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DETECTION AND GENOTYPING OF CLASSICAL SWINE FEVER VIRUS ISOLATES IN SERBIA

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Classical swine fever (CSF) is a highly contagious disease of pigs leading to significant economic losses worldwide. Classical swine fever virus can be classified into three genogroups, each consisting of three or four subgroups. However, there is a lack of knowledge on the genotypes of CSFV isolates in Republic of Serbia. This study, based on the sequences analysis of partial E2 gene and 5' non coding region (NCR) of 15 CSFV isolated during 2006–2008 from domestic pigs, revealed that all were clustered into genetic group 2.3. Additionally, we showed that the two most often used real time RT-PCR assays were able to detect all local CSF viruses circulated in Serbia in the last years during intensive vaccination campaign against CSF.

Key words: Classical swine fever virus, genotyping, real time RT-PCR, Serbia

INTRODUCTION

Classical swine fever (CSF) is often fatal and highly contagious multisystemic disease of pigs (Edwards, 2000). It is caused by the Classical Swine Fever Virus (CSFV), a member of the family *Flaviviridae*, genus *Pestivirus* (Heinz *et al.*, 2000). CSFV virus is enveloped and its genome is composed of single-stranded RNA of positive polarity (Meyers *et al.*, 1989). Natural hosts of CSFV are domestic pigs and wild boars (Blome *et al.*, 2010). Infection usually occurs via oronasal route although other routes are also possible (Paton and Greiser-Wilke, 2003). After the primary replication in the epithelial crypts of the tonsil, the virus may be carried to local lymph nodes and into the bloodstream causing a severe leucopenia, immunosuppression, widespread thrombosis and endothelial damage (Paton and Greiser-Wilke, 2003). The disease severity depends on the age and, probably, the breed of the pigs, the virulence of the virus and some other, still unidentified factors (Paton and Greiser-Wilke, 2003). The disease appears in the classic form characterized by high fever, generalized illness, hemorrhagic lesions, immunosuppression, pronounced lymphocytopenia and a high mortality, as well as in the chronic course and "late onset" form which is of great importance as far

as virus surviving and maintaining in nature are concerned (Thiel *et al.*, 1996). Considering that CSFV easily passes through the placental barrier (Dewulf *et al.*, 2001), infection of fetuses is common in pregnant sows. The outcome of foetal infection depends on the gestation stage and virulence of the virus (Moennig and Plagemann, 1992).

CSF is widespread throughout the world. The greatest virus diversity is seen in Asia where CSF is endemic while the situation in Africa is uncertain (Paton and Greiser-Wilke, 2003). Australia, North America and most of Europe are CSF free (Paton and Greiser-Wilke, 2003). However, EU Member States have been periodically experiencing reintroduction of CSFV into domestic pigs via wild boars (Greiser-Wilke *et al.*, 2000). Based on the nucleotide sequences of at least two regions, 5'NCR and partial E2 coding gene, CSF viruses can be divided into 3 genetic groups and several subgroups whereas only one serotype has been found (Blome *et al.*, 2010).

It has been shown that almost all analyzed viruses isolated in Europe during the period 1920-1970 cluster in Group 1. Most of them are of subgroup 1.1. Subgroup 1.2. was found in Brescia, Italy, while no subgroup 1.3 viruses have been identified in Europe (Paton *et al.*, 2000). Nevertheless, in 1970 group 1 has been very rarely reported in Europe. In the following years, a genetic shift from group 1 to group 2 was observed throughout Europe, with evident prevalence of the 2.3 subgroup (Paton *et al.*, 2000). The earliest appearance of group 2.3 was in Germany in 1982 and later on in Italy, France, Belgium, Great Britain, Austria, Switzerland, Hungary, Czech Republic, Poland, and Slovak Republic. Viruses of subgroup 2.2 have been found in Central Europe, in Austria, Czech Republic, Italy, Germany, Romania and Hungary, from 1985 onwards (Greiser-Wilke *et al.*, 1998), whereas subgroup 2.1 viruses have been only sporadically reported in Europe (Paton *et al.*, 2000). Subgroups 2.2 and 2.3 were commonly derived from wild boars (Paton *et al.*, 2000). Recently, genetic typing of viruses isolated in Eastern Europe countries has shown that, with the exception of two isolates (one from Croatia belonging to 2.1. and the other one from Macedonia to 2.2 subgroup), all isolates were members of subgroup 2.3 (Blome *et al.*, 2010). 5'NCR sequences analysis of all available 2.3. Eastern European isolates have shown clustering in two clades designated as 2.3.1 and 2.3.2 (Blome *et al.*, 2010). The 2.3.1. clade was composed of isolates from Bulgaria, collected during the period 1997-2007, Germany, Poland, Czech Republic, Slovak Republic and Croatia (Blome *et al.*, 2010). All Romanian isolates were in clade 2.3.2., as well as isolates from Croatia (period 2006-2007), Kosovo and Austria (Blome *et al.*, 2010).

