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ANTIGENIC, BIOLOGICAL AND MOLECULAR CHARACTERIZATION OF THE CUBAN CSFV ISOLATE "MARGARITA"

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ABSTRACT: **"Margarita" CSF virus isolate (Cuba, 1958) was characterized from the antigenic, biological and molecular point of view; adapted on PK-15 and SK-6 cell lines with an infective titer of $10^{7.5}$ $DICT_{50}$, and biologically cloned by limit dilution keeping its pathogenicity in pigs. Genotyping of this virus was made and its phylogenetic relationship with another strain deposited at the GenBank was determined keeping located in the 1.2 sub-group very close related with the field isolates from the 1993-2006 epidemic in Cuba. Its antigenic behavior was similar to the "Alfort" strain, showing no reactivity to MAbss against vaccine strains. The complete sequence of the gene for the E2 protein deposited in the EMBL with number AJ704817 is shown, also the a.a. deduced sequence showing all the residues of cysteine highly conserved in the pestivirus genome is given being part of the different types of conformational epitopes of the glycoprotein E2. From these results, an isolate of a Cuban-native CSF virus fully characterized is available, which can be used as reference material for several purposes.**

(Key words: CSF; CSF reference strain; antigenic biological and molecular characterization)

CARACTERIZACIÓN ANTIGÉNICA, BIOLÓGICA Y MOLECULAR DEL AISLADO CUBANO "MARGARITA" DEL VIRUS DE LA PESTE PORCINA CLÁSICA

RESUMEN: **Con el objetivo de disponer de una cepa como material de referencia de Peste Porcina Clásica (PPC) se realizó la caracterización desde el punto de vista antigénico, biológico y molecular del aislado "Margarita" (La Habana, 1958) del virus de la PPC mantenido en cerdos. Se adaptó al crecimiento en células PK-15 y SK-6 con un título infectivo de $10^{7.5}$ $DICT_{50}$, se clonó biológicamente por dilución límite manteniendo su patogenicidad para el cerdo. Se realizaron estudios filogenéticos con otras cepas informadas en el GenBank quedando ubicada en el sub-grupo 1.2 muy estrechamente relacionada con los aislados de campo de la epizootia cubana desde 1993 hasta la fecha. Se determinó que su comportamiento antigénico es similar al de la cepa "Alfort" y mostró no reactividad frente a anticuerpos monoclonales dirigidos contra cepas vacunales. La secuencia completa del gen que codifica para la proteína E2 fue depositada en el EMBL con número de acceso AJ704817 y se discute la secuencia de aminoácidos deducida, la cual mostró todos los residuos de cisteína que se encuentran altamente conservados en los genomas de los pestivirus y que forman parte de los diferentes epítopos conformacionales de la glicoproteína E2. A partir de los resultados obtenidos se dispone de un aislado autóctono cubano del virus de la PPC completamente caracterizado que sirve como material de referencia para diferentes propósitos.**

(Palabras clave: PPC; cepa de referencia PPC; caracterización antigénica biológica y molecular)

INTRODUCTION

Classical swine fiver (CSF) is a highly contagious viral disease affecting domestic and wild pigs. It is considered as one of the most severe diseases affecting the Pork World Industry, both from the economical and sanitary point of view (25).

The etiological agent is a virus, with an icosahedric symmetry of 40 to 60 nm in diameter and with a lipid envelope (38). CSF virus (CSFV), together with the

bovine viral diarrhoea virus (BVDV) and the border disease virus (BDV) conform the Genus *Pestivirus*, Family *Flaviviridae* (16).

The disease affects the swine immune system, induces immune suppression associated to important haematological changes such as leucopoenia, thrombocytopenia, coagulation disorders, thyme and bone marrow atrophy (11).

Specific viral antibodies are not detected until three weeks post infection (35) probably due to the strong leucopoenia induced by the virus. Pigs recovered from the infection generally develop neutralizing antibodies persisting during their whole life.

The severity of clinical signs mainly depends on the virulence of the viral strain, also influenced by the age and the immunological state of the animal. (25). Thus, the CSF clinical form and severity are very variable. Nevertheless, their clinical form has been classified as: i) post natal infections, including the hyper acute, acute and chronic forms, ii) trans-placentary infections, which produces foetal and neonatal affections and iii) persistent infections (8, 33).

Nowadays, CSF is enzootic in Central America and the Caribbean area, South America, Southeast Asia and Eastern Europe (7, 8, 25). The EU is considered as a re emergency high risk zone for the disease, due to the high density of swine population, to the non vaccination policy currently followed and the nearness to the Eastern Europe countries.

The presence of wild pigs with CSF endemic infections in some of the state members (17) is one of the problems which has been associated to the disease re-emergency, as occurred in Germany between 1990 and 1998. These re-emergencies have taken place in spite of the solid control programs which include the sanitary slaughter of all affected animals, pig movements and international trade restrictions (34,6).

