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Molecular Identification of Hog Cholera Viruses from Southern China

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Abstract: 17 HCV infected tissue samples collected from Southern China between 1996 and 2000 were identified with a RT-PCR and sequencing, PCR primers used in this study were designed according to the sequence of E2 gene published in GeneBank.15 out of them were positive when detected by a RT-PCR, all the samples were parallel to recover virus in vitro, 5 were positive and two isolates were confirmed to be virulent with rabbit inoculation test. Sequence analysis revealed that HCV isolates from Southern China collected from different years homologous to different strains reported in GeneBank, among them, strain 529 similar to a Italian field strain PR/98/dp, 829 and HCV lapinized strain at the same group, 1026 was similar to Alfort strain, isolates 580and 856 at the same group, whereas GS-YZ isolates, a virulent strain from mainland China was at a separate group.

Key words: Hog cholera virus (HCV), RT-PCR, Sequencing

Introduction

Classical swine fever (CSF) or hog cholera is a highly contagious and often fatal disease of pigs, which is characterized by fever and hemorrhages and run either an acute or a chronic course. Outbreak of the disease can cause severe economic losses. The causative agent of the disease is classical swine fever virus (CSFV) or hog cholera virus (HCV), a member of the Pestivirus genus of the Flaviviridae family (Wengler *et al*., 1995). Pestiviruses are small, enveloped positive stranded RNA viruses and genome, varying in length from 9.5 to 12.5kb, contains a single large open reading frame (ORF). The ORF is translated into a polyprotein that is processed into mature protein by viral ad host cell protease (Rumenapf *et al*., 1993). The envelop of pestiviruses contains three glycoprotein Erns, E1 and E2 (Thiel *et al*., 1991). E2 is its immunogenic gene and induces high virus neutralizing titers of serum antibodies (Hulst *et al*., 1993; Rijin *et al*., 1996; Zijl *et al*., 1991 and Bouma *et al*., 1999).

CSF is on the list a disease of the office International des Epizootic (OIE), since the first introduction of CSF in the swine population, several vaccines have been developed. The Chinese strain C strain is one of them, which is a live attenuated lapinized vaccine strain that effectively protect pigs against this disease (Terpstra *et al*., 1990). However, pigs that are vaccinated with the C strain can not be serological differentiated from those infected with HCV field strain, moreover, although vaccination programs had been implemented in Southern China pig farms for many years, cases with mild symptoms or even severe outbreak were frequently reported, it is still an endemic disease in China, also highly prevalent among swine in other Asia countries, central and south America and in some wild boar populations in the EU (Gennip *et al*., 2002). Detailed molecular characterization of different strains could highlight the cause of high incidence in Southern China, facilitating the control of this disease.

In this study, infected tissue samples from South China during 5 years were collected, all the samples were detected by a RT-PCR and the PCR products were further sequenced and analyzed with other HCV strain sequences reported in GeneBank. Resulting phylogentic tree information revealed the genotype of those HCV field strain isolated from South China.

Materials and Methods

Tissue Samples and Cells: 11 frozen tissue samples of lung, liver, spleen, tonsil, blood and lymph node were provided by Hong Kong Agricultural and Fishery Department, the other four were collected from south China pig farms. All these samples had been demonstrated to be HCV positive with a fluorescent staining assay. PK15 cell line was purchased from ATCC and grown in Eagle's medium, supplemented with 10% fetal calf serum. Virus was isolated on PK15 cells as described (Widjojoatmodjo *et al*., 2000).

RNA Extraction and RT-PCR: Viral RNA was extracted from infected tissue samples or infected cell cultures with Trizol (GiBcoBRL), cDNA synthesis were performed as the instruction of superscript pre-amplification system (GiBcoBRL). A pair of primers (F02 and H06) flanking the E2 gene of HCV was designed and synthesized by GiBcoBRL, The predicted size of the PCR product was 1.25kb in length, The sequence of the primers used for PCR and sequencing were listed as follows:

Primer name	Sequence	Strand
F01	ataggatccatgaaagccctattggcatgggcagtgatag	+
F02	attggctccatgcggctagcctgcaaggaagattac	+
H06	cccgggaattccatgaaacagcagtagtatccatttctt	-
SQF01-1	gcctccaaataaagattgcg	+
SQH06-1	gctggaatagctgtccttgg	-
SQH06-1a	gctggaaatagctgtccttgg	-
SQF01-2	acccaggccaggaatagacc	+
SQF06-2	gataacgacgccatatctgt	-
SQF01-3	gaagacgggaagattcttcatg	+
SQF06-3	caaagtcatctcccatctcctc	-
Gp55-U	atatatgctcaagggcgagt	+
Gp55-L	acagcagtagtatccatttcttta	-

After reverse transcription, 25ul PCR mixture was prepared (ddH_2O 19.45ul, 10XPCR buffer 2.5ul, 25mm $Mgcl_2$ 0.75ul, 10mm dNTP 0.5ul, Primer-1 0.25ul, Primer-2 0.25ul, cDNA 1ul, Taq DNA polymerase 0.3ul), amplification was carried in 94^0C 3min, 1cycle, 30 cycle at 94^0C 0.5min, 52^0C 2min, 72^0C 2min and 1 cycle at 72^0C 10min.

