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SPREČAVANJE ŠIRENJA KLASIČNE KUGE SVINJA U POGRANIČNOM REGIONU KROZ POBOLJŠANJE SANITARNIH STANDARDA I EDUKACIJU FARMERA (STOP – KKS)

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“PREVENTION OF CLASSICAL SWINE FEVER IN THE BORDER REGIONCROATIA – SERBIA (STOP – CSF)”
„SPREČAVANjE ŠIRENjA KLASIČNE KUGE SVINjA U POGRANIČNOM REGIONU HRVATSKA – SRBIJA (STOP – KKS)“

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SEQUENCING AND TYPING OF CSFV ISOLATES FROM THE REPUBLIC OF SERBIA

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Abstract

Classical swine fever (CSF) is a globally significant disease of domestic pigs and wild boars caused by virus (CSFV). In spite of attempts to control and eradicate CSF, outbreaks of the disease keep happening and causing problems worldwide. Genetic typing of CSFV isolates can help in understanding the epidemiology of disease and trace down the source of outbreak. With the aim to gain some knowledge about the CSFV epidemiology in Serbia, genomic characterization of RT-PCR amplified products of 5'-UTR genome region of 9 CSFV strains isolated from CSF outbreaks in Serbia during 2006 was conducted. Nucleotide sequences of Serbian isolates from 2006 were aligned and phylogeneticaly analyzed with the corresponding sequences of 63 CSFV isolates and reference strains from 24 countries, as well as with 3 previously characterized CSFV isolates from Serbia, obtained from NCBI gene bank and CSFV database in Hannover. All 9 Serbian isolates from 2006 were typed as genotype 2 and subtype 2.3 of CSFV. Despite the fact that all analyzed Serbian isolates are grouped in the same virus subtype, they can be separated in 5 different groups or clusters. The relationship between these Serbian isolates and outbreaks, as well as a possible relationship between these isolates and isolates collected in other European countries were analyzed.

Keywords: CSFV; 5'-UTR; molecular typing; Serbia

Introduction

Classical swine fever (CSF) is a highly contagious, economically important and OIE notifiable viral disease of domestic pigs and wild boars. In spite of attempts to control and eradicate CSF such as the use of vaccination strategy in some countries or a strict stamping out policy as in the European Union are implemented, outbreaks of the disease keep happening and causing problems worldwide (Edwards et al., 2000; Paton et al., 2000; Jemeršić et al., 2003).

The causative agent of CSF is a member of the genus *Pestivirus* within family *Flaviviridae* (Lowings et al., 1996; Heinz et al., 2000). CSF virus (CSFV) has an enveloped virion incorporating glycosylated membrane proteins and an icosahedral nucleocapsid. The genome is a single positively polarized RNA strand of about 12300 nucleotides in length flanked by highly conserved untranslated region at either end, 5'-UTR and 3'-UTR. The genome consists of a single open reading frame (ORF) encoding about 4,000 amino acid long polyprotein which is processed into structural and nonstructural viral proteins (Meyers and Thiel, 1996). CSFV is antigenically and genetically closely related to other members of the

Pestivirus genus such as bovine viral diarrhea viruses (BVDV) and border disease viruses (BDV). These pestiviruses occasionally infect pigs but usually do not spread efficiently. Despite close similarities to BVDV and BDV, CSFV form a distinctive group that can be differentiated serologically or on the basis of genetic similarities (Paton et al., 2000).

These pestiviruses may cross-react when conventional serological and virological techniques are used. Considering that the CSF and its control have serious economic consequences, CSFV has to be rapidly identified and distinguished from other pestiviruses (Terpstra and Wensvoort, 1988; Vilček and Belak, 1996; Stadejek et al., 1997). During the 90s of the last century, RT-PCR based methods became available for pestivirus differentiation. The application of molecular methods has significantly improved the laboratory detection and characterization of CSFV. Gel based RT-PCR has been widely used for a highly specific detection and differentiation of CSFV and other pestiviruses (Lowings et al., 1994; Vilček et al., 1996; Sandvik et al., 1997). For that purpose 5'-UTR of the viral genome is frequently used as a target for detection of CSFV, since 5'-UTR is highly conserved among all pestiviruses (Meyers and Thiel, 1996). RT-PCR can detect CSFV in blood samples of the infected pigs on average 2.8 days earlier than "Gold Standard" virus isolation method (Dewulf et al., 2004). These methods are highly sensitive but they are not able to discriminate between different CSFV isolates. The ability to discriminate between CSFV strains is essential for studies of the spread of outbreaks and of virus evolution (Stadejek et al., 1997).

The nucleotide sequencing data generated by the amplified RT-PCR products of CSFV genome have been used for the comparative sequence analysis and interpretation of genetic relatedness among CSFV isolates. Genetic typing of the CSFV isolates is performed by sequencing of different regions of the viral RNA followed by phylogenetic analysis. The most frequently used gene fragments for this are the 5'-untranslated region (5'-UTR), the E2 glycoprotein and NS5B coding genes (Lowings et al., 1996; Meyers and Thiel, 1996; Stadejek et al., 1997; Becher et al., 1997; Greiser-Wilke et al., 1998; Bjorklund et al., 1999; Paton et al., 2000; Jemeršić et al., 2003). According to previous studies (Lowings et al., 1996; Stadejek et al., 1997; Greiser-Wilke et al., 1998; Paton et al., 2000) three major genetic groups of CSFV are known. Groups 1 and 2 are divided in three subgroups (1.1, 1.2, 1.3 and 2.1, 2.2, 2.3) and genetic group 3 contains four subgroups (3.1, 3.2, 3.3 and 3.4). Members of group 1 are mostly comprised of old European isolates together with old and recent American isolates and some vaccine strains, while isolates in group 2 are currently found worldwide. Isolates from group 3 are derived from different parts of Asia (Paton et al., 2000; Lowings et al., 1996; Vilček et al., 1996). The most recent CSFV outbreaks in the European Community are associated essentially with isolates that cluster in the genotype 2 (Pol et al., 2008; Leifer et al., 2010).

In Serbia the disease is endemic for more than 20 years. During that period disease outbreaks were observed in almost all parts of the country, but the epidemiological studies do not clearly demonstrate the relationships between the outbreaks in the different regions. The vaccination policy, as state funded program with the C-strain vaccine, with the exception of few years, was introduced few times to control and eradicate CSFV during these years, and it is still ongoing. The last

CSFV outbreak was in 2010 when the disease occurred on one commercial pig farm and in a few backyards in the village near that farm. The stamping out method was used and the disease was stopped in about 3 weeks. More than 8000 pigs were killed during the eradication.

As a step ahead in understanding the CSFV epidemiology in Serbia, the aim of this study was to characterize field viruses that were isolated in Serbia during 2006 by molecular typing, to map the spread of CSFV and to analyze the relationship between these isolates, as well as to find a possible relationship between these isolates and isolates collected in other European countries. The present paper describes the genomic characterization of RT-PCR amplified products of 5'-UTR genome region of 9 CSFV strains isolated from CSF outbreaks in Serbia during 2006.

Materials and methods

CSFV isolates

CSFV strains were isolated from blood and/or tissues samples of succumbed or euthanized pigs obtained during the outbreaks of disease on northern part of Serbia in 2006. Data about the name of isolates, geographic origin, date of samples reception for analysis, and type of pig production where disease outbreak happened is presented in Table 1. CSFV positive samples were obtained from 4 epizootiologic area (district) in Serbia (Severnobački, Južnobački, Sremski and Srednjebanatski). The CSF viruses were isolated on PK15 cell line, but for molecular characterization both, isolated virus strains from cell culture and viruses in original tissue samples were used in parallel, to exclude any possibility of cross contamination or virus mutation during passage on cell culture.