Clinical diagnosis of CSF is very difficult particularly in older animals and when the involved virus strain is of low or moderate virulence. Many other diseases having similar symptoms (Done *et al.*, 2000) make clinical diagnosis of CSF additionally difficult and prolonge the time until the disease is confirmed (Paton, 2002). Because of the lack of pathognomonic clinical signs of CSF, laboratory confirmation of the disease is necessary, even for secondary cases during the large outbreaks (Paton and Greiser-Wilke, 2003).

Laboratory diagnosis based on virus isolation (VI) or the detection of viral antigen, specific antibodies and viral RNA is essential for accurate confirmation of

suspicious cases (Van Oirschot *et al.*, 1999). CSFV detection, regardless the clinical stage of infection, from early infection, during the incubation and all along the clinical phase, is possible using reverse-transcription PCR (RT-PCR) and real-time RT-PCR (Handel *et al.*, 2004). Furthermore, detection of viral RNA is possible regardless the course of CSF, even when mild forms of CSF occur, as well as at the seroconversion phase, in the presence of antibodies (Handel *et al.*, 2004). Assays for the detection of CSFV genome are mainly based on 5'-NCR (Barlic-Maganja and Grom, 2001) and 3'-NCR (Vilcek *et al.*, 1999) sequences as well as genes for N^{pro}, C, E^{rns} (Barlic-Maganja and Grom, 2001), E2 (Vilcek *et al.*, 1996) and NS5B proteins (Vilcek *et al.*, 1996). Also, assays for the differentiation of CSFV from other Pestiviruses are developed: nested RT-PCR (Katz *et al.*, 1993), restriction analysis (Parchariyanon *et al.*, 1999; Vilcek and Belak, 1998), RT-PCR-ELISA (Barlic-Maganja and Grom, 2001) and real time RT-PCR (McGoldrick *et al.*, 1999; Risatti *et al.*, 2003). Currently, real time RT-PCR is being recommended for confirmatory diagnosis in individual animals, as well as for herds surveillance (Handel *et al.*, 2004). Although it is considered that higher RT-PCR sensitivity had been achieved by assays harmonisation, contamination remains the main problem (Paton *et al.*, 2000a). Validation of commercial kits is done according to OIE recommendations. However, a many of the published and in house assays do not provide this documentation (Blome *et al.*, 2006).

Control of CSF in Serbia has been done by C strain mass vaccination. According to National Directive on Classical Swine Fever (Anonymous, 2009), compulsory vaccination is requested for all piglets 45-60 days of age, gilts and sows at least 15 days before each insemination, and boars every 6 months. During the last decade, 1381 CSF outbreaks were recorded in Serbia, with the peak in 2006 when more than 400 outbreaks were reported (Milicevic *et al.*, 2009).

The main objective of this study was genotyping of 15 CSFV isolates in Serbia between 2006 and 2008 based on the partial nucleotide sequences of the E2 glycoprotein gene and 5'NCR. In addition, the effectiveness of real time RT-PCR for the detection of CSFV from clinical specimens was evaluated.

MATERIAL AND METHODS

CSFV isolates and sequences

Tissue samples (15 tonsils and lymph nodes) uniformly representing spatial and temporal CSF occurring during the 2006-2008 were selected for the study.

The reference strains of classical swine fever viruses, genotypes 1.1. (CSF0902/gt1.1.), 1.3. (CSF0653/gt1.3.), 2.1. (CSF0849/gt2.1.), 2.2. (CSF0018/gt2.2.), 2.3. (CSF0621/gt2.3.), bovine viral diarrhoea virus type 1 (C24V) and 2 (UK502643) and border disease virus (MoreDun) were used, as well. Sample IDs, including the region virus has been isolated from, the year of isolation, as well as the reference materials data, are given in Table 1.