After some years of relative calm, some outbreaks of the disease in domestic swine farms had been notified by Germany from March 1st, 2006.

All these elements make debatable the policy of no vaccination followed by the EU.

For disease control, different live vaccines, very efficient concerning protection against the disease, as well as a great quantity of new generation vaccines which have served as model for studying the immunological mechanisms related to the induced protection against the CSFV have been developed. The characteristics and uses of these vaccination strategies have been widely discussed (10).

Concerning Cuba, CSF was maintained under control until 1993 through vaccination which was applied since 1965 using a lapinized live strain (Labiofam) obtained from the strain "C" (7). This vaccine was successfully applied until 1993 when the disease re emerged and it is still in use as part of the measures established in the National Control Program against CSF. "Margarita" isolate (Havana-Cuba,1958), is used since almost 40 years ago, as challenge strain in the potency assays of the CSF vaccines in Cuba and there is a strong evidence on the direct responsibility of this strain on the outbreaks occurred in the West part of the Island during the epizootic started in 1993.(4). Thus, the decision of the characterization of "Margarita" isolate (given by the State Control Laboratory, IMV) was taken; and also to use it as the virus to begin the studies with the aim to obtain a virus adapted to cell cultures to be used as candidate for the new vaccination strategies in Cuba in the control of CSF as well as reference material for the diagnostic assays.

MATERIALS AND METHODS

Production of the virus

The viral isolate "Margarita", multiplied by successive passes in pig with a titer of $10^{7,4}$ lethal dose $_{50}$ (DL_{50}) /mL, was used.

To multiply the viral isolate "Margarita" in cell cultures, firstly a pig blood preparation with a titer of $5x10^5$ DL_{50} in pig was used. From this preparation, PK-15 recently grown cells were inoculated at an infection multiplicity (IM) of 0,3 DL_{50} /cell. The supernatant and cells were taken at 48 hours post infection (HPI), and they were submitted to three freezing-thawing cycles. The supernatant recovered after the sedimentation of cell residues was used for other two successive passes following the previous conditions. The viral suspension finally obtained was titered in PK-15 cells, detecting the presence of the virus by direct immunoperoxidase assay in plate (PLA) (39). For carrying out PLA, cells were incubated for 1 hour at 37º C with a hyperimmune serum (named a-VPPC serum) against CSFV (32). The viral titer was calculated from the last dilutions of the virus in which specifically stained infectious foci were observed using Reed and Muench's method (1938) (30). The result was expressed as the viral dose which produces infection in the 50% of the inoculated monolayers ($DICT_{50}$)/mL.

This viral suspension was cloned by limit dilution, and the virus recovered from it was amplified in PK-15 cells using an IM of 0,4 which is an infective dose in tissue cultures $_{50}$ ($DICT_{50}$)/ cell and named c-Margarita.

On the other hand, using the same IM, there were made 11 passes in PK-15 cells and other 2 in SK6 cells. This viral suspension was titered with the same way of the rest of viral preparations used (nc-Margarita).

The strain NADL (given by Dr. Aynaud, INRA, France) of CSFV was grown on MDBK cells at an IM of 0,2 $DICT_{50}$/ cell, while strain Alfort (same origin of the previous one) was grown on PK-15 cells at a IM of 0,1.

Antigenic characterization

Direct and indirect immunofluorescence (DIF-IIF) and direct and indirect immunoperoxidase (DIP-IIP)

For carrying out DIF and IIF, the protocols described by other authors were used respectively (2, 37). For characterizing the antigenic reactivity of the virus "Margarita", 5 monoclonal antibodies (mAb), which recognized glycoprotein E2 of CSFV or BVDV, as well as others against the non-structural protein NS3 of CSFV, were used. The hyperimmune serum a-CSFV conjugated with FITC was used. The assays IIF and DIP with these mAb were carried out using laminar antigens prepared from PK-15 cells infected or not with the virus "c-Margarita", the Alfort strain of CSFV and MDBK cells infected or not with NADL strain of BVDV. The mAb used were the following:

- anti- protein E2 of CSFV: **a18** at a dilution of 1:20 (36) (given by Dr. Emilia Campos, CISA-INIA, Spain;
- **HC/34/3p1**; anti- protein NS3 of CSFV, **C 16/1/1-M**; anti-E2 of BVDV: **CA/3/2/22** and **CA 34+1+5** (given by Dr. Greiser-Wilke, CSF World Reference Center, Hannover, Germany);
- **WH211** (*Central Veterinary Laboratory*, Weybridge, UK).

The pig hyperimmune serum a-CSFV conjugated with fluorescein isothiocyanate (FITC) was used for DIF (Dako, Denmark) (32).

For carrying out the DIP, the laminar antigens and the negative controls were incubated with the following mAb against the protein E2 of CSFV, conjugated with peroxidase: 21.2, 44.3 and 63.19 (Ceditest, Lelystad, Netherland). In the case of the IIP, cells were incubated with the hyperimmune serum a-CSFV.