Table 1: Data on the Serbian CSFV isolates that were obtained during 2006

No.	CSFV isolate name	Epizootiologic area	Municipality	Settlement	Date of reception	Type of pig prod.*
1.	234/06	Sremski	Stara Pazova	Vojka	17.01.06.	B
2.	1058/06		Indjija	USAOJ	16.02.06.	F
3.	6287/06	Južnobački	Novi Sad	Stepanovićevo	08.09.06.	B
4.	6168/06				04.09.06.	B
5.	6263/06		Temerin	Temerin	07.09.06.	B
6.	6250/06		Bač	Plavna	07.09.06.	B
7.	1533/06		B. Petrovac	B. Petrovac	02.03.06.	B
8.	5885/06	Severnobački	Senta	Senta	18.08.06.	B
9.	5882/06	Srednjebanatski	Zrenjanin	Žitište	21.08.06.	B

B – Backyard pigs – extensive pig production
F – Industrial – Farm pig production

RNA extraction

Total RNA was extracted directly from 250 µl of 10% suspension of tissues samples (tonsil, spleen, limphonodes and kidney) in PBS from dead and euthanized moribund animals, and from supernatants of tissue cultures infected with third

passage of CSFV isolates. RNA extraction was performed using TRIzol® reagent (Invitrogen) according to the manufacturer recommendations. Briefly, 750 µl of TRIzol® reagent was mixed with 250 µl of sample. After 10 min. 200 µl of chloroform was added, mixed and the suspension was centrifuged for 15 min at 14000g at 4 °C. The RNA containing aqueous phase was removed and precipitated with 500 µl of isopropanol, maintained at room temperature for 10 min, and centrifuged for 10 min at 14000g. The RNA pellet was washed with 500 µl of cold 75% ethanol, centrifuged for 5 min at 12000g, dried, and resuspended in 40 µl of diethyl pyrocarbonate (DEPC) treated water and stored at < - 60 °C until examination or was immediately included in RT-PCR.

RT-PCR

Amplification of CSFV nucleic acid in samples was done by RT-PCR, following the procedures described by Barlič-Maganja and Grom (2001) and Toplak (2002), which targeted the 5'-UTR of the viral genome. The "one-step RT-PCR" assay was performed by using reagents supplied in a commercial "Access RT-PCR system", (Promega Corporation, UK) according to the manufacturer`s instruction. A highly conserved sequence within 5'UTR of the CSFV RNA was amplified by panpestivirus primers P1U (sense) and Pest2-L (antisense) described by Barlič-Maganja and Grom (1999) (Table 2). In reactions, a 6µl of RNA sample was added to a 44µl reaction mixtures containing AMV/*Tfl* 1 x reaction buffer, dNTP mix (10mM), 25 pmol of sense and antisense primer, 1 mM of $MgSO_4$, 5 U of AMV RT and 5 U of *Tfl* DNA polymerase. The RT-PCR cycling conditions were as follows, 45 min at 48 °C for RT, 94 °C for 2 min for AMV RT inactivation and RNA/cDNA/ primer denaturation, 40 cycles of 30 s at 94 °C, 1 min at 60 °C and 2 min at 68 °C, and a final extension step at 68 °C for 7 min. Amplified products were visualized on 1.5% agarose gel stained with ethidium bromide.

Table 2. Details of oligonucleotide primers used for RT-PCR

Primer*	sequence (5' - 3')	genome position**	size and location
P1-U [a]	AGA GGC TAG CCA TGC CCT TAG T	79-100	294
Pest2-L [b]	TCA ACT CCA TGT GCC ATG TAC	353-373	5'-UTR

[*] described by Barlič-Maganja and Grom, 1999

** according to the genome position of Alfort/Tubingen - Stadejek et al., 1997

[a] modified after Boye *et al.*, 1991; [b] similar to the 326 primer described by Vilček *et al.*, 1994

Sequencing of 5`-UTR

DNA bands, of the calculated sizes, were excised from the gels, purified using Wizard PCR prep chemistry and spin columns (Promega, USA) following the manufacturer's instructions. The purified PCR products were used as templates in direct cycle sequencing reactions primed with internal primers for 5'UTR described in Table 3, and terminated by fluorescently labelled dideoxynucleotides (BigDye Terminator Cycle Sequencing Kit, PE Biosystems, USA). All PCR products were sequenced in both directions. The resulting products were analysed on an automated nucleic acid analyzer (ABI PRISM 310 Genetic Analyzer, PE Biosystems, USA).

Table 3. Details of oligonucleotide primers used for sequencing reactions

primer[a]	sequence (5' - 3')	genome position*	size and location
104F	GCTAGCCATGCCCTTAGTAGGACT	83-106	289 bp
402R	CAACTCCATGTGCCATGTACAGCA	349-372	5'-NCR

[a] Barlič-Maganja i Grom (2001)

* according to the genome position of Alfort/Tubingen

Phylogenetic analysis

Nucleotide sequence comparisons and phylogenetic analysis of the 5'-UTR were done with DNASTAR program package (DNASTAR Inc., USA). Nucleotide sequences were assembled and proof read using the SeqMan™II and EditSeq programs. After assembly of consensus sequences, 150 nucleotide long regions of Serbian isolates were aligned using the MegAlign™ and ClustalW program (Thompson et al., 1994) with corresponding sequences of 66 CSFV isolates and reference strains obtained from NCBI GenBank (http://www.ncbi.nlm.nih.gov/) and from CSFV Community Reference laboratory (CRL) database in Hanover (http://viro08.tiho-hannover.de) (Greiser-Wilke et al., 2000). The obtained sequences belongs to the CSFV strains originated from 24 countries (4 from Austria, 2 from Hungary, 2 from Slovak Republic, 2 from Czech Republic, 2 from Switzerland, 2 from United Kingdom, 2 from France, 13 from Germany, 1 from Belgium, 3 from Romania, 2 from Bulgaria, 7 from Croatia, 1 from Bosnia and Herzegovina, 1 from Spain, 1 from The Netherlands, 4 from Italy, 1 from Poland, 1 from Estonia, 2 from USA, 1 from China, 2 from Honk Kong, 2 from Japan, 2 from Malaysia, and 3 from Korea, as well as with 3 previously sequenced (1999) strains from Serbia), representing the most of the currently known genetic subgroups of CSFV. Details of those CSFV strains are presented in Table 4. The phylogenetic tree was constructed using neighbour – joining method based on bootstrap of 1000 replicates (using MEGA version 4).

Results and Discussion

The alignment of 150 bases long 5'-UTR nucleotide sequences of 9 Serbian isolates (Table 1) and corresponding sequences of 66 reference strains and isolates from different and surrounding countries (Table 4) are performed. The 5'-UTR region of CSFV genome was already previously described and confirmed as very sensitive and suitable for detection as well as for a genetic typing of the CSFV isolates (Hofmann et al., 1994; Vilček et al., 1996; McGoldrick et al., 1998). For more accurate typing of CSFV isolates from Serbia, the representatives of all known genotypes and most common subtypes of CSFV taken from the gene banks (NCBI GenBank and CSFV CRL database from Hannover) were included in molecular typing and phylogenetic analysis (Table 4). Special attention was paid to isolates from neighbouring countries (Austria, Hungary, Slovakia, Czech Republic, Romania, Bulgaria, Croatia and Bosnia and Herzegovina). Since vaccination policy is still ongoing in Serbia and was also present during 2006, the China strain (AY805221) is also included in analysis. It was already confirmed that the Chine strain from attenuated vaccine „Kilapin", that is in use in Serbia, belongs to 1.1

CSFV subtype and that is in 254 nucleotides long 5'-UTR sequence 100% similar to the China strain AY805221 (data is not shown).

Table 4: Details of 66 CSFV strains that was included in genetic typing with Serbian CSFV isolates from 2006.

