VETERINARSKI ARHIV 86 (5), 623-640, 2016

The genetic heterogeneity of equine infectious anaemia virus field strains in Croatia

Vladimir Stevanović[1]*, Ivana Lojkić[2], Ljubo Barbić[1], Snježana Kovač[1], Vesna Mojčec-Perko[1], Andreja Ambriović-Ristov[3], Suzana Hađina[1], Lejla Velić[4], Matko Perharić[1], and Vilim Starešina[1]

[1]*Department of Microbiology and Infectious Diseases with Clinic, Faculty of Veterinary Medicine, University of Zagreb, Zagreb, Croatia*

[2]*Department of Virology, Croatian Veterinary Institute, Zagreb, Croatia*

[3]*Laboratory for Genotoxic Agents, Division of Molecular Biology, Ruđer Bošković Institute, Zagreb, Croatia*

[4]*Department of Epizootiology, Faculty of Veterinary Medicine, University of Sarajevo, Sarajevo, Bosnia and Herzegovina*

STEVANOVIĆ, V., I. LOJKIĆ, LJ. BARBIĆ, S. KOVAČ, V. MOJČEC-PERKO, A. AMBRIOVIĆ-RISTOV, S. HAĐINA, L. VELIĆ, M. PERHARIĆ, V. STAREŠINA: The genetic heterogeneity of equine infectious anaemia virus field strains in Croatia. Vet. arhiv 86, 623-640, 2016.

ABSTRACT

Equine infectious anaemia (EIA) is an infectious disease of equids caused by lentivirus. Equine infectious anaemia virus (EIAV) causes lifelong infection after integration of the proviral DNA into the host cell genome. Due to the obligatory slaughter of infected animals in most countries of the world, EIA is one of the most significant infectious diseases of horses. Control of EIA is based on antibody detection, but today the focus in diagnostics is aimed at PCR based methods. In this study, 98 AGID (agar gel immunodiffusion) positive sera samples, three spleen samples and cell cultures infected with EIA were tested for the presence of viral RNA or proviral DNA, using two nested PCR protocols. Out of all the samples 27 partial gag sequences of viral RNA were amplified. The Croatian isolates showed high diversity within the group of other available European sequences. Phylogenetic analysis showed that at least three gag gene subtypes are presently circulating in Croatia. The results of this study showed the limited efficiency of two PCR methods in terms of diagnostics, although the primers used for amplification targeted part of the viral RNA that was regarded as one of the most preserved. The high variability of the Croatian isolates obtained highlighted the necessity for determining more EIAV sequences to allow detection of less variable areas of the viral genome, to be used for amplification with universal primers.

Key words: equine infectious anaemia, diagnostics, field isolates, phylogenetic analysis, Croatia

*Corresponding author:

Vladimir Stevanović, PhD, DVM, Department of Microbiology and Infectious Diseases with Clinic, Faculty of Veterinary Medicine, University of Zagreb, Heinzelova 55, 10000 Zagreb, Croatia, Phone: +385 1 2390 211; Fax: +385 1 2390 211; E-mail: vladimir.stevanovic@vef.hr

ISSN 0372-5480
Printed in Croatia

Introduction

Equine infectious anaemia (EIA) is an infectious disease of equids, caused by the equine infectious anaemia virus (EIAV). The disease is transmitted by biting insect vectors, mainly horseflies and stable flies, as well as iatrogenically (STEIN et al., 1942; MORE et al., 2008; MARESCA et al., 2012). Equine infectious anaemia virus is a lentivirus, a member of the *Retroviridae* family and, like other members of this genus, causes persistent, lifelong infection (ISSEL et al., 1982; CHEEVERS and McGUIRE, 1985). The virus presents with two copies of single stranded RNA, surrounded by a proteinaceous matrix and entrapped in a conical viral core. On the viral surface there is a lipid membrane with glycoprotein projections (MATHEKA et al., 1976; WEILAND et al., 1977; QUINLIVAN et al., 2013). Viral RNA is about 8.2 kbp long and consists of three genes (gag, pol and env) that arise in two open reading frames (ORF) (PERRY et al., 1992; COOK et al., 1998). On both ends of the viral genome there are long terminal repeats (LTR). After infection, viral RNA undergoes reverse transcription, producing proviral DNA, which integrates into the cell genome.