Detection of the protein E2 of CSFV by electrophoresis in SDS-PAGE and immune *Western blot*

The proceeding described by Sambrook *et al.* (31) was used with some modifications briefly described here. Cells PK-15 infected and not infected with the virus "c-Margarita" were used. Such cells were washed twice with PBS, and 100 ml lysis tampon (50mM Tris-HCl pH 8; 1% NP-40; 150 mM NaCl; 2mM EDTA) supplemented with 1mM PMSF (SIGMA) were added as protease inhibitors. Subsequently, a volume of protein dissociative buffer (0,5 M Tris-HCl pH 6,8; 10% SDS; 10% glycerol; 14,2 M 2-mercaptoetanol; 0,1% bromophenol blue) was added. Samples were charged in a gel of 10% polyacrylamide (SDS-PAGE), and the low molecular weight marker (28-111 kDa) (BioRad) was included (31). Electrotransference to nitrocellulose membrane was carried out (31). It was blocked with 5% skimmed milk; PBS-0,05% Tween 20, after being incubated for an hour at 37ºC with the mAb a18 (aE2). After three washes, membranes were incubated for an hour at 37ºC with anti- mouse'IgG goat serum (SIGMA). Samples were revealed using 0,005 g of diaminobenzidine/10ml of TBS (50mM Tris-HCl; 150mM NaCl pH 7.4) and 9% hydrogen peroxide (SIGMA) as chromogen.

Biological characterization. Experimental infection in pigs

In order to check if "nc-Margarita" virus multiplied in cultures and the virus bilogically cloned in PK-15 cells kept the pathogenicity characteristics in their natural target, 9 commercial domestic pigs (Landrace x Large White) from 6 to 8 weeks old (free of antibodies against CSFV) were used. They were kept in separated cages in three groups of three pigs each ones with water and commercial feedstuff *ad libitum*. All of them were inoculated by deep intramuscular route in the neck with their respective viral suspensions. In group 1, pigs were inoculated with 10^5 DL_{50} of "Margarita" virus adapted to the pig used as positive control to infection; the pigs from group 2 were inoculated with 10^5 $DICT_{50}$ of "c-Margarita" virus; and those from group 3 with 10^4 $DICT_{50}$ of "nc-Margarita" virus.

Before inoculation and during the 14 days post-infection, the rectal temperature was daily recorded and there was a pursuit of the disease clinical signs.

Necropsy and a deep anatomopathological analysis were carried out after the death or euthanasia of the pigs. Samples from tonsils, spleen, gastrohepatic and mesenteric ganglia and kidney were taken for the detection of the virus.

Molecular characterization

CSFV RNA extraction

Tripure® was used for viral RNA extraction following the manufacture´s indications (SIGMA). Samples of viral suspension, supernatant of infected cells, macerated from organs, serum and mononuclear cells

TABLE 1. Oligonucleotides used for amplifying and sequencing the gene of glycoprotein E2./ *Oligonucleótidos usados para la amplificación y secuenciación del gen de la glicoproteína E2*

Oligonucleotide	Sequence (5´→ 3´)	Positions in the genome [a]
F190 (sense)	TC(G/A)(A/T)CAACCAA(T/C)GAGATAGGG	2467-2487
R190 (antisense)	CACAG(C/T)CC(A/G)AA(T/C)CC(A/G)AAGTCA	2718-2738
SE2 (sense)	GTGAGGATCCGCCACCATGATAAAAGTATTAAGAGGACAGGTCGT	2359-2384
S3a (sense)	GTATTAAGAGGACAGGTCCGT	2364-2384
A4b (antisense)	GCCGTGCACTCTTATAACACC	3311-3331
S5 (sense)	GATGGATGATGACTTCGG	2709-2726
AE2 (antisense)	TAGCTCTAGATTAATGGAACAGCAGTAGTATCCATTTCTT	3661-3687

[a] Numbering corresponds to that Publisher for gene E2 of Brescia Srtain (Moormann *et al.*, 1990)./ *La numeración corresponde a la publicada para el gen E2 de la cepa Brescia (Moormann et al., 1990).*

of peripheric blood (MCPB) or 100 ml of "c-Margarita" virus were processed.

a) Oligonucleotides used for amplification by RT-PCR of the gene E2

The oligonucleotides couple F190 and R190 described by Lowings *et al*. (19) was used for amplification and latter sequencing and phylogenetic analysis of CSFV isolates (Table 1). These oligonucleotides allow amplifying a DNA fragment of 272 pb of the extreme 5´ of the gene E2.

The primers design for the complete amplification of the gene E2 was carried out using the sequences corresponding to Brescia strain of CSFV (26). This strain was selected by its strong phylogenetic relation with "Margarita" isolate (4). Brescia strain sequence was aligned with those corresponding to Alfort (20) and Weybridge (40) strains, to determine the flanking regions of the gene E2 conserved among CSFV isolates of different genogroups. Taking into account this criterion, the oligonucleotides indicated in Table 1 were selected.