Name of the virus strain	NCBI or Genebank Hanover*	Sub type	Country / Year of isolation	Name of the virus strain	NCBI or Genebank Hanover*	Sub type	Country / Year of isolation
Alfort-Tuebingen	J04358	2.3	Germany	Novska 5-02	CSF0821	2.3	Croatia 2002
Switzerland 1 /93	AF045068	2.3	Switzerland 1993	Nadas Zarand-04	CSF0837	2.3	Romania 2004
V 694	CSF0005	2.3	Germany 1984	Zalau 1/ 2004	CSF0846	2.3	Romania 2004
V 744	CSF0007	2.3	Germany 1984	4944-06	CSF0852	2.3	Bulgaria 2006
SF 86/8	CSF0009	2.3	GrBritain 1987	V21-2	XXX0246	2.3	Croatia 2006
Spreda/Han94	CSF0113	2.3	Germany 1994	Sch 194	CSF0020	2.2	Germany, 1989
1042/92	CSF0085	2.3	Hungary 1992	JIHLAVA 97325-91	CSF0076	2.2	Czech R 1991
1043/92	CSF0086	2.3	Hungary 1992	SP 4985/2	CSF0122	2.2	Austria 1994
Wingene 93/358	CSF0099	2.3	Belgium 1993	5089 VA/97	CSF0400	2.2	Italy 1997
Kaernten/95 933	CSF0126	2.3	Austria 1995	Fukushima-80	AB019661	2.2	Japan
V 1381/94	CSF0219	2.3	Germany 1994	Paderborn	AY072924	2.1	Germany 1997
D4889 I/82/NA	CSF0279	2.3	Italy 1982	Switzerland 2 /93	AF045069	2.1	Switzerland 1993
518/94	CSF0300	2.3	Estonia 1994	SP 10549/13	CSF0150	2.1	Austria 1993
2/1	CSF0317	2.3	Poland 1991	VRI 2277	CSF0305	2.1	Malaysia 1986
2101/8	CSF0333	2.3	Germany 1997	NLB3	CSF0361	2.1	Espana 1997
3823/97	CSF0369	2.3	Czech R. 1997	S-168	CSF0669	2.1	Croatia 1997
V 2104/95	CSF0437	2.3	Germany 1995	18-Cr	CSF0670	2.1	Croatia 1997
NWS 3581/95	CSF0464	2.3	Germany 1995	Lelystad	CSF0938	2.1	Netherlands 1992
Runow1	CSF0523	2.3	Germany 1998	KP2002N4	DQ452388	2.1	Korea 2002
25668/98	CSF0527	2.3	Slovakia 1998	Alfort-187	X87939	1.1	France 1968
29	CSF0577	2.3	France 1997	Baker A	CSF0932	1.1	USA
3-4/99	CSF0608	2.3	Bulgaria 1999	Eystrup	AF326963	1.1	Germany 1964
CSFV p3Debeljača	CSF0615	2.3	Serbia 1999	331	CSF0918	1.1	USA 1969
CSFV p3 Niš	CSF0616	2.3	Serbia 1999	Glentorf	U45478	1.1	Germany 1968
CSFV p3 Vrsac	CSF0617	2.3	Serbia 1999	C-strain (China)	AY805221	1.1	China
24/Cr	CSF0672	2.3	Croatia	Brescia	M31768	1.2	Italy 1945
1180/98	CSF0722	2.3	Slovakia 1998	VRI 4167	CSF0306	1.3	Malaysia 1986
Sp1795	CSF0733	2.3	Austria 2000	97-7446/4	CSF0643	1.3	HN 1996
VA 2046	CSF0734	2.3	Italy 2000	HCV 31	CSF0653	1.3	HN 1992
ARAD	CSF0800	2.3	Romania 2002	Kongenital tremor	CSF0410	3.1	Gr. Britain 1964
Tomislavgrad BiH	CSF0817	2.3	Bosnia 2003	JJ9811	XXX0095	3.2	Korea
Nova Gradiska-02	CSF0818	2.3	Croatia 2002	NS9811	XXX0096	3.2	Korea
Novska-02	CSF0819	2.3	Croatia 2002	Kanagawa	CSF0309	Outg	Japan 1974

* Greiser-Wilke et al., 2000 (http://viro08.tiho-hannover.de)

The results of sequencing and phylogenetic analysis of Serbian CSFV isolates in 5'-UTR genome region is presented in phylogenetic tree (Fig. 1). The results show that all analysed CSFV isolates from Serbia belongs to CSFV genetic

group 2 and into subgroup 2.3. Representatives of this virus subtype appeared in Europe at the end of the last and in the beginning of this century. This subtype is highly prevalent in Europe, particularly in central and southern area of the continent and is the most frequently detected virus subtype in Europe in the last 20 years. Despite the fact that all analyzed Serbian isolates are grouped in the same virus subtype and the similarities that are found with the isolates from other European countries, certain differences are also observed, as well as connections between isolates in relation to the sources and spread of infection.

Regarding the differences found between Serbian isolates, they can be separated in different groups or clusters (Fig. 1). If we take into account the earlier sequenced isolates CSFV p3 Nis, CSFV p3 Vrsac and CSFV p3 Debeljača (taken from the CSFV database in Hanover), all analyzed Serbian CSFV isolates from 2006 can be divided into 5 clusters. Belonging to a particular group or cluster, directly define the molecular epidemiology characteristics of isolates, and define their origin, directions of spreading, and the linkages between individual outbreaks. For example, isolates 234/06, 5882/06, 6263/06 and 1058/06, as well as previously typed isolate CSFV p3 Niš (detected in 1999) can be grouped in one cluster with 100% nucleotide identity. This data suggests that their genomic sequences are very similar, even identical to each other, which undoubtedly indicates their connection. On that basis we can assume with great probability that an outbreak of classical swine fever in the areas of municipalities Inđija (isolate 1058/06), Zrenjanin (isolate 5882/06) and Temerin (isolate 6263/06) was associated with the outbreak of classical swine fever in the area of Stara Pazova (isolate 234/06 - the first detected one), and that the source of infection is the same for all mentioned places. The fact that CSFV isolate p3 Niš detected in 1999 is in the same cluster indicates that the virus of such genetic characteristics at least six years circulate in Serbia. From epidemiological point of view, in the past all of the mentioned isolates in that cluster have the same origin.

As could be clearly seen from the phylogenetic tree, those isolates from Serbia are very similar or even identical with some isolates from Croatia, Bosnia and Herzegovina, Hungary, Romania, Austria and Switzerland, and only partly related to some isolates from Slovakia and the Czech Republic. The analyzed viruses from these countries are isolated during 90s of the last century, and from 2000 to 2006, which directly points to their common origin with Serbian isolates and spreading of the infection in the past, as well as to the connection of classical swine fever outbreaks in Serbia and those from neighboring countries.

The strain CSFV p3 Vrsac from 1999 (sequence taken from the CSFV database in Hanover) is at the same position in the phylogenetic tree where the first cluster is, but on the separate branch. This finding points to a different nucleotide sequence and different origin of isolate CSFV p3 Vršac compared to the other analyzed isolates from Serbia, and compared to isolates from other European countries, whose sequences were analyzed in this study. This indicates that the CSFV p3 Vrsac is possibly the representatives of the second CSFV cluster in Serbia.

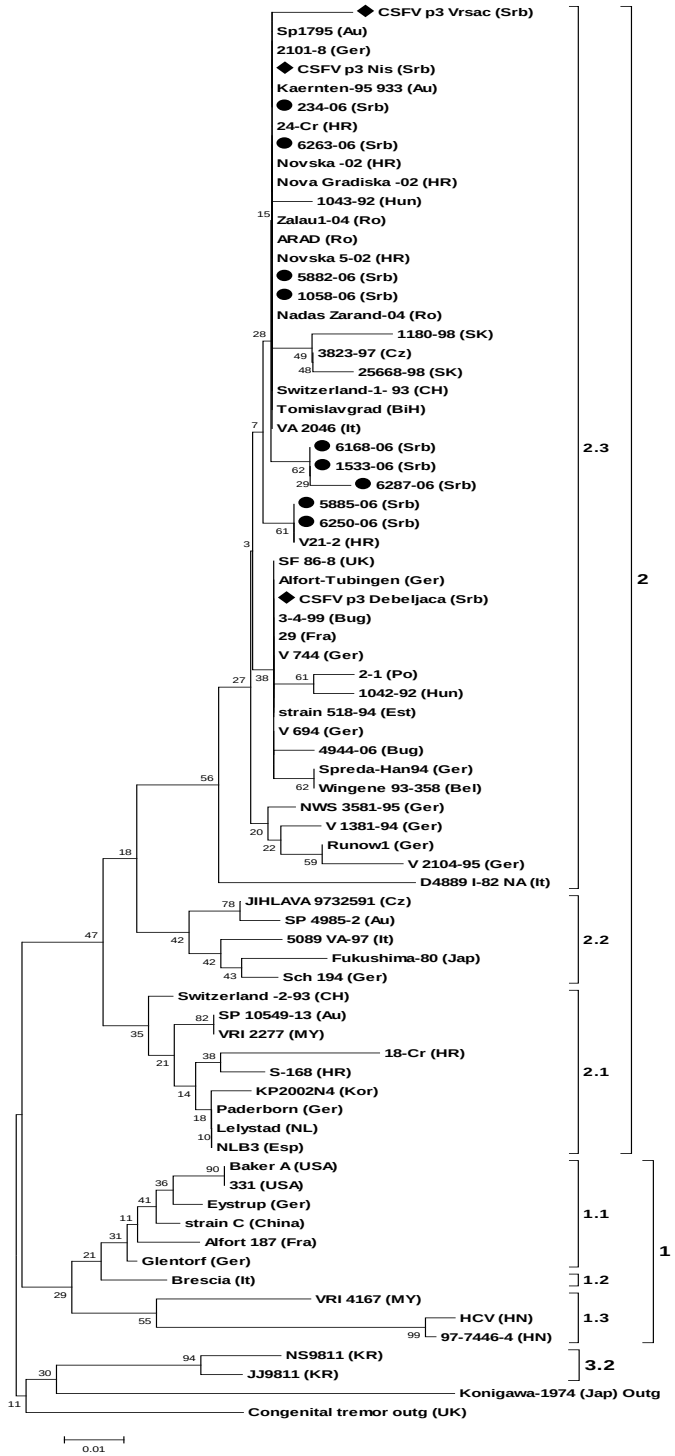

Fig. 1: Phylogenetic tree constructed from 150 long 5'-UTR nucleotide sequences of 9 Serbian CSFV isolates from 2006 (marked with black circle) and 63 nucleotide sequences of CSFV isolates and reference strains from 24 counties and 3 previously typed isolates from Serbia (marked with black rhombus) obtained from NCBI GenBank and from CSFV Reference laboratory database in Hanover.