Virus Isolation, Purification and Electronic Microscope Observation: Tissue samples was disrupted and lyses by 3 cycles of freezing and thawing, centrifuged at 2000rpm 4^0C for 15min, the supernatant was isolated and 1000u/ml penicillin and streptomycin were added, kept it at 4^0C for 30min, infected PK15 at serial dilutions (1:10—1:200), blind passage each sample for 3 times, harvested and used it for re-infection, virus was purified and examined under electronic microscope.

Differentiate Virulent HCV by Using a Rabbit Inoculation Test: Lapinized HCV vaccine can induce fever in rabbit, whereas virulent HCV can't (Smit *et al.*, 2000). This phenomenon could be used in swine fever diagnosis. In this study, 6 rabbit (2-2.5kg) was randomly divided into 3groups, 2 rabbit per each, designated as group 1,2 and 3. Group 3 was setup as the control, measuring body temperature of rabbit 3 days before test and calculate its average body temperature, after that, inoculate 1ml

Infected cell culture into rabbit through ear vein, measuring body temperature of rabbit every 8hrs, 1 week later, inoculate rabbit with the laprinized HCV vaccine in three groups, The result was obtained as follows:

Test samples	Fever response after first injection	Fever response after second injection	Results
1	-	-	Virulent HCV
2	+	-	Vaccine HCV
3	+	+	Other pathogen

The rational of the judgment is, in first case, since the rabbit has been immunized by virulent HCV after first inoculation, there is no response to the consequently vaccine inoculation due to neutralization. In second case, rabbit has fever response to vaccine injection first time, but no response to the second injection also due to the neutralization.

Sequencing of the PCR Product: Before the product was used as the template for cycle sequencing, they were purified with a Geneclean II kit; using a DNA sequencing kit purchased from Pekin Elmer performed sequencing reaction. Pekin Elmer ABI310 sequencer performed automatic sequencing.

Results and Discusion

RT-PCR was performed by using a pair of primers F02 and H06, the size of PCR product were about 1.25kb in length, 5ul PCR product was used for agarose gel electrophoresis, 15 tissue samples and 5 infected cell cultures were positive.

Isolation HCV from infected tissue samples: 5 out of 15 samples were positive when subjected to recover virus in vitro, typical virus were observed under electronic microscope. (Table 1)

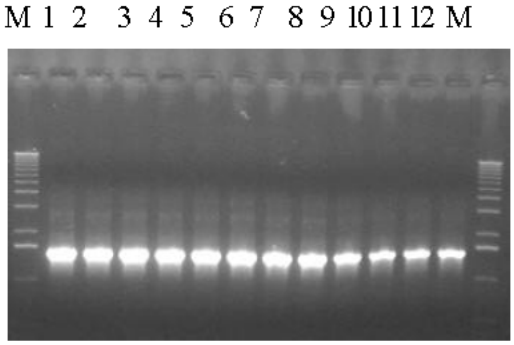

Fig. 1:Ditection of HCV from tissue sampels with a RT- PCR

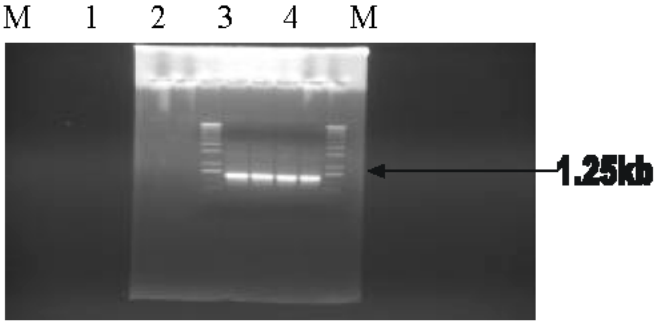

Fig. 2: Ditection of HCV fromcell culture with a RT- PCR

Isolation HCV from Infected Tissue Samples: 5 out of 15 samples were positive when subjected to recover virus in vitro, typical virus were observed under electronic microscope. (Table 1)

Table 1: RT-PCR results and virus isolation from tissue samples

Sample code	source	RT-PCR Detection	Virus isolation
525/96	Hong Kong	+	+
628/96	Hong Kong	+	-
708/96	Hong Kong	+	-
812/96	Hong Kong	+	-
9/97	Hong Kong	+	-
10/97	Hong Kong	+	-
529/99	Hong Kong	+	-
580/99	Hong Kong	+	-
828/99	Hong Kong	+	+
856/99	Hong Kong	+	+
1026/99	Hong Kong	+	+
GZ1	Guangzhou	+	+
GZ2	Guangzhou	+	-
GZ3	Guangzhou	+	-
GZ4	Guangzhou	+	-

Sequences were analyzed by a software package DNAstar (DNAstar, Co), the result showed that 529 was at the same group with a Italian field strain, 828 was homologous to HCV laprinized vaccine strain, 1026 was similar to Alfort strain, 580 and 856 was different from other strains since they were at the same group, this may explain why they were virulent when tested with rabbit inoculation test, whereas GS-YZ strain was at a separate group, it was totally different from Hong Kong isolates.