It is estimated that EIA is enzootic in more than 23% of countries worldwide (BICOUT et al., 2006). The first case of EIA in Croatia was described in 1934 (BOSNIĆ, 1936). In the last few decades EIA has been considered enzootic in our country, with occasional outbreaks (PETROVIĆ et al., 1975; ŽUPANČIĆ and JUKIĆ, 1981; STAREŠINA, 2004). One of the largest outbreaks was documented in 2002 when large number of horses died suffering from a peracute illness (STAREŠINA, 2004).

Control of EIA in Croatia and in most countries is based on detection of antibodies to EIAV and identification of clinically healthy carrier animals that constitute the vast majority of positive animals. For decades, agar gel immunodiffusion (AGID), or the Coggins test, was considered to be the only reliable serological indicator of EIAV infection (COGGINS et al., 1972). Since the mid-1980s, as an alternative to AGID, enzyme linked immunoassay (ELISA) and immunoblotting (IB) have been introduced (ISSEL et al., 1987; MATSUSHITA et al., 1989). All serological tests for EIAV have recognized limitations. The main disadvantage of serodiagnostics is that these tests rely on detection of antibodies and not the causative agent. To obtain positive results by serological tests the animal has to produce a certain amount of antibodies, depending on the test sensitivity. In the early course of the infection the humoral response is still immature. Moreover, it has been shown that some horses appear unable to produce an antibody titre detectable by serological testing (COGGINS et al., 1972; ISSEL et al., 1982; ISSEL et al., 1990). In field conditions a period as long as 157 days has been recorded from infection to the first AGID positive testing (CULLINANE et al., 2007).

Today, the focus in direct assays is on PCR based methods. There are two main problems in the development of diagnostic PCR. After the initial viraemia the viral burden in the blood and tissues becomes low, especially in the invisible carrier state (HARROLD

et al., 2000). Therefore diagnostic PCR has to be extremely sensitive. Successful implementation of PCR is directly influenced by the primer sequence, that must be largely homologous to the viral genome. In addition, not only the level of homology but also the position of the nonhomologous nucleotide within the primer is crucial. This is a major problem when working with lentiviruses which have one of the most variable genomes (BAKHANASHVILI and HIZI, 1993; LEROUX et al., 2004). In addition to these variations, geographically distinct isolates of EIAV *in vivo* exist as complex populations of related, but not identical, viral genotypes or quasispecies (LEROUX et al., 2004). To date, most of the published PCR protocols target the viral gag gene (COOK et al., 2002; NAGARAJAN and SIMARD, 2007; QUINLIVAN et al., 2007; CAPPELLI et al., 2011; DONG et al., 2012). It was previously believed that most of variations take place within the env gene and that EIAV gag and pol genes were relatively conserved (SALINOVICH et al., 1986; ZHENG et al., 1997; QUINLIVAN et al., 2007). However, recent studies have suggested that in addition to env, there may be significant variations between isolates in other major viral genes, such as gag (QUINLIVAN et al., 2007). As a prerequisite for development of successful diagnostic PCR, it is essential to identify the highly conserved regions within the EIAV genome. The goal of this study was to determine the applicability of the two PCR methods described for detection of EIAV in Croatian field isolates. The results of this study revealed genetic and amino acid variability in EIAV isolates and place them in correlation with other published EIAV sequences. These results represent the first genetic characterization of Croatian EIAV isolates.

Materials and methods

In this study, 98 AGID positive sera were used. Samples were obtained as part of an EIA surveillance programme between 1985 and 2014. In 2014 eight samples of whole blood, originating from positive animals, were received by the Laboratory for Infectious Anaemia of the Faculty of Veterinary Medicine, University of Zagreb and were also included in this study. Additionally, three spleen samples originating from seropositive animals were collected from positive horses at the slaughter house. Isolates of EIAV from the outbreak in 2002 adapted, to grow in ED cell culture, were also used.

Viral RNA was extracted from samples using a QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Total cell DNA for the detection of proviral DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany).