Table 1. Detailed description of samples and reference materials

SAMPLE ID	ORIGIN
BELGRADE 2006	INSTITUTE OF VETERINARY MEDICINE OF SERBIA
BELGRADE 2007	
KRALJEVO 2006	
KRALJEVO 2007	
ZRENJANIN 2006	
JAGODINA 2006	
JAGODINA 2007	
NIS 2006	
NIS 2007	
PANCEVO 2006	
PANCEVO 2007	
POZAREVAC 2006	
SABAC 2006	
SABAC 2007	
ZAJECAR 2008	
CSF gt 1.1.	
CSF gt 1.3.	
CSF gt 2.1.	
CSF gt 2.2.	
CSF gt 2.3.	
BDV	OIE REF.LAB FOR BVD (Veterinary Laboratories Agency: VLA Weybridge)
BVDV 1	
BVDV 2	

Viral RNA isolation

Tissues samples were prepared as 10% homogenates in PBS. Suspensions were centrifuged for 10 minutes at 2000 g and supernatants were used for RNA extraction.

Viral RNA was isolated using the commercial kit, QIAamp® Viral RNA Mini Kit (Qiagen, USA) according to the manufacturer's recommendations from 140 µL of supernatant.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

For phylogenetic analysis, two regions, 150 nt fragment of the 5' NTR and 190 nt fragment of the E2 gene, were amplified using previously published primers (Paton *et al.*, 2000) in concentration 0.6 pmol/µL. Both RT-PCR were run as 50 µL reactions using the Qiagen OneStep RT-PCR Kit (Qiagen, USA) according to the manufacturer's specifications, in an Eppendorf Master Cycler

(Eppendorf, USA). Thermocycling was performed under following conditions: 5°C for 30 min; initial denaturation at 95°C for 15 min, followed by 35 cycles of denaturation at 95°C for 45 sec; 1 min of annealing at 50°C (5'NCR)/55°C (E2), 1 min and extension for 1 min at 72°C,) with a 5 min final elongation step at 72°C. Products were separated on a 2% agarose gel stained with ethidium bromide.

TaqMan real time RT-PCR was carried out as 25 µL reaction using TaqMan® One-Step RT-PCR Master Mix Reagents Kit (Applied Biosystems, Warrington, UK) according to the manufacturer's instructions. Primers and probe, previously published by Hoffmann *et al.* (2005), were used in concentrations of 250 nmol/µL and 50 nmol/µL, respectively. The PCR program was set up as follows: 48°C 30 min, 95° for 10 min followed by 50 two step cycles (94°C for 15 sec; 1 min at 60°C).

A single tube SYBR Green real time RT-PCR assay in a 25 µL reaction mix was performed, using the SYBR® Green PCR Master Mix and RT-PCR Reagents, according to manufacturer's recommendations (Applied Biosystems, Warrington, U.K) including set of primers (Vilcek *et al.*, 1994), in concentrations 250 nmol/µL. The thermo profile was set up as follows: 48°C for 30 min; 10 min at 95°C, 50 two step cycles (95°C for 15 sec; 1 min at 60°C). RT-PCR was coupled with the high-resolution melting analysis, in order to determine specific amplification.

Nucleotide sequencing and sequence analysis

The amplicons were purified using the QIAquick PCR Purification Kit (Qiagen) according to manufacturer's instructions. Purified products were cycle-sequenced in both directions using Big Dye Terminators (Applied Biosystems, Warrington, UK) in ABI 310 genetic analyzer (Applied Biosystem, Warrington, UK). Distances of sequences were calculated by the Kimura-2 parameter method and trees were generated by Neighbor Joining using MEGA 4.0 software (Tamura *et al.*, 2007).

RESULTS

Comparing partial E2 and 5'NCR nucleotide sequences of Serbian CSFV isolates and reference CSF viruses and other Pestiviruses, it was shown that all viruses from Serbia belonged to 2.3 subgroup. Genotyping was based on 150 nt partial E2 encoding gene and 190 nt 5' NCR. Phylogenetic tree was constructed using Neighbor Joining method, for both analyzed regions (Figures 1 and 2).