Compared with the previous report, several groups had described reverse transcription and subsequent PCR for the detection and differentiation of HCV and BVDV-BDV by utilizing primers derived from the structural or non-structural

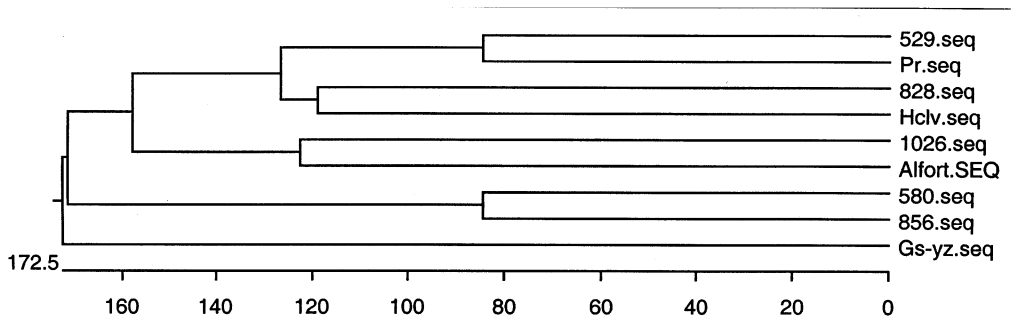

Fig. 3: Dendrogram of the phylogenetic tree of HCV isolates from Southern China, which was inferred from the gene by DNA star.

Phylogentic tree analysis of HCV E2 gene of South China isolates (Fig.3.)

Strain	Genebank accession number	Source
1026	AF171637	HongKong
828	AF171636	HongKong
580	AF171635	HongKong
529	AF171634	HongKong
856	AF171633	HongKong
Alfort	AF182866	France
HCLV	AF531433	Mainland China
PR/98/dp	AJ312876	Italy
GS-YZ	AF143089	Mainland China

genes (Barli *et al*., 2000). In this study, a pair of primer based on the structural gene E2 of the classical swine fever virus (CSFV) were designed, we were able to detect 15 tissue samples and 5 cell associated HCV isolates with that. Although 15 suspected tissue samples were positive detected by RT-PCR, only 5 isolates were recovered I in PK15 cell, this may due to the duration of sample storage, half of the samples had been kept in freeze over 1-3years, HCV contained in the samples may have been inactivated and lost its infectivity.

Although HCV can be propagated on PK15 cell line, its replication is restricted in the cytoplasm of the cell (Mosser *et al*., 1998), most of the HCV does not result in a cytopathogenicity effect, so far, less than 30 CPE associated HCV had been reported, among them, alfort strain was a typical one, Meyers reported defective interfering particles was responsible for the cytopathogenicity effect of HCV (Meyers *et al*., 1995; Moser *et al*., 1999; Tautz *et al*., 1999). In this study, HK isolates 580 was interestingly to have such effect, but its appearance was various according to the duration post-infection, including rounding and clump, granularity, foamy appearance, thickness and tearing of the cell, CPE was also confirmed by a plaque forming technique (Data was not shown).

In order to demonstrate fidelity of the PCR products, E2 sequence were determined, the result showed a high homology of Hong Kong isolates with other strains, but differences was also observed. Some groups found particular strain of HCV was very stable even passed for 20 years, whereas in our study, the progressively mutation was observed within 5 years. Maybe this can highlight the frequently vaccination failure in this area. Laboratory diagnosis of HCV is based on the detection of viral antigen, virus isolation or demonstration of viral antibody. Rabbit inoculation test can be used to distinguish virulent strain from vaccine strain, which is based on the principle that laprinized vaccine strain has the ability to induce fever and antibodies producing in rabbit after intravenous inoculation, HCV isolates 525 and 580 had been confirmed to be virulent HCV through this method.

HCV isolates were also examined under a electronic microscope, although we observed 40-60nm diameter virion under Philips EM300 electron microscope at a magnificent of 50K, the amount of the virus was not as much as that of the alfort strain and there was still some impurities in it. This maybe due to the low passage of the isolates, as usual, isolate was not so good adapt to the cell line in the preliminary isolation compared with the vaccine strain, secondly, it is related to the growth temperature of the isolates, we propagated the isolates at 37^0C, while alfort strain was propagated at 39^0C, moreover, we harvested isolates 48hrs post-infection.

In summary, we have isolated 5 HCV from historical and recent samples collected south China pig farms, PCR sequencing demonstrated it has promising as a rapid sensitive and specific test for the detection of HCV, these advantages are especially obvious to the samples which is difficult to isolate virus. As the sequence of Hong Kong isolates were highly homologous with strains isolated from European country and hyper variable with isolate from

mainland China, it implicated they may have a different origin although they are so adjacent. HCV is only subjected to 3 genotypes at present, the resulting information of this study suggested new genotype may emerge in the near future, therefore, south China pig farms should select their genotype-matched vaccine to control this economic important disease.

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