For detection of EIAV, primers for nested PCR as described by NAGARAJAN and SIMARD (2007), were used. For the first round PCR, the following primers (F: 5'-GGGACAGCAGAGGAGAACTTAC-3'; R: 5'-CCTCTAATAAATCTTGCTGTC-3') were used to amplify a 769 bp fragment (nt 454-1222) of gag gene. The second round

of PCR targeted the region within the first PCR product with the following primer pair (F: 5'-CIITGACATGGAGCAAIGCG-3'; R: 5'-TGICTGTATGGTATTIACCCA-3') to amplify a 449 bp fragment (nt 535-983).

In the first step of nested PCR, amplification of viral RNA QIAGENOneStep RT-PCR Kit (Qiagen, Hilden, Germany) was used. The RT-PCR reaction was performed in a final volume of 25 µl under the following conditions: 50 °C for 30 minutes (reverse transcription) and 95 °C for 15 minutes (polymerase activation) followed by 35 cycles: 94 °C, 30 s (DNA denaturation), 54 °C, 30 s (annealing) 72 °C, 45 s (elongation) and 72 °C for 10 minutes for the final elongation of DNA products. Amplification of the 769 bp fragment of the proviral DNA was made using Platinum® TaqPolimerase (Invitrogen, Life Technologies, Grand Island, New York, USA). Reaction conditions were the same as for RT-PCR, except that the initial phase of reverse transcription was omitted and initial polymerase activation at 95 °C lasted 2 minutes. The same Platinum® TaqPolimerase commercial kit was used for nested PCR. Reaction conditions were: 95 °C for 2 minutes and 30 cycles of denaturation at 94 °C for 30 s, annealing at 54 °C for 30 s, polymerization at 72 °C for 30 s and final elongation at 72 °C for 10 minutes.

The second protocol used for detection of the EIAV RNA and proviral DNA was an adjusted protocol described by CAPPELLI et al. (2011). For the first round PCR, the primers (F: 5'- GACATGGAGCAAAGCGCTCA-3'; R: 5'- CTGCCCAGGCACCACATCTA-3') were used to amplify a 547 bp fragment of the gag gene. The inner set of primers (F: 5'-TGTGGGCGCTAAGTTTGGTG-3'; R: 5'- TTTCTGTTTCCAGCCCCATC-3') were used to amplify a 313 bp fragment of the first round PCR product. The first PCR reaction conditions were: initial polymerase activation at 94 °C for 2 minutes, followed by 30 cycles: 94 °C, 30 s (DNA denaturation), 66 °C, 30 s (annealing) 68 °C, 30 s (elongation) and 68 °C for 10 minutes for the final elongation of DNA products. Amplification of the inner fragment was done under the following conditions: 94 °C for 2 minutes, followed by 30 cycles: 94 °C, 20 s (DNA denaturation), 64 °C, 30 s (annealing) 68 °C, 30 s (elongation) and 68 °C for 7 minutes for the final elongation of DNA products. For detection of the viral RNA reverse transcription an additional step was used in the first round at 50 °C for 30 minutes. The same kits and ingredients were used as those already described in the first PCR/RT-PCR protocol.

The PCR products generated with sets of inner primers were purified using the PCR Purification Kit (Qiagen, Hilden, Germany). Both DNA strands were sequenced with an ABI3730xl DNA Analyzergene analyser, using the same primers as for the nested PCR reaction.

Sequences were aligned using the ClustalW algorithm, implemented in the Mega6 software package (TAMURA et al., 2013). The same software was used for molecular and phylogenetic analyses of the compared sequences. Maximum likelihood (ML)

phylogenetic analysis was performed on 315-nt gag sequences, using Tamura-3 as the best-fit model estimated by jModelTest V.0.1.1. (POSADA, 2008). The reliabilities of phylogenetic relationships were evaluated using nonparametric bootstrap analysis, with 1000 replicates for ML analysis.

A total of 35 gag sequences, originated from different geographical regions, were retrieved from the GenBank and compared with the 27 Croatian gag sequences.

Ethical and animal research. Ethical review was not required due to fact that this study is based on laboratory archive sera samples and samples collected from horses subjected to euthanasia due to their disease status, according to the legislation of Republic of Croatia.

Results

To compare protocols, regarding primer sets or viral RNA/proviral DNA detection, the eight whole blood samples obtained in 2014 were tested first. The specific PCR product from viral RNA was amplified using the protocol described by NAGARAJAN and SIMARD (2007) in two of the eight whole blood samples (25%), while the same primer set did not result in amplification of a specific PCR product from proviral DNA in all eight samples. At the same time, the modified protocol described by CAPPELLI et al. (2011) did not result in amplification of specific PCR fragments, either for viral RNA or proviral DNA, from all eight whole blood samples.