Three other oligonucleotides (S3a, A4b and S5) inside Brescia strain E2 sequence were designed. They were used in sequencing the product obtained. All primers used were synthetized by Isogen Bioscience.

b) Amplification, by RT-PCR, of the regions 5' of the gene E2 and for the complete gene E2.

The DNA fragment, corresponding to the positions 2467-2738 of the extreme 5´of the protein E2 gene of "c-Margarita" isolate, was amplified using oligonucleotides F190 and R190. The DNA amplified was purified and used for determining the sequence of 190 nucleotides comprised between the position 2508 and 2697. Amplifications were carried out following the protocol described by Díaz de Arce *et al*. (4), in which 2 ml of the viral DNA obtained from the suspension with the virus "Margarita" and "c-Margarita" were included. Two hundred ng of the antisense oligonucleotide (R190) and 10 U of the transcriptase reverse enzyme of the Avian Myeloblastosis virus (Seikagaku America, Inc) were also added in a final volume of 100 ml. After 40 min at 42ºC, 200 ng of the sense oligonucleotide (F190) and 2,5 U of the Tag polymerase enzyme were added (Perkin-Elmer).

The primers design for the amplification of the protein E2 of the virus "Margarita", of unknown sequence, was carried out using the strain Brescia, because it belongs to its genogroup. Sequences CSFVs belonging to other genogroups were also taken into account for the identification of conserved regions among CSFVs. For amplifying "c-Margarita" virus E2 sequence, 1,5ml of RNA extracted from the virus purified were used. Reaction, in a final volume of 100ml included 200 ng of each specific primer SE2 and AE2 (Table 1). DNA was amplified by 35 cycles with the following steps: 1 min of denaturation at 94ºC, 1 min of annealing at 37ºC and 2 min of extension at 72ºC. Samples were finally incubated for 10 min at 72ºC.

c) Determination and comparison of sequences

RT-PCR products corresponding to the region 5' of the gene E2 were purified by the kit *Wizard PCR Preps system* (Promega) and sequenced using the kit *fmol DNA Sequencing System* (Promega) and the oligonucleotides F190 and R190 according to the protocol described by Díaz de Arce *et al*. (4). In this way, the sequence of 190 nucleotides was analyzed from the position 2508 to 2697 of the gene E2, corresponding to one of the regions used for phylogenetic comparison among different CSFV isolates (19).

Gene E2 sequence was determined using oligonucleotides SE2, S3a, A4b, S5 and AE2, and the sequencing equipment *ABI automated DNA sequencer* (Perkin Elmer). The results obtained were analyzed by the program *Chromas (http://www.technelysium.com.au/chromas.html).*

For comparison of sequences, the ones used were those corresponding to CSFV isolates of different genogroups, according to the classification of Lowing *et al.* (19), available in EMBL databases and the CSF World Reference Center in Hannover.

RESULTS

Growth on cell cultures

"Margarita" isolate is the one used for the CSF vaccine potency assay. This isolate (1957) was obtained by successive experimental infections from organs of a natural infected animal and never multiplied on cell cultures before (Naranjo, P. 1995, comunicación personal[1]).

To facilitate the virus amplification and to allow its biological cloning and characterization, the initial virus preparation was used to inoculate PK-15 cells.

As it is often seen with CSFV isolates, the inoculated cells did not show cytopatic effect.

However, the monolayer stain with a polyclonal hyperimmune serum against CSFV using the DIP technique, allowed the virus detection in the infected cells and a titer estimation of 10^5 $DICT_{50}$/ml. for the initial "Margarita" isolate preparation; also a biological clone was obtained by limit dilution with a titer of $10^{5,6}$ $DICT_{50}$/mL.

A not biological cloned viral suspension was also obtained after 11 passes on PK-15 cells and two passes on SK6 cells; this suspension reached a titer of $10^{6,.9}$ $DICT_{50}$/mL.

Antigenic characterization and molecular weight estimation of E2 protein

DIF and IIF results demonstrate that both, a-CSFV serum conjugated with FITC and mAb C 16/1/1-M directed to anti-NS3 protein which recognize different pestiviruses, had positive reaction against laminar antigens of "c-Margarita" and "nc-Margarita" viruses (Fig. 1). The reactivity observed was similar to the one with strains Alfort and NADL of BVDV.