Differences rate for E2 region ranged from 0.000 (Jagodina 2006 – Nis 2006 – Nis 2007 – Pancevo 2007 – Sabac 2007 – Kraljevo 2007) to 0.069 per nucleotide site (Pozarevac 2006 – Sabac 2006). Overall distance between E2 sequences was 0.021 per nucleotide site. Even less variable results were observed for 5'NCR region where overall distance was 0.07.

Based on identical E2 and 5'NCR sequences of some isolates (Jagodina 2006 – Kraljevo 2007 – Nis – 2007 – Sabac 2007) the same source of infection can be considered. But some isolates identical in 5'NCR were the most divergent in E2 region (Pozarevac 2006 – Sabac 2006) additionally confirming that for molecular epidemiology sequence analysis and genotyping based on of one single region is not enough.

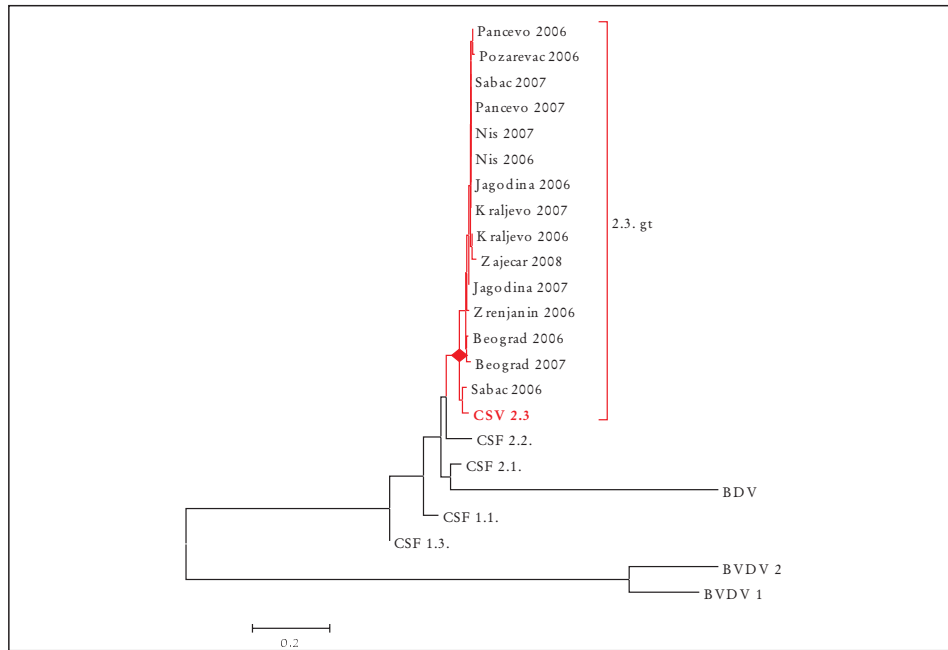


Figure 1. Phylogenetic tree constructed for partial E2 gene, Neighbor-Joining method