Out of 98 AGID positive sera samples, EIAV RNA was detected in 24 (24.49%) and in one of three spleen samples using the modified protocol described by NAGARAJAN and SIMARD (2007) (Table 1). The oldest positive sera sample dated back to 1998. An RT-PCR positive spleen was sampled from a horse that gave a positive RT-PCR result in the blood sample (C-178). A partial viral genome was also amplified using RT-PCR in samples of persistently infected ED cell cultures. Cell culture was used for growing field isolates originating from one of the diseased animals in the major outbreak in 2002 (CRO1-2002). Surprisingly none of the sera, whole blood, spleen or cell cultures gave positive results when the adjusted protocol described by CAPPELLI et al. (2011) was used.

To compare the sensitivity of the two described protocols and of RNA/DNA detection, 14 RT-PCR positive samples were randomly selected. Proviral DNA was detected in two of the 14 tested sera samples (14.29%) using NAGARAJAN and SIMARD's (2007) modified protocol (Table 1). Again we were unable to amplify viral RNA as well as proviral DNA using the second set of primers (CAPPELLI et al., 2011).

Table 1. RT-PCR positive samples when modified protocol described by NAGARAJAN and SIMARD (2007) was used

Isolate	Year	Sample tested	Proviral DNA
CRO1-2002	2002	Cell culture	+
C-105	2005	Serum	-
C-112	2007	Serum	-
C-117	2009	Serum	-
C-120	2010	Serum	-
C-126	2001	Serum	NT
C-129	2007	Serum	+
C-132	1998	Serum	-
C-134	2002	Serum	-
C-135	2013	Serum	-
C-137	2013	Serum	-
C-141	2005	Serum	NT
C-143	2005	Serum	NT
C-144	2002	Serum	NT
C-151	2004	Serum	NT
C-153	2002	Serum	NT
C-154	2005	Serum	NT
C-168	2005	Serum	NT
C-173	NA	Serum	NT
C-178	2014	Blood, Serum, Spleen	+ (spleen)
C-179	2014	Blood, Serum, Spleen	-
C-182	NA	Serum	NT
C-185	2009	Serum	+
C-190	2009	Serum	-
C-192	2009	Serum	-
C-196	2007	Serum	-

Name of the isolates are listed with year of horse sampling and results of proviral DNA amplification using same set of primers as for RT-PCR. (″NA″- not available, ″+″- positive, ″-″- negative, ″NT″- not tested)

All 27 nested PCR products were sequenced and phylogenetically analysed. The overall homology of Croatian nucleotide sequences with the Wyoming strain was between 75.6 and 81.7%, and on amino acid (aa) the level was from 77.8 to 88.3%. Identity of nucleotide and the predicted aa sequence of Croatian strains and the most closely related Italian strain (Ita-87) was 79.0-92.5% and 74.0-86.5%, respectively. With Slovenian strains nucleotide homology was 72.5-92.2% and 75.0-93.2% at aa level.

Table 2. EIAV sequences available in GeneBank used for phylogenetic analysis

Acc. Numb	Name	Origin
KF977819	SLO-22	Slovenia
KF977820	SLO-19	Slovenia
KF977804	SLO-26	Slovenia
HM177438	Ita74	Italy
HM177437	Ita73	Italy
HQ888861	Ita87	Italy
HQ888862	Ita90	Italy
GQ229581	Rom-1	Italy
EU375543	Ita-2	Italy
EU375544	Ita-3	Italy
EU741609	Ita-4	Italy
EU240733;	Ita-1	Italy
JX480631	F2	Ireland
AF016316	EIAuk	UK
AF033820	Wyoming	USA
AY742249	614-5	USA
AF170894	EIAV-TX	USA
EF418581	Arg1	Argentina
EF418582	Can1	Canada
EF418575	Can2	Canada
EF418576	Can4	Canada
EF418577	Can5	Canada
EF418578	Can6	Canada
EF418584	Can7	Canada
EF418579	Can8	Canada
EF418590	Can9	Canada
AB008196	V70	Japan
AB008197	V26	Japan
AF327877	Liaoning	China
AF327878	DLV	China