The tree was constructed using neighbour – joining method based on bootstrap of 1000 replicates (using MEGA version 4). The scale beneath the tree measures the distance between sequences.

Isolates 1533/06 from area of settlement Bački Petrovac and isolates 6168/06 and 6287/06 from area of settlement Stepanovićevo can be classified in the third CSFV cluster in Serbia pointing to the fact that outbreaks of classical swine fever in these two settlements were connected and at the same time not associated with other outbreaks of classical swine fever in Serbia during 2006. Isolates 1533/06 and 6168/06 have identical nucleotide sequence but isolate 6287/06 differs from them though it is on the same branch of the tree. This data indicates that in the settlement Stepanovićevo in 2006 there was at least two sources of infection, one of which is connected with those from Bački Petrovac, while the other source of infection is unknown. None of the isolates from this cluster shows any higher similarity to the other analyzed isolates from Serbia or to the analyzed isolates from other countries in the region and beyond. This fact indicates that this viral cluster had in a while a separate evolution in Serbia.

Isolates 5885/06 from area of settlement Senta and isolates 6250/06 from the area of settlement Bač can be classified to the fourth CSFV cluster in Serbia. Their genomic sequences were identical to each other indicating the spread of CSFV infection from area of Senta to the area of settlement Bač. Also, their sequences were identical to the sequence of the V21-2 virus that was isolated in Croatia in 2006, indicating a strong connection between CSFV outbreaks in these two countries during 2006. Besides the similarity to the isolate V21-2 from Croatia, higher similarity of these isolates with other isolates in other countries was not observed. Since higher similarity of these strains with the other Serbian isolates from 2006 and 1999 was not found, it could be assumed that this is either a virus originating in Croatia or, like in the previous case, the viral cluster which had a separate evolution for some period in Serbia.

The only representative of the possible fifth cluster in Serbia is CSFV isolate p3 Debeljača from 1999, whose sequence was taken from the CSFV database in Hanover. This strain is completely different from all other isolates from Serbia (located on separate branch), pointing to its highest distinction. This strain is not similar to any of the isolates from 2006 and 1999 from Serbia, but is similar to some of the analyzed isolates from France, Estonia, Germany, Bulgaria, Great Britain and Poland isolated in the last 20 years indicating their common origin. Particularly interesting is its similarity to the sequence of isolate 3-4-99 from Bulgaria from the same year. This fact indicates the high probability of connection between the outbreak of classical swine fever in Serbia and Bulgaria in 1999. Since isolate CSFV p3 Debeljača differs from the other analyzed Serbian CSFV isolates from 1999 and 2006, there is a possibility that the origin of this virus isolate is from Bulgaria, or it is a virus strain with longer separate evolution in Serbia or even is it a virus which in the past infected animals in both countries. Currently we cannot give the correct answer to this question due to the relatively limited number of analyzed isolates from Serbia and Bulgaria from 1999.

Besides all previously mentioned, it should be stressed that none of the analyzed Serbian CSFV isolates was similar to the China vaccine strain that is in use in Serbia for the control of CSFV.

Though the present study is a preliminary epidemiological investigation involving only a few CSFV isolates, it provides a piece of information about the possible genotypes and subtypes of CSFV that was recently present in Serbian pig population. Conducted data analysis and given explanations and assumptions were, except performed laboratory and molecular-epidemiology tests, enabled also with a large number of previously typed classical swine fever viruses and works on this subject (Vilček et al., 1996; Stadejek et al., 1997, Paton et al., 2000, Edwards et al., 2000; Biagetty et al., 2001; Jemeršić et al., 2003). Further analysis, as well as the answers on many open questions of origin, connection and diversity of CSFV isolates from Serbia will be enabled with the examination of numerous virus isolates from past and recent years.

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SEKVENCIONIRANJE I TIPIZACIJA CSFV IZOLATA IZ REPUBLIKE SRBIJE

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Kratak sadržaj

Klasična kuga svinja (CSF) je značajno oboljenje domaćih i divljih svinja od globalnog značaja koje je izazvano virusom (CSFV). Uprkos pokušajima da se kontroliše i iskoreni CSF, epizootije bolesti se i dalje dešavaju uzrokujući probleme širom sveta. Genetska tipizacija izolata CSFV može pomoći u razumevanju epizootiologije bolesti i pratiti epizootiju do izvora infekcije. U cilju razumevanja epizootiologije klasične kuge svinja u Srbiji izvršena je genetska karakterizacija umnoženih RT-PCR produkata 5'-UTR dela genoma 9 izolata virusa izolovanih u slučajevima izbijanja CSF u Srbiji tokom 2006. godine. Nukleotidne sekvence CSFV izolata iz Srbije iz 2006. godine su upoređene i filogenetski analizirane sa odgovarajućim sekvencama 63 izolata i referentnih sojeva CSFV iz 24 države, kao i sa 3 predhodno tipizirana izolata CSFV iz Srbije, preuzetih iz banke gena NCBI i baze podataka virusa CSF u Hanoveru. Svih 9 srpskih izolata CSFV iz 2006. godine su tipizirani kao CSF virusi genotipa 2 i podtipa 2.3. I pored činjenice da su se svi analizirani izolati iz Srbije grupisali u isti podtip virusa, oni se mogu podeliti u 5 različitih grupa, odnosno klustera. U radu je analiziran odnos između izolata virusa CSFV iz Srbije i izbijanja bolesti, kao i moguća povezanost između ovih izolata i izolata CSFV iz drugih evropskih zemalja.

Ključne reči: CSFV, 5'-UTR, molekularna tipizacije, Srbija

Uvod

Klasična kuga svinja (CSF) je visoko kontagiozno i ekonomski značajno virusno oboljenje domaćih i divljih svinja koje je obavezno za prijavljivanje medjunarodnoj kancelariji za epizootije (OIE). I pored pokušaja kontrole i eradikacije CSF kao što je korišćenje strategije vakcinacije u nekim zemljama ili striktna regulativa *stamping out* metoda koja se primenjuje u Evropskoj Uniji, izbijanja bolesti se i dalje dogadjaju uzrokujući probleme širom sveta (Edwards i sar., 2000; Paton i sar., 2000; Jemeršić i sar., 2003).

Uzročnik CSF je virus koji spada u rod *Pestivirus* i u familiju *Flaviviridae* (Lowings i sar., 1996; Heinz i sar., 2000). CSF virus (CSFV) se sastoji od virusnog omotača u koji su ugradjeni glikolizovani membranski proteini i ikozaedralnog nukleokapsida. Genom virusa predstavlja jednolančana pozitivno orjentisana RNK dužine od oko 12300 nukleotida koja sa obe strane ima visoko kozervisane netranslatorne regione, 5'-UTR i 3'-UTR. Genom se sastoji od samo jednog rama za

očitavanje (open reading frame - ORF) koji kodira sintezu jednog poliproteina od oko 4000 aminokiselina koji se postranslatorno cepa u više strukturnih i nestrukturnih proteina (Meyers i Thiel, 1996). CSFV je antigeno i genetski veoma blizak sa drugim predstavnicima roda *Pestivirus* kao što je virus govedje virusne dijareje (BVDV) i virus Border bolesti ovaca (BDV). Ovi pestivirusi povremeno inficiraju svinje ali se najčešće ne šire efikasno na taj način. I pored velike sličnosti sa BVDV i BDV, CSFV formira odvojenu grupu virusa koja se može diferencirati serološki ili na bazi genetskih sličnosti (Paton i sar., 2000).