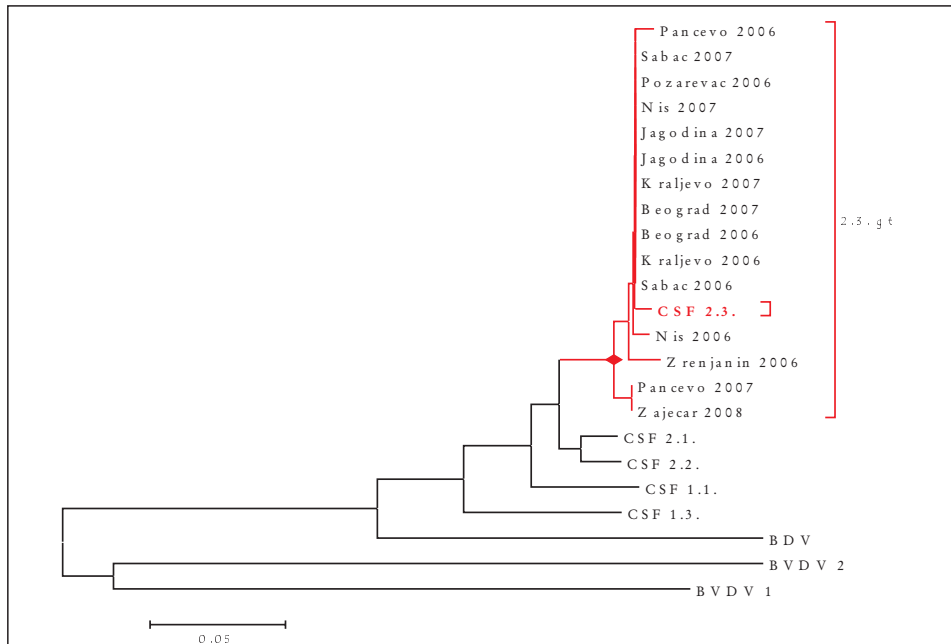


Figure 2. Phylogenetic tree constructed for 5'NCR, Neighbor-Joining method

DISCUSSION

Evaluating applicability of recommended real time RT-PCR assays with Serbian isolates, it was shown that all target nucleotide sequences were detected using TaqMan and SYBR Green. Positive amplification was observed with reference strains of classical swine fever virus, while other Pestiviruses were detected only by panpesti SYBR Green real time RT-PCR.

After 2000 CSF situation in Serbia become very difficult since CSFV was spread throughout the whole country. The most common transmission rout was illegal trade (Milicevic *et al.*, 2009). Additionally, vaccination coverage was very low due to the poor economic status of pig holders and their inability to cover vaccination costs. After 2006. vaccination has been funded by the Budget of the Republic of Serbia and since then CSF incidence has been decreasing continuously. The last outbreak was reported in 2010 (World Organization for Animal Health-OIE). During this period, the large economic loss was estimated at 137 million euros (Milicevic *et al.*, 2009).

The real time RT-PCR is a powerful diagnostic tool suitable for the detection of CSFV infection in animals. In order to obtain the most reliable results by real time RT-PCR method, it is necessary to analyze as many as possible genome sequences of the CSF virus which have been circulating in the local swine population. Although CSF in Serbia was common in the past, very few sequences are available in gene banks which imply that assays currently in use have to be evaluated with local isolates. While some assays are recommended by CSF CRL, submission of Serbian isolates to CSF CRL for confirmatory and further testing was not common practice in the past and validation of these assays should not be accepted *a priori*.

By conducting the program of CSF eradication, Serbia approaches the moment when vaccination against CSFV will be ceased. Before the late phase of eradication, when the chronic course of the disease and infections with low virulent strains are common, it is very important that the evaluation of different diagnostic methods is completed. To support very difficult clinical diagnosis in this phase, laboratory diagnostic has to be most accurate. Assays evaluated in this study have been chosen in order to harmonize diagnostic procedures of CSF with standards applied in EU countries. Using both assays we were able to detect local CSF viruses which circulated in Serbia in the last five years during the intensive vaccination campaign against CSF. In order to maintain a reliable diagnostic of CSF, it is necessary to monitor on the virus genome for new CSF isolates in Serbia, as well as in other countries, and to confirm each negative result by other tests due to viral mutagenic potential. Moreover, since in the next phase of the disease eradication, vaccination against CSF will be stopped, it would be interesting to follow the future expansion of this virus and its genome changes in susceptible (unvaccinated) swine populations.

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DETEKCIJA I GENOTIPIZACIJA IZOLATA VIRUSA KLASIČNE KUGE SVINJA U SRBIJI

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SADRŽAJ

Klasična kuga svinja (CSF) je visoko kontagiozno oboljenje svinja koje do-
vodi do značajnih ekonomskih gubitaka širom sveta. Na osnovu genetske struk-
ture, virus klasične kuge svinja podeljen je u tri genogrupe, od kojih svaka ima tri ili
četiri podgrupe. Nedostaju podaci o tome koji genotipovi virusa klasične kuge
svinja na teritoriji Republike Srbije cirkulišu u prijemljivoj populaciji. Sekven-
cioniranjem dela E2 gena i 5'nekodirajućeg regiona (NCR) 15 izolata virusa

klasične kuge svinja prikupljenih u periodu od 2006-2008 godine, poreklom od domaćih svinja, dokazano je da svi pripadaju genetskoj grupi 2.3. Uz to, dokazano je i da je upotrebom dva najčešće korišćena real time RT-PCR protokola moguće detektovati sve lokalne izolate virusa klasične kuge svinja koji cirkulišu u Srbiji poslednjih godina, u kojoj se uporedo vršila i intenzivna vakcinacija protiv ove bolesti.