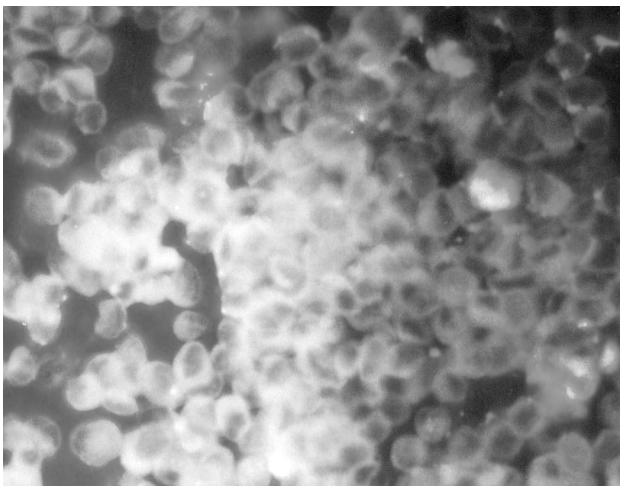

FIGURE 1. Positive immunofluorescence of PK-15 cells infected with "c-Margarita" virus. Mab anti-NS3 (pan-pestivirus) was used./ *Inmunofluorescencia positiva de las células PK-15 infectadas con el virus c-Margarita. Se empleó el Mab anti-NS3 (pan-pestivirus).*

The IIF with mAb A18, HC/34/3p1 and WH211, directed against different neutralizing epitopes of E2 from CSFV was positive when laminar antigens infected with "c-Margarita", "nc-Margarita" viruses and with Alfort strain were used. The CSFV anti-E2 mAb used did not react with NADL strain. On the contrary, the specific mAb in front of BVDV CA/3/2/22 and CA 34+1+5, reacted with NADL strains and not with Alfort strains or with "c-Margarita" and "nc-Margarita" viruses. None of the mAb used recognized the antigens prepared from non infected PK15 and MDBK cells.

The DIP technique was used to analyze the reactivity of "c-Margarita" virus and Alfort and NADL strains with mAb 21.2 (which detect field and vaccinal strains of CSFV), 44.3 (which differentiate CSFV vaccinal strains from field strains) and 63.19 (which differentiate vaccinal strains from field CSFV strains).

Both, Alfort strain and Maragarita virus showed positive reaction with mAb 21.2 and 44.3; NADL strain did not react with any of them. McAb 63.19 did not react with the three viruses analyzed.

The SDS-PAGE and Western blot results indicated that E2 protein appeared as a band of approximately 55 kDa, migrating parallel to the corresponding protein of Alfort strain, used as positive control (Fig. 2). The specificity of the reaction was confirmed by the absence of staining in the control lane charged with a protein extract of non infected PK-15 cells.

Naranjo, P. Laboratorio de control Estatal del IMV.

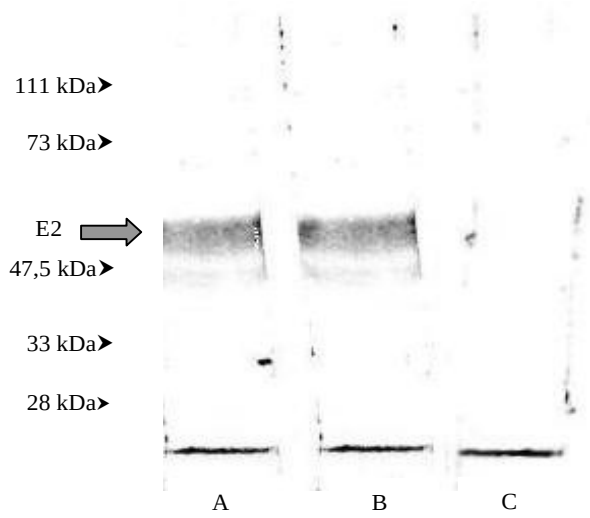

FIGURE 2. Detection of glycoproteine E2 of CSFV by immuno *Western-blot.* The results obtained with lysates of PK-15 cells are shown: A) infected with Alfort strain; B) infected with "c-Margarita" virus; C) no infected (control cells)./ *Detección de glicoproteína E2 del VPPC mediante inmuno Western-blot. Se muestran los resultados obtenidos con lisados de células PK-15: A) infectadas con la cepa Alfort; B) infectadas con el virus c-Margarita; C) no infectadas (células control).*

Genomic characterization

The targeted nucleotide sequence is variable among different virus isolates, and enough conserved to count with phylogenetic quality which makes it broadly used for CSFV sequence comparison and analysis (4, 5, 13, 18, 19).

Similarly, the corresponding sequencies from the parental "Margarita" isolate and the viral preparation grown from it were determined, during three passes in PK-15 cells, and it was used for obtaining the biological clone from which the Margarita virus came from.

Non nucleotide change was found among the three determined sequences.

With the aim to determine the phylogenetic relationship among Margarita virus and other CSFV isolates, the sequence determined was used to obtain the phylogenetic tree which is shown in Fig. 3 including the sequences corresponding to isolates representing different CSFV genogroups.