Ovi pestivirusi mogu unakrsno reagovati kada se koriste konvencionalne serološke i virusološke tehnike. S obzirom da CSF i njena kontrola imaju veoma ozbiljne ekonomske konsekvence, CSFV se mora brzo identifikovati i razlučiti od drugih pestivirusa (Terpstra i Wensvoort, 1988; Vilček i Belak, 1996; Stadejek i sar., 1997). Metode za diferencijaciju pestivirusa bazirane na reverznoj transkripciji – lančanoj reakciji polimeraze (RT-PCR) su postale dostupne 90-tih godina prošlog veka. Uvodjenje molekularnih metoda je značajno unapredilo laboratorijsku detekciju i karakterizaciju CSFV. Klasičan RT-PCR metod baziran na detekciji produkata na gelu je široko upotrebljavan za visoko specifičnu detekciju i diferencijaciju CSFV i drugih pestivirusa (Lowings i sar., 1994; Vilček i sar., 1996; Sandvik i sar., 1997). U tu svrhu je, kao ciljni region za CSFV detekciju, najčešće korišćen netranslatorni (nekodirajući) region genoma sa njegovog 5' kraja (5'-UTR), s obzirom da je 5'-UTR visoko konzervisan kod svih pestivirusa (Meyers i Thiel, 1996). RT-PCR može detektovati CSFV u uzorcima krvi inficiranih svinja u proseku 2,8 dana pre nego metoda izolacije virusa koja predstavlja tzv "zlatni standard" (Dewulf i sar., 2004). Pomenuti molekularni metodi su visoko osetljivi ali oni ne omogućavaju razlikovanje izmedju pojedinih isolata CSFV. Mogućnost utvrdjivanja razlika izmedju sojeva CSFV je neophodna u istraživanjima širenja virusa, epizootija i evolucije virusa (Stadejek i sar., 1997).

Podaci dobijeni sekvencioniranjem umnoženih RT-PCR produkata genoma CSFV su korišćeni u uporednim analizama nukleotidnih sekvenci i interpretaciji genetske sličnosti i zavisnosti izmedju CSFV izolata. Genetska tipizacija izolata CSFV je sprovodjena sekvencioniranjem različitih regiona RNK genoma uz naknadnu filogenetsku analizu. Najčešće korišćeni delovi genoma u ovu svrhu su 5'-nekodirajući region (5'-UTR), deo genoma koji kodira sintezu glikoproteina E2 i NS5B kodirajući geni (Lowings i sar., 1996; Meyers i Thiel, 1996; Stadejek i sar., 1997; Becher i sar., 1997; Greiser-Wilke i sar., 1998; Bjorklund i sar., 1999; Paton i sar., 2000; Jemeršić i sar., 2003). Prema predhodnim istraživanjima (Lowings i sar., 1996; Stadejek i sar., 1997; Greiser-Wilke i sar., 1998; Paton i sar., 2000) poznate su tri glavne genetske grupe (genotipovi) CSFV. Grupa 1 i 2 su dalje podeljene u tri genetska podtipa (1.1, 1.2, 1.3 and 2.1, 2.2, 2.3), a genetske grupa 3 ima četiri podtipa (3.1, 3.2, 3.3 and 3.4). Pripadnici genotipa 1 su najčešće stari evropski izolati, kao i stari i skorašnji amarički izolati i neki vakcinalni sojevi, dok se izolati virusa genotipa 2 trenutno mogu naći širom sveta. Izolati genotipa 3 potiču iz različitih delova Azije (Paton i sar., 2000; Lowings i sar., 1996; Vilček i sar., 1996). Najskorije epizootije CSFV u Evropskoj Uniji su suštinski povezane sa izolatima koji se svrstavaju u genotip 2 (Pol i sar., 2008; Leifer i sar., 2010).

U Srbiji je oboljenje endemskog karaktera više od 20 godina. Tokom tog perioda epizootije bolesti su utvrdjene u skoro svim krajevima zemlje ali

epizootiološka istraživanja nisu bila u mogućnosti da jasno demonstriraju odnos i zavisnost izmedju epizootija u različitim regionima. Upotreba vakcinacije sa Kina sojem vakcinalnog virusa (C-soj), kao deo državno finansiranog programa, sa izuzetkom u nekoliko godina, je u nekoliko navrata korišćen metod za kontrolu i eradikaciju CSFV i još uvek se primenjuje. Poslednja epizootija je bila 2010. godine kada se oboljenje pojavilo na jednoj komercijalnoj farmi svinja i u nekoliko dvorišta u selu u blizini farme. Bolest je zaustavljena *stamping out* metodom u roku od 3 nedelje. Preko 8000 svinja je neškodljivo uklonjeno tokom eradikacije.

Kao korak napred u razumavanju epidemiologije CSFV u Srbiji, cilj ovog ispitivanja je bio karakterizacija terenskih virusa koji su bili izolovani u Srbiji tokom 2006 godine putem molekularne tipizacije, mapiranje širenja CSFV i analiza odnosa i povezanosti izmedju ovih izolata, kao i utvrdjivanje moguće povezanosti ovih izolata sa izolatima utvrdjenim u drugim Evropskim zemljama. U ovom radu je predstavljena genetska karakterizacija RT-PCR metodom umnoženih produkata 5'-UTR regiona genoma 9 CSFV sojeva izolovanih u epizootijama CSF u Srbiji tokom 2006. godine.

Materijal i metodi rada

CSFV izolati

CSF virusi su izolovani iz uzoraka krvi i/ili tkiva uginulih ili eutanaziranih svinja uzorkovanih tokom epizootija bolesti na području severnog dela Srbije tokom 2006. godine. Podaci vezani za naziv izolata, njegovo geografsko poreklo, datum prijama uzorka za ispitivanje, kao i način uzgoja svinja u kojima se pojavila bolest su prikazni u Tabeli 1. Uzorci pozitivni na prisustvo CSFV su prikupljeni sa 4 epizootiološka područja u Srbiji (Severnobački, Južnobački, Sremski i Srednjebanatski okrug). CSF virusi su izolovani na kulturi ćelija PK15 ali su u molekularnu karakterizaciju paralelno bili uključeni i izolovani virusi sa kulture ćelija, kao i originalni uzorci tkiva iz kojih su ti virusi izolovani, da bi se isključila bilo kakva mogućnost unakrsne kontaminacije ili virusne mutacije tokom subkultivacije na kulturi ćelija.

Table 1: Podaci o CSFV izolatima iz Srbije izolovanih tokom 2006. godine

Br.	Naziv CSFV izolata	Epizootiološko područje	Opština	Naseljeno mesto	Datum prijema	Tip proizvod. svinja*
1.	234/06	Sremski	Stara Pazova	Vojka	17.01.06.	B
2.	1058/06		Indjija	USAOJ	16.02.06.	F
3.	6287/06	Južnobački	Novi Sad	Stepanovicevo	08.09.06.	B
4.	6168/06				04.09.06.	B
5.	6263/06		Temerin	Temerin	07.09.06.	B
6.	6250/06		Bač	Plavna	07.09.06.	B
7.	1533/06		B. Petrovac	B. Petrovac	02.03.06.	B
8.	5885/06	Severnobački	Senta	Senta	18.08.06.	B
9.	5882/06	Srednjebanatski	Zrenjanin	Žitište	21.08.06.	B

B – Dvorišni način držanja – ekstenzivna proizvodnja svinja

F – Industrijski – Farmska proizvodnja svinja

Ekstrakcija RNK

Kompletna RNK je estrahovana direktno iz 250 µl 10% suspenzije uzoraka tkiva (tonzile, slezina, limfni čvorovi i bubreg) u PBS-u poreklom od uginulih ili eutanaziranih životinja ii z supernatanta cultura ćelija inficirane sa trećom pasažom CSFV izolata. Ekstrakcija RNK je vršena upotrebom TRIzol® reagensa (Invitrogen) po uputstvu proizvodjača. Ukratko, 750 µl TRIzol® reagensa je pomešano sa 250 µl uzorka. Nakon 10 min. dodato je 200 µl hloroforma, promešano i centrifugirano tokom 15 min na 14000g na 4 °C. Gornja vodena faza koja je sadržavala RNK je pažljivo odvojena u nove mikrotube i precipitirana sa 500 µl izopropanola na sobnoj temperaturi tokom 10 min. i naknadno centrifugirana 10 min na 14000g. Talozi (pellet) RNK su ispirani sa 500 µl hladnog 75% etanola, centrifugirani 5 min na 12000g, osušeni i resuspendovani u 40 µl vode tretirane dietil pirokarbonatom (DEPC) i skladišteni/čuvani na < - 60 °C do ispitivanja ili su odmah uključivani u RT-PCR.