Isolates of EIAV from different geographical regions are listed with NBI accession number, name of the isolate and country of origin

Phylogenetic analysis of Croatian sequences with corresponding gag sequences from Europe, North and South America and Asia, revealed their assortment based on their geographical origin (Table 2). There was clear clustering of North American, UK and Argentinian strains (Fig. 1). Slovenian strains, together with the majority of Croatian

isolates and the Italian strain Ita-87 were grouped together. Isolates from outbreaks in Italy and Ireland in 2006, as well as three other Italian isolates comprised another group of European isolates, more closely related to Chinese strains than the rest of the European ones. Interestingly, isolate C-120, together with Italian Ita-90, comprise a divergent group of isolates, separate from the rest of European isolates, more closely related to North American and UK strains than to the Chinese strains.

When compared to each other, Croatian partial gag sequences were 77.5-99.3% identical on the nt level and 77.8-99.0% on the aa level. C-126 and C-144 are the most divergent of the Croatian sequences (77.5% identity with other Croatian sequences). A high level of genetic diversity was also confirmed in four isolates (C-135, C-137, C-178 and C-179) originating from the same horse farm. The isolates C-135 and C-137 were obtained from the outbreak in 2013, and C-178 and C-179 during the outbreak in 2014. The level of identity of these four isolates at nucleotide and predicted aa level is shown in Table 3. The phylogenetic tree of Croatian isolates suggests there were at least three gag gene subtypes (Fig. 2). One group covered most of the obtained gag sequences, and the second and third comprised two isolates each. The largest group had isolates dating from 1998 to 2014. The second group had EIAV isolates from two consecutive years, 2001 and 2002, (C-126 and C-144) but the third had isolates dated from 2007 and 2009 (C-129 and C-192).

Table 3. Percent of identity between isolates from same horse far at amino acid and nucleotide level

Isolate	135	137	178	179
C-135	-	*99.60*	*98.70*	*98.00*
C-137	**99.00**	-	*99.00*	*98.30*
C-178	**96.10**	**97.00**	-	*97.40*
C-179	**96.10**	**97.00**	**94.10**	-

Bold numbers indicate percentage of identity between isolates at amino acid level and italic percentage of identity at nucleotide level

Since the PCR protocol used (NAGARAJAN and SIMARD, 2007) amplifies the gag sequence corresponding to aa 18-119, it was possible to track changes in the epitope clusters EC1, partially, and EC2 in total (Fig. 3). Comparing the epitope of EC1 cluster we could not find any conserved region. Amino acids 77-119 comprise the CTL recognizing the EC2 cluster. Substantial genetic variations were noticed in all obtained sequences and there was no single conserved region. In as many as four isolates (C-112, C-120, C-182 and C-185) deletions were found. Isolates C-112, C-120 and C-185 had deletion of threonine at position 112 only, and C-182 had deletion of three amino acids at positions 113-115.

