據病毒基因 3 端未轉譯區核酸序列而設計，採用生物素標識引子進行單步驟 RT-PCR 反應，隨後與固定在高分子塑膠晶片上的探針進行雜合反應 (Hybridization)。本方法可精確地將豬瘟病毒區分為三種主要基因型，並可同時區別野外毒及疫苗毒。傳統 RT-PCR 方法可檢測豬瘟野外毒之最低力價為 10 $TCID_{50}$/mL，DNA 晶片可檢測之最低病毒力價為 1 $TCID_{50}$/mL，DNA 晶片相較於 RT-PCR 方法敏感性高 10 倍。RT-PCR 結合 DNA 探針雜合技術可提高檢測敏感性，可快速鑑定臨床檢體中豬瘟病毒的基因型別，並區別野外毒及疫苗毒。

關鍵詞：豬瘟病毒、DNA 晶片、探針、反轉錄聚合酶鏈反應

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