RT-PCR

Umnožavanje nukleinskih kiselina CSFV u uzorcima je izvedeno pomoću RT-PCR metode sa 5'-UTR delom virusnog genoma kao ciljnim delom umnožavannja, po proceduri opisanoj od strane Barlič-Maganje i Groma (2001) i Toplaka (2002). Jednostepani tzv "one-step RT-PCR" test je izvodjen korišćenjem reagenasa iz komercijalnog kita "Access RT-PCR system" (Promega Corporation, UK), po uputstvu proizvodjača. Visoko konzervisana sekvenca u okviru 5'-UTR dela CSFV RNK je umnožena pomoću panpestivirusnih oligonukleotidnih prajmera P1U (*sense*) i Pest2-L (*antisense*) opisanih ood strane Barlič-Maganje i Groma (1999) (Tabela 2). U reakciji je 6µl uzorka estrahovane RNK dodavano na 44µl reakcione mešavine koja je sadržavala AMV/*Tfl* reakcioni pufer, dNTP miks (10mM), 25 pmol oba prajmera, 1 mM $MgSO_4$, 5 jedinica enzima AMV RT i 5 jedinica enzima *Tfl* DNA polimeraze. Temperaturni ciklus RT-PCR reakcije je bio sledeći, 45 min na 48 °C za reverznu transkripciju (RT), 94 °C tokom 2 min za inaktivaciju enzima AMV RT i RNA/cDNA/prajmer denaturaciju, 40 ciklusa 30 s na 94 °C, 1 min na 60 °C i 2 min na 68 °C, kao i krajnje izduživanje umnoženih lanaca na 68 °C tokom 7 min. Umnoženi produkti su vizuelno detektovani na 1,5% agaroznom gelu obojenom etidium bromidom.

Tabela 2. Detalji o oligonukleotidnim prajmerima korišćenim za RT-PCR

Prajmer*	sekvenca (5' - 3')	pozicija u genomu**	veličina i lokacija
P1-U[a]	AGA GGC TAG CCA TGC CCT TAG T	79-100	294
Pest2-L[b]	TCA ACT CCA TGT GCC ATG TAC	353-373	5'-UTR

* opisanih od strane Barlič-Maganje i Groma, 1999

** prema poziciji u genomu soja virusa Alfort/Tubingen - Stadejek i sar., 1997

[a]modifikovano posle Boye i sar.,1991; [b]slično prajmeru 326 opisanom od Vilčeka i sar., 1994

Sekvencioniranje 5`-UTR dela genoma

Bendovi DNK specifične veličine su isecani iz gela i prečišćavani pomoću "Wizard PCR prep" reagenasa i spin kolona (Promega, USA) prema uputstvu proizvodjača. Prečišćeni PCR produkti su korišćeni kao matrica u reakciji direktnog

sekvencioniranja vodjenu internim oligonukleotidnim prajmerima za 5'-UTR opisanim u Tabeli 3, uz upotrebu fluorescentno obeleženih nukleotida (BigDye Terminator Cycle Sequencing Kit, PE Biosystems, USA). Svi PCR produkti su sekvencionirani u oba smera. Dobijeni produkti su analizirani na automatskom nukleokiselinskom analizatoru (ABI PRISM 310 Genetic Analyzer, PE Biosystems, USA)

Tabela 3. Detalji oligonukleotidnih prajmera korišćenih u reakciji sekvencioniranja

prajmer[a]	sekvenca (5' - 3')	pozicija u genomu*	veličina i lokacija
104F	GCTAGCCATGCCCTTAGTAGGACT	83-106	289 bp
402R	CAACTCCATGTGCCATGTACAGCA	349-372	5'-NCR

[a] Barlič-Maganja i Grom (2001)

* prema poziciji u genomu virusa Alfort/Tubingen

Filogenetska analiza

Komparacija nukleotidnih sekvenci i filogenetska analiza 5'-UTR delova genoma je izvedena sa DNASTAR programskim paketom (DNASTAR Inc., USA). Nukleotidne sekvence izolata virusa su poredjane i prekontrolisane (*proof read*) upotrebom SeqMan^{TM}II i EditSeq programa. Nakon uporednog poredjenja sa naspramnom (*consensus*) sekvencom, 150 nukleotida dugačke sekvence CSFV izolata iz Srbije su poravnate korišćenjem MegAlign^{TM} i ClustalW programa (Thompson i sar., 1994) sa odgovarajućim sekvencama 66 CSFV izolata i referentnih sojeva prikupljenih iz NCBI banke gena (*NCBI GenBank*) (http://www.ncbi.nlm.nih.gov/) i baze podataka Evropske referentne laboratorije za CSFV (CRL) iz Hanovera (http://viro08.tiho-hannover.de) (Greiser-Wilke i sar., 2000). Preuzete nukleotidne sekvence koje su bile uključene u ispitivanje pripadaju CSFV sojevima poreklom iz 24 države (4 iz Austrije, 2 iz Madjarske, 2 iz Slovačke, 2 iz Češke, 2 iz Švajcarske, 2 iz Ujedinjenog Kraljevstva, 2 iz Francuske, 13 iz Nemačke, 1 iz Belgije, 3 iz Rumunije, 2 iz Bugarske, 7 iz Hrvatske, 1 iz Bosne i Hercegovine, 1 iz Španije, 1 iz Holandije, 4 iz Italije, 1 iz Poljske, 1 iz Estonije, 2 iz SADa, 1 iz Kine, 2 iz Honk Konga, 2 iz Japana, 2 iz Malezijea, 3 iz Koreje, kao i 3 već predhodno sekvencionirana (1999) izolata iz Srbije), reprezentujući na taj način većinu do sada poznatih genetskih tipova i podtipova CSFV. Detalji pomenutih sojeva CSFV su prikazani u Tabeli 4. Filogenetsko stablo je konstruisano pomoću *neighbour – joining* metoda baziranom na *bootstrap*-u od 1000 ponavljanja (korišćenjem programa MEGA verzija 4).

Rezultati i Diskusija

U radu je izvršeno poredjenje 150 baza dugačkih 5'-UTR nukleotidnih sekvenci 9 izolata iz Srbije (Tabela 1) i odgovarajućih sekvenci 66 referentnih sojeva i izolata CSFV iz različitih i okolnih država (Tabela 4). 5'-UTR region genoma CSFV je već predhodno opisan i potvrdjen kao veoma osetljiv i pogodan deo genoma za detekciju kao i za genetsku tipizaciju izolata CSFV (Hofmann i sar., 1994; Vilček i sar., 1996; McGoldrick i sar., 1998). U cilju preciznije tipizacije

CSFV izolata iz Srbije, u molekularnu tipizaciju i filogenetsku analizu su uključeni predstavnici svih poznatih genotipova i najčešćih podtipova CSFV preuzetih iz banki gena (NCBI GenBank i baze podataka CRL za CSFV iz Hanovera) (Tabela 4).

Table 4: Podaci o 66 CSFV sojeva koji su bili uključeni u genetsku tipizaciju sa CSFV izolatima iz Srbije iz 2006. godine.