The tree topology reveals, with highly significant *boostrap* values, the broadly accepted genogroup classification for CSFV. Inside the tree, the Margarita sequence was located, with an adequate significant

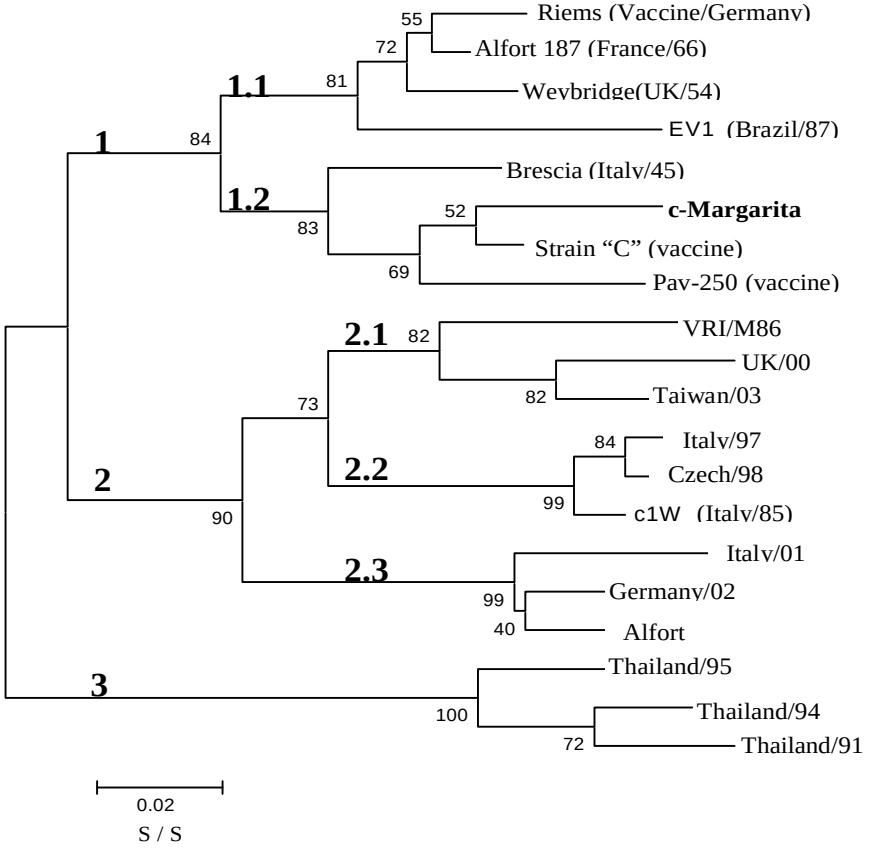

FIGURE 3. Phylogenetic tree obtained from 190 nucleotides of the gen E2 of CSFV. It incluyes "c-Margarita" virus sequences and isolates representing the three genogroups and their corresponding subgroups in black, described for CSFV (Lowing *et al.*, 1996; Paton *et al.*, 2000). */Árbol filogenético obtenido a partir de los 190 nucleótidos del gen E2 del VPPC. Incluye las secuencias del virus c-Margarita y de aislados representativos de los tres genogrupos y sus diferentes subgrupos, descritos para el VPPC (Lowing et al. 1996; Paton et al., 2000), indicados en negritas.*

level (*boostrap* value 83), in the branch including the CSFV isolates belonging to the genogroup 1.2.

Study on the culture cell multiplied Margarita virus pathogenicity in swine

Pigs of control group (inoculated with "Margarita" virus adapted to pigs) developed high temperature from the 4th day post inoculation. Animals inoculated with "c-Margarita" virus showed high temperature similarly, while the ones inoculated with 10^4 $DICT_{50}$ of "NC Margarita" virus started from the 5th day post inoculation (Fig.4). From the 7th day all animals showed anorexia, depression and other clinical signs described for CSF, such as dehydration, conjunctivitis, constipation followed by diarrhoea, nervous disorders, skin cyanosis and prostration. All animals in the control group died between the 11th and 13th day post infection. In the other groups, one pig died in each. (Fig.4). The animals which remained alive at the 14th day were slaughtered taking into account the deterioration due to the disease.

In the necropsy, typical lesions in target organs were observed in a CSF acute case (8) as marginal infarcts in the spleen, haemorrhages in lungs, kidneys and ganglia and enteritis.

The virus was isolated in PK-15 cells from the organs obtained by necropsy in every inoculated pigs. Positive results were also obtained from all the samples when its RNAs were extracted and amplified by RT-PCR using CSFV1 and CSFV2 oligonucleotides (3) corresponding with the gene codifying the non structural protein NS5B, usually used for detecting the RNA of VPPC (data not shown).

Amplification of E2 gene by RT-PCR

The amplification strategy involved all genes described by Moorman *et al.* (26), including the existing RTM at the C-terminal extreme of the molecule. The amplification of purified viral RNA by RT-PCR using SE2 and AE2 oligonucleotides, allowed the obtainment of a DNA fragment of 1361 pb. The fragment contained a restriction site in the 5´ extreme to BamH I, enzyme, the Kozak consensus sequence, an AUG codon and the 1329 nucleotides corresponding to the aa 667-1109 from the E2 gene, carrying in its 3´extreme a terminal codon of the translation followed by the sequence for the restriction enzyme XbaI.