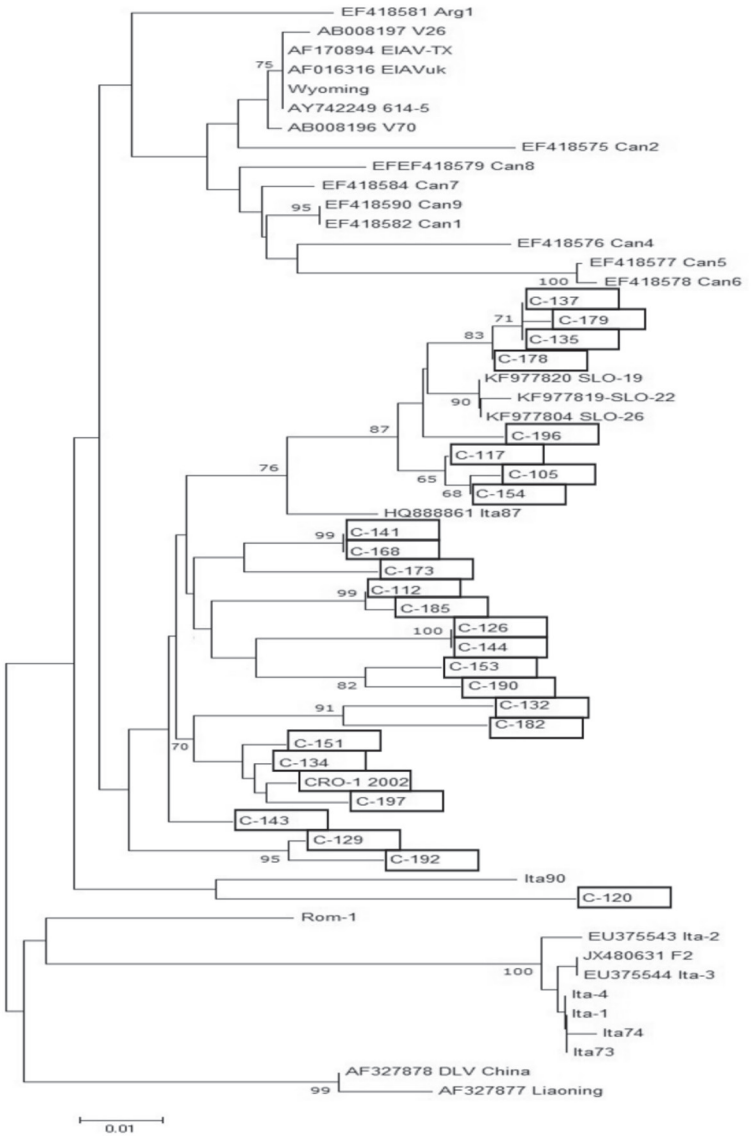


Fig. 1. Phylogenetic analysis of the 315-nt gag sequence of 27 Croatian and 35 sequences originated from different geographical regions.

The tree is inferred using maximum likelihood analysis with Tamura-3 evolutionary model. The sequence identifiers include the NCBI accession number and isolate name. Croatian isolate is indicated by its name. Bootstrap supports (n = 1000) are indicated on branches.

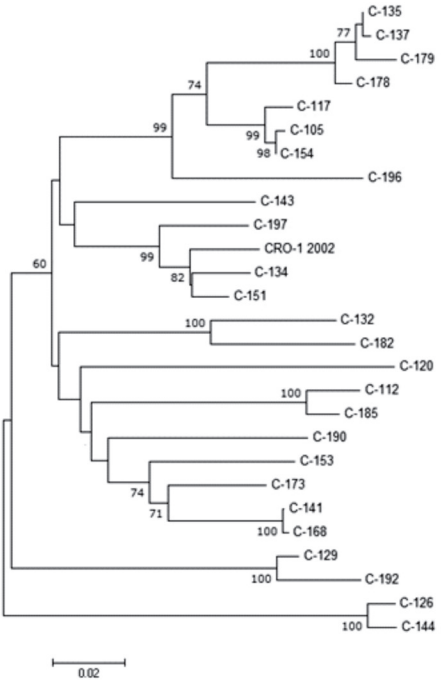


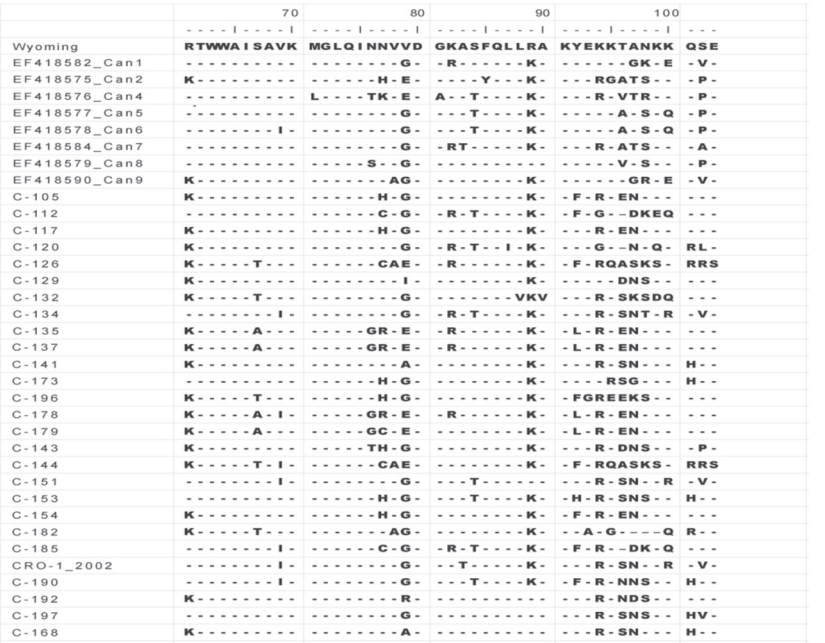
Fig. 2. Phylogenetic analysis of the 315-nt gag sequence of 27 Croatian EIAV isolates.