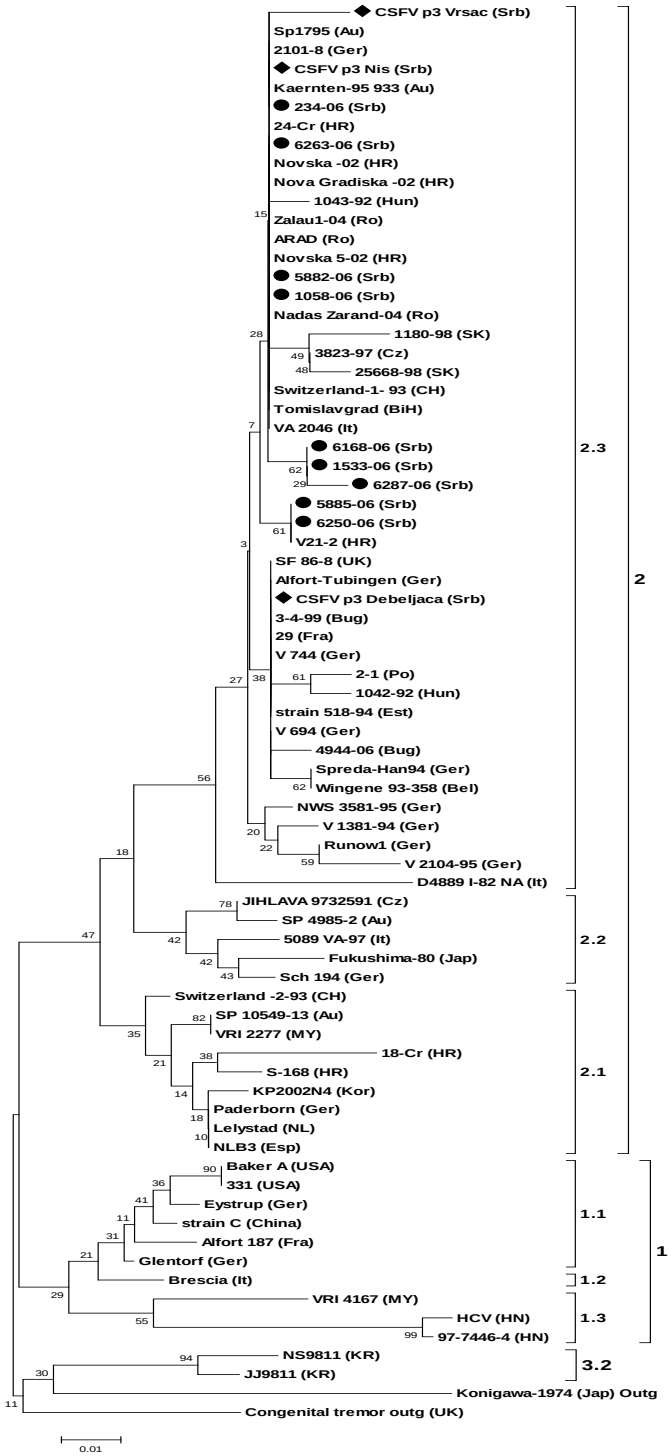
Ime virusnog soja / izolata	NCBI ili Genebank Hanover*	Pod tip	Država / godina izolacije	Ime virusnog soja / izolata	NCBI ili Genebank Hanover*	Pod tip	Država / godina izolacije
Alfort-Tuebingen	J04358	2.3	Germany	Novska 5-02	CSF0821	2.3	Croatia 2002
Switzerland 1 /93	AF045068	2.3	Switzerland 1993	Nadas Zarand-04	CSF0837	2.3	Romania 2004
V 694	CSF0005	2.3	Germany 1984	Zalau 1/ 2004	CSF0846	2.3	Romania 2004
V 744	CSF0007	2.3	Germany 1984	4944-06	CSF0852	2.3	Bulgaria 2006
SF 86/8	CSF0009	2.3	GrBritain 1987	V21-2	XXX0246	2.3	Croatia 2006
Spreda/Han94	CSF0113	2.3	Germany 1994	Sch 194	CSF0020	2.2	Germany, 1989
1042/92	CSF0085	2.3	Hungary 1992	JIHLAVA 97325-91	CSF0076	2.2	Czech R 1991
1043/92	CSF0086	2.3	Hungary 1992	SP 4985/2	CSF0122	2.2	Austria 1994
Wingene 93/358	CSF0099	2.3	Belgium 1993	5089 VA/97	CSF0400	2.2	Italy 1997
Kaernten/95 933	CSF0126	2.3	Austria 1995	Fukushima-80	AB019661	2.2	Japan
V 1381/94	CSF0219	2.3	Germany 1994	Paderborn	AY072924	2.1	Germany 1997
D4889 I/82/NA	CSF0279	2.3	Italy 1982	Switzerland 2 /93	AF045069	2.1	Switzerland 1993
518/94	CSF0300	2.3	Estonia 1994	SP 10549/13	CSF0150	2.1	Austria 1993
2/1	CSF0317	2.3	Poland 1991	VRI 2277	CSF0305	2.1	Malaysia 1986
2101/8	CSF0333	2.3	Germany 1997	NLB3	CSF0361	2.1	Espana 1997
3823/97	CSF0369	2.3	Czech R. 1997	S-168	CSF0669	2.1	Croatia 1997
V 2104/95	CSF0437	2.3	Germany 1995	18-Cr	CSF0670	2.1	Croatia 1997
NWS 3581/95	CSF0464	2.3	Germany 1995	Lelystad	CSF0938	2.1	Netherlands 1992
Runow1	CSF0523	2.3	Germany 1998	KP2002N4	DQ452388	2.1	Korea 2002
25668/98	CSF0527	2.3	Slovakia 1998	Alfort-187	X87939	1.1	France 1968
29	CSF0577	2.3	France 1997	Baker A	CSF0932	1.1	USA
3-4/99	CSF0608	2.3	Bulgaria 1999	Eystrup	AF326963	1.1	Germany 1964
CSFV p3Debeljača	CSF0615	2.3	Serbia 1999	331	CSF0918	1.1	USA 1969
CSFV p3 Niš	CSF0616	2.3	Serbia 1999	Glentorf	U45478	1.1	Germany 1968
CSFV p3 Vrsac	CSF0617	2.3	Serbia 1999	C-strain (China)	AY805221	1.1	China
24/Cr	CSF0672	2.3	Croatia	Brescia	M31768	1.2	Italy 1945
1180/98	CSF0722	2.3	Slovakia 1998	VRI 4167	CSF0306	1.3	Malaysia 1986
Sp1795	CSF0733	2.3	Austria 2000	97-7446/4	CSF0643	1.3	HN 1996
VA 2046	CSF0734	2.3	Italy 2000	HCV 31	CSF0653	1.3	HN 1992
ARAD	CSF0800	2.3	Romania 2002	Kongenital tremor	CSF0410	3.1	Gr. Britain 1964
Tomislavgrad BiH	CSF0817	2.3	Bosnia 2003	JJ9811	XXX0095	3.2	Korea
Nova Gradiska-02	CSF0818	2.3	Croatia 2002	NS9811	XXX0096	3.2	Korea
Novska-02	CSF0819	2.3	Croatia 2002	Kanagawa	CSF0309	Outg	Japan 1974

* Greiser-Wilke i sar., 2000 (http://viro08.tiho-hannover.de)

Posebna pažnja je tom prilikom poklonjena izolatima virusa iz susednih zemalja (Austrije, Madjarske, Slovačke, Češke, Rumunije, Bugarske, Hrvatske i Bosne i Hercegovine). S obzirom da se u Srbiji još uvek sprovodi program vakcinacije i da je bio prisutan i tokom 2006. godine, u molekularnu analizu je uključen i vakcinalni Kina soj (C-soj) virusa (AY805221). Već je predhodno utvrdjeno da Kina soj virusa CSFV iz atenuirane vakcine „Kilapin“, koja se koristi u Srbiji, pripada podtipu 1.1 CSFV i da je u 254 nukleotida dugoj 5'-UTR sekvenci 100% sličan originalnom Kina soju virusa koji je prijavljen u banku gena AY805221 (ovi podaci nisu prikazani).

Rezultati sekvencioniranja i filogenetske analize CSFV izolata iz Srbije u 5'-UTR regionu genoma su prikazane u filogenetskom stablu (Fig. 1). Rezultati pokazuju da svi analizirani izolati iz Srbije spadaju u CSFV genotip 2 i podtip 2.3. Predstavnici ovog podtipa virusa su se pojavili na području Evrope krajem prošlog i početkom ovog veka. Ovaj podtip virusa je izuzetno rasprostranjen na području Evrope, naročito na njenom centralnom i južnom području i predstavlja najčešće detektovani virusni podtip u Evropi tokom poslednjih 20 godina. I pored činjenice da su svi analizirani izolati iz Srbije grupisani u isti virusni podtip i sličnosti koje su utvrdjene sa izolatima iz drugih evropskih država, utvrđene su i različitosti, kao i povezanosti izolata u odnosu na izvore i širenje infekcije.