Comparison of the protein E2 sequence of the Margarita virus.

The nucleotide sequence determined allowed to deduce the aa sequence of E2 from the "c-Margarita" virus, and to do an alignment with 15 different pestivirus strains. The sequence obtained for gene E2 from "c-Margarita" virus was deposited on the EMBL database with the access number: AJ704817.

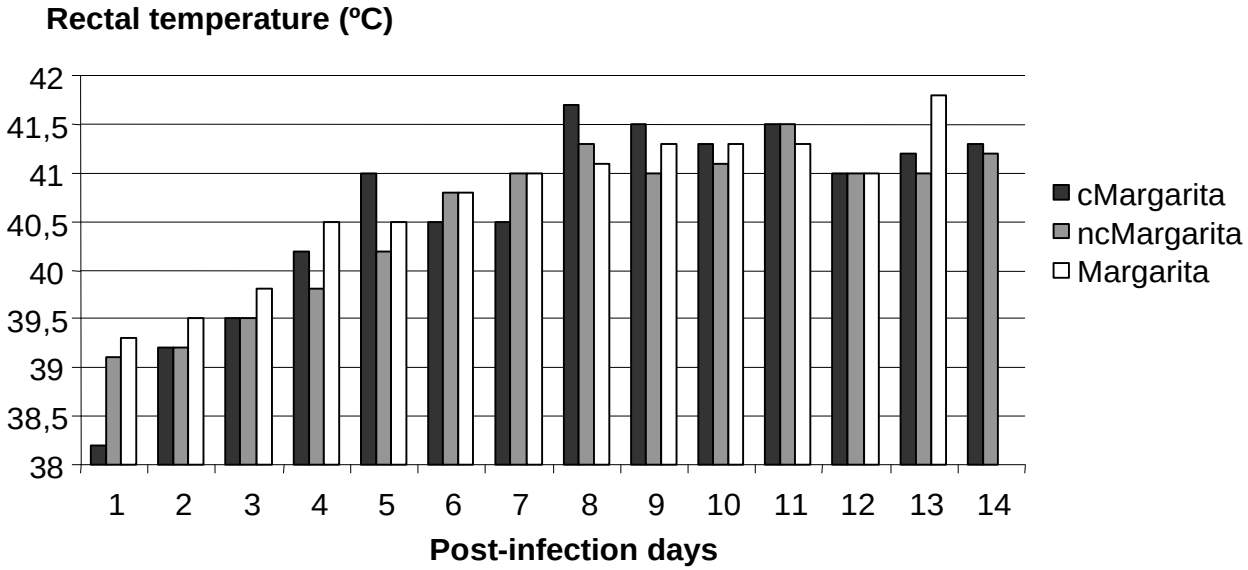

FIGURE 4. Pyrexia developed by the three groups of pigs inoculated with the different suspensions of "Margarita" virus. Each bar represents the average temperatura of each group./ *Pirexia desarrollada por los tres grupos de cerdos inoculados con las diferentes suspensiones del virus Margarita. Cada barra representa la media de temperatura corporal de cada grupo.*

As it is shown in Table 2, the E2 sequence of Brescia and Glentorf CSFV strains were, from all analyzed, the ones showing higher homology (93 %) with the "c-Margarita" virus sequence. These strains are on the CSFV genogroup 1.2. For Alfort strain, on the genogroup 2, the homology was 90%. With the Asiatic strains LPS and GSJC (genotype 3), the homology percents were 85 and 89 % respectively. The lowest homology percent was obtained with strain Giessen1 from BVDV type I (55%).

TABLE 2. Homology percentage of E2 aminoacids between "c-Margarita" virus and CSFV, BVDV and BDV./ *Porcentajes de homología de aminoácidos de E2 entre el virus c-Margarita y aislados de VPPC, VDVB y VBD*

Pestivirus strain[a]	*% Homology regarding "c-Margarita"*
Brescia	93
Glentorf	93
Alfort187	92
Weybridge	92
Shimen	92
ALD	92
GPE$^-$	92
Cepa China "C"	91
Riems	91
Alfort	90
LPC	85
GSJC[b]	89
X1818 (VEF)	68
New York (VDVBII)[c]	61
Giessen 1 (VDVB I)	55

[a] Four hundred forty three aa corresponding to the positions 667-1110 of Brescia strain were analized (Moorman *et al.,* 1990)./ *Se analizaron 443 aa correspondientes a las posiciones 667-1110 de la cepa brescia (Moorman et al., 1990).*

[b] Three hundred eighty seven aa available in EMBL database were compared. Four hundred twelve aa available in EMBL database were compared./ Se compararon los 387 aa disponibles en la base de datos del EMBL[c]. Se compararon los 412 aa disponibles en la base de datos del EMBL.

On the other hand, the aminoacid sequences deduced for the complete E2 from c-"Margarita" virus presented all the cysteine residues which are highly conserved in the pestivirus genomes being part of the different conformational epitopes of Glycoprotein E2.