I pored toga što svi izolati CSFV iz Srbije spadaju u isti podtip virusa, među njiima su utvrđene značajne razlike po kojima se mogu svrstati u grupe, odnosno klustere (Slika 1). Ako uzmemo u obzir i ranije sekvencionirane izolate CSFV p3 Niš, CSFV p3 Vršac i CSFV p3 Debeljača (preuzete iz baze podataka iz Hanovera), svi izolati CSFV iz Srbije se mogu svrstati u 5 klustera, odnosno utvrđeno je 5 različitih virusnih sekvenci. Pripadnost pojedinoj grupi, odnosno klusteru, direktno definišu molekularno epizootiološku odrednicu izolata, odnosno definišu njihovo poreklo, pravce širenja, kao i povezanost pojedinih epizootija. Primera radi, izolati 234/06, 5882/06, 6263/06 i 1058/06, kao i ranije tipiziran izolat CSFV p3 Niš (sekvenca preuzeta iz banke gena) se mogu svrstati u jedan kluster sa 100% sličnosti nukleotidnih sekvenci. Ovaj podatak ukazuje na činjenicu da su njihove analizirane genomske sekvence veoma slične, čak identične jedna drugoj, što nesumljivo ukazuje na njihovu međusobnu povezanost. Na osnovu toga možemo sa velikom verovatnoćom predpostaviti da su izbijanja klasične kuge svinja na području naseljenih mesta Inđija (izolat 1058/06), Zrenjanin (izolat 5882/06) i Temerin (izolat 6263/06) bila povezana sa izbijanjem klasične kuge svinja na području naseljenog mesta Stara Pazova (izolat 234/06 – najranije ustanovljen), odnosno da je izvor infekcije za sva pomenuta mesta isti. Činjenica da se u istom klusteru nalazi izolat CSFV p3 Niš iz 1999. godine ukazuje da se virus takvih genetskih karakteristika nalazi bar šest godina na području Srbije. Epizootiološki gledano u prošlosti svi pomenuti izolati u klusteru imaju zajedničko poreklo. Kao što se iz filogenetskog stabla jasno može videti, pomenuti izolati iz Srbije su veoma slični ili čak i identični sa nekim izolatima iz Hrvatske, Bosne i Hercegovine, Mađarske, Rumunije, Austrije i Švajcarske, a samo delimično slični nekim izolatima iz Slovačke i Češke. Analizirani virusi iz pomenutih zemalja su izolovani tokom 90-tih godina prošlog veka, kao i od 2000. do 2006. godine, što direktno ukazuje na njihovo zajedničko poreklo i širenje infekcije u prošlosti, kao i na povezanost izbijanja klasične kuge svinja u Srbiji i zemljama u okruženju.



Slika 7: Filogenetsko stablo - analiza 75 genomskih sekvenci CSFV u dužini od 150 nukleotida 5'-UTR genoma u neighbor – joining programu baziranom na bootstrap testu od 1000 ponavljanja (n=1000) (program MEGA verzija 4). U filogenetsku analizu sekvenci je uključeno 9 CSFV izolata sa područja Srbije (označeni punim krugom) izolovanih tokom 2006. godine i 66 već tipiziranih izolata i

referentnih sojeva virusa iz 24 zemalje među kojima su i 3 ranije tipizirana izolata sa područja Srbije (obeleženi punim rombom) čije su nukleotidne sekvence preuzete iz banki gena: NCBI GenBank i baze podataka CRL za CSFV iz Hanovera.

Na istom ogranku filogenetskog stabla na kojem je prvi kluster, nalazi se i izolat CSFV p3 Vršac iz 1999. godine (sekvenca preuzeta iz baze podataka iz Hanovera), medjutim on se od izolata iz prvog klustera grana odvojeno (dužina horizontalnih linija u filogenetskom stablu označava veličinu razlike u analiziranom delu genoma, dok vertikalne linije samo vizuelno razdvajaju izolate i nisu rezultat razlika u analiziranom delu genoma). Ovakav nalaz ukazuje na drugačiju nukleotidnu sekvencu i drugačije poreklo izolata CSFV p3 Vršac bilo u odnosu na izolate iz Srbije, bilo u odnosu na izolate iz drugih evropskih zemalja, čije su sekvence analizirane u ovom radu. To ukazuje na CSFV p3 Vršac kao predstavnika drugog klustera CSFV na području Srbije.

U treći CSFV kluster u Srbiji se mogu svrstati izolati 1533/06 sa područja naseljenog mesta Bački Petrovac i 6168/06 i 6287/06 sa područja naseljenog mesta Stepanovićevo ukazujući na činjenicu da su izbijanja klasične kuge u ova dva naseljena mesta međusobno povezana a i da istovremeno nisu povezana sa ostalim izbijanjima klasične kuge svinja tokom 2006. godine. Izolati 1533/06 i 6168/06 imaju identičnu sekvencu ali se izolat 6287/06 razlikuje od njih iako se grana na istom ogranku stabla. Ovaj podatak ukazuje na činjenicu da su na području naseljenog mesta Stepanovićevo tokom 2006. godine postojala bar dva izvora infekcije, od kojih je jedan povezan sa onim iz Bačkog Petrovca, dok je drugi izvor infekcije nepoznat. Nijedan izolat iz ovog klustera ne pokazuje veću sličnost sa drugim analiziranim izolatima iz Srbije niti sa analiziranim izolatima iz drugih zemalja u okruženju i šire. To govori u prilog tome da je ovaj virusni kluster tokom nekog vremena imao zasebnu evoluciju na području Srbije.

U četvrti kluster u Srbiji se mogu svrstati izolati 5885/06 sa područja naseljenog mesta Senta i 6250/06 sa područja naseljenog mesta Bač. Njihove genomske sekvence su bile identične ukazujući na širenje infekcije CSFV sa područja Sente na područje Bača. Takođe, njihove sekvence su bile identične sekvenci virusa V21-2 izolovanom u Hrvatskoj 2006. godine ukazujući na sigurnu povezanost epizootija CSFV na području Srbije i Hrvatske tokom 2006. godine. Osim sa izolatom V21-2 iz Hrvatske nije uvrđena sličnost izolata iz ovog klustera sa drugim izolatima u drugim zemljama. S obzirom da nije utvrđena veća sličnost ovih izolata sa ostalim izolatima od 2006. i 1999. godine iz Srbije, može se predpostaviti da je to ili virus poreklom iz Hrvatske ili je kao i u predhodnom slučaju virusni kluster koji je imao zasebnu evoluciju u dužem vremenu na području Srbije.

Jedini predstavnik mogućeg petog klustera na području Srbije je izolat CSFV p3 Debeljača iz 1999. godine, čija sekvenca je preuzeta iz banke podataka iz Hanovera. Ovaj izolat se potpuno odvojeno grana od svih ostalih izolata iz Srbije, ukazujući na njegovu najveću različitost. Ovaj izolat nije sličan ni sa jednim izolatom iz 2006. i 1999. godine sa područja Srbije, već je sličan sa nekim analiziranim izolatima iz Francuske, Estonije, Nemačke, Bugarske, Velike Britanije i Poljske izolovanim poslednjih 20-tak godina ukazujući na njihovo zajedničko poreklo. Naročito je interesantna njegova sličnost sa 3-4-99 izolatom iz Bugarske iz iste 1999. godine. Ova činjenica sa velikom verovatnoćom ukazuje na povezanost

izbijanja epizootija kuge u Srbiji i Bugarskoj tokom 1999. godine. S obzirom da se CSFV p3 Debeljača razlikuje u odnosu na ostale analizirane CSFV izolate iz Srbije iz 1999. i 2006. godine, postoji mogućnost da je poreklo ovog izolata iz Bugarske ili da je u pitanju virusni kluster sa dužom zasebnom evolucijom u Srbiji ili je pak u pitanju virus koji je u daljoj prošlosti inficirao životinje na području obe zemlje. Trenutno se ne može dati tačan odgovor na ovo pitanje zbog relativno ograničenog broja analiziranih izolata sa područja Srbije i Bugarske iz 1999. godine.

Pored svega predhodno rečenog, posebno je potrebno naglasiti da nijedan od analiziranih izolata CSFV iz Srbije nije bio sličan vakcinalnom Kina soju virusa koji se koristi u programu kontrole CSFV u Srbiji.

Iako sprovedena ispitivanja, koja su obuhvatila tipizaciju samo nekoliko CSFV izolata, predstavljaju samo preliminarno epizootiološko istraživanje, ona pružaju deo informacija o mogućim genotipovima i podtipovima CSFV koji su u skorašnjem periodu bili prisutni u populaciji svinja u Srbiji. Sprovedena analiza i data objašnjenja i predpostavke su, pored sprovedenih laboratorijskih i molekularno epizootioloških testova, omogućene i velikim brojem već ranije tipiziranih virusa klasične kuge svinja i radova na tu temu (Vilček i sar. 1996; Stadejek i sar. 1997; Paton i sar. 2000; Edwards i sar. 2000; Biagetty i sar. 2001; Jemeršić i sar. 2003). Dalje analize, kao i odgovori na mnoga otvorena pitanja porekla, povezanosti i raznolikosti CSFV izolata iz Srbije će biti omogućena dataljnim ispitivanjima većeg broja izolata virusa iz ranijih i skorašnjih godina.

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