DISCUSSION AND CONCLUSIONS

CSF is a serious problem for pig industry, in spite of the enormous efforts carried out during the last decades to eradicate the disease. Many factors make difficult the disease control, such as globalization and the increasing on trading and pig movements, the constantly growing pig populations with increased high-density areas and the increased wild pig populations acting as reservoir of the disease. Likewise, the emergency of the diseases with clinical signs related to porcine reproductive-respiratory syndrome (PRRS) and the syndrome of swine dermatitis and nephropathy complicate the diagnostic of the disease (25).

The control of the CSF in endemic areas is done by vaccination with attenuated virus strains, which not allow the differentiation between vaccinated and infected animals.

As complementary or alternative measure, depending on the severity of the outbreak and the epidemiological situation, eradication campaigns can be carried out through the policy of slaughtering animals around the points where the disease appears, together with the restriction of animal movements, as in the case of the EU countries, including Spain (24).

The CSF epizootic occurred in Europe in 1997 (7) was a very illustrative example of the considerable economic looses caused by the disease, mainly due to the great quantity of non infected animals slaughtered and the restriction on trading and animal movement.

The main goal approached was the growing of the "Margarita" isolate in Pk-15 cells, to facilitate its production, biological cloning and further antigenic and molecular characterization. The virus inoculation in cell monolayers did not produce detectable cytopatic effect. This situation is habitual with CSFV isolates, in which the existence of the so-called cytopatic biotype is very infrequent (21), being obtained, mainly, after several passes in cell cultures (22). Due to the lack of cytopatic effect, the virus multiplication became evident by the IIP technique using a hyperimmune serum against the virus (24). After the biological cloning by limit dilution, the recovered virus from a "c-Margarita" virus was amplified, and antigenic and genetically characterized.

The sequence amplification of CSFV by RT-PCR strategies combined with the sequencing of the products obtained allows an easy and accurate characterization of the isolates (4, 5,14, 15,18, 29). Thus, this sequence was determined for the "c-Margarita" virus amplified in cell cultures and for the parenteral virus, the "Margarita" isolate, resulting identical for both viruses.

This information allows to confirm that the "c-Margarita" virus is included in the subgroup 1.2, together with other viruses isolated in Cuba between 1993 and 1997 (4, 5).

Inside the genogroup 1.2, vaccinal strains as "C" and Pav-250 are included, and also recent isolates in Ukraine, Malasia isolates from the 80s and other CSFV strains isolated in USA and in the European continent before 1987; as it is the case of Brescia strain (28). Inside the sequences corresponding to the genogroup 2, strain Alfort and isolates from the epizooties occurred in Europe during the last years are found. (12,13).

The genogroup 3 constitutes an independent branch including the viruses isolated in Asia during the last years (27). The classification of the CSFV in three different genogroups facilitates the epidemiological studies for determining the origin and evolution of an outbreak of CSF (25), in spite of the high percentage of genetic homology that all CSFV isolates show among them (20).

As it is said, glycoprotein E2 contains the antigenic sites of CSFV recognized by neutralizing antibodies (1, 36) and it is the only protein of the virus capable of inducing the protection against the disease when it has been used in different vaccination strategies (9).

Due to the interest in using glycoprotein E2 of "c-Margarita" virus in developing a new recombinant vaccine, the antigenic characterization of this virus was carried out.

The "c- Margarita" virus was recognized by a mAb that recognizes isolates of different viruses belonging to the Genus Pestivirus (23), (37) directed against the non structural protein NS3 (more conserved among Pestiviruses). The use of mAb specifically recognizing the E2 protein from CSFV isolates, allows to confirm that "c-Margarita" virus presented equal recognizing pattern than Alfort strain, being only recognized by the specific mAb from field isolates, and not by the used specific mAb from vaccine strains.

The characterization of "c-Margarita" virus included, also, an analysis of the pathogenicity induced in pigs, after the cloning and/or amplification in PK-15 cells. The results confirmed that both, "c-Margarita" and "nc-Margarita" viruses produce acute CSF clinical signs in 6 to 8 week pigs. The virus adapted to cultures is, therefore, a highly virulent CSFV strain taking into account the classification of Mittelholzer *et al.* (22), due to the fact that all the pigs presented severe clinical signs of the disease, characterised by fever, progressive deterioration and death before the 15th day post infection.

To conclude, it is ratified the great interest of the results, and justify the use of the "c-Margarita" virus for the development of new vaccination strategies against the virus in Cuba. Besides, the possibility of using CSF virus adapted to grow in cell cultures as strain for challenge in the vaccine potency test in Cuba, is open; thus guaranteeing a higher stability and safety, simplifying the obtainment and titration of the virus.

Finally, and not less important, these results have allowed to have a complete characterized native strain to be used as reference material for different purposes either in the diagnostic or for obtaining non conventional vaccine candidates .

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