The tree is inferred using maximum likelihood analysis with Tamura-3 evolutionary model. Croatian isolate is indicated by its name. Bootstrap supports (n = 1000) are indicated on branches.

	10	20	30	40	50	60
Wyoming	VTVOGSQKLT	TGNCNWALSL	VDLFHDTNFV	KEKDWQLRDV	IPLLEDVTQT	LSGQEREAFE
EF418582_Can1	----------	I---------	--------Y-	----------	----------	----------
EF418575_Can2	---------S	V---------	-------KY-	-----H---I	-------S--	---K------
EF418576_Can4	----------	S---------	--------YA	----------	----------	----------
EF418577_Can5	-----A----	----------	--------YM	-A--------	----D--IL-	-T--------
EF418578_Can6	-----A----	----------	-------SYM	-A-----K--	----D--IL-	-T--------
EF418584_Can7	----------	I--------N-	--------YT	----------	----D-----	-----K----
EF418579_Can8	----------	I---------	---Y----YT	----------	----------	-----K----
EF418590_Can9	----------	I---------	--------Y-	----------	-------A--	----------
C-105	---M------	S---------	----------	--------E-	----------	-----KD---
C-112	----------	-S--------	---L---S-M	--------S-	----G--S--	---P-K----
C-117	---L------	S---------	----------	--------E-	----------	-----KD---
C-120	-----A----	P---------	-------SY-	-A---P--E-	-------S--	-----K----
C-126	--------M-	V---------	-------TY-	---------L	-------S--	---R-K----
C-129	---------N	A--L------	----------	---------I	-------S--	---P-KD---
C-132	----------	V---------	--IL---D-S	-Q--------	--------R-	-T---K----
C-134	-M--------	----------	---------M	----------	----------	-----K----
C-135	---L------	SS--------	---Y------	---------I	----------	------D---
C-137	---L------	SS--------	---Y------	---------I	V---------	------D---
C-141	----------	I---------	-------T--	----------	----G---E-	-----K----
C-173	-K--------	I---------	--------L-	----------	----------	---P-K----
C-196	---------S	N---------	--------Y-	----------	----------	------D--V
C-178	---L------	SS--------	----------	----------	V---------	------D---
C-179	---L--R---	SS--------	---Y------	---------I	V---------	------D---
C-143	-M--------	----------	---L------	----------	----------	-----K----
C-144	--------M-	A---------	-------TY-	---------L	-------S--	---R-K----
C-151	----------	----------	-------T-M	----------	----------	----------
C-153	-----N----	----------	-------TYM	-----P----	-------S--	-T--------
C-154	---L------	S---------	----------	--------E-	----------	-----KD---
C-182	----------	I---------	-------DL-	-Q----I---	-------A--	---P-K----
C-185	----------	-S--------	---L---S-L	----------	-------S--	---P-K----
CRO-1_2002	----------	----------	---------M	----------	----------	-----K----
C-190	----------	----S-----	---L---T-M	---------I	-------S--	-T---K---D
C-192	--------R-N	A--L------	---Y---D-G	---------I	-------S--	--EP--D--D
C-197	---H------	I---------	----------	----------	----D--I--	-----K----
C-168	----------	I---------	-------T--	----------	----G---E-	-----K----

Fig. 3. Amino acid alignment of the partial gag protein sequence (aa 18-119) of Croatian EIAV isolates in comparison with American and Canadian isolates.

Predicted amino acid sequence is corresponding to part of Gag CTL epitope cluster EC1 (aa 1-35) and complete epitope cluster EC2 (aa 77-119). Dots indicate identical amino acids as that of prototype EIAV Wyoming (USA); dashes indicate deletions.



	70	80	90	100	
Wyoming	RTWWAISAVK	MGLQINNVVD	GKASFQLLRA	KYEKKTANKK	QSE
EF418582_Can1	----------	--------G-	-R------K-	------GK-E	-V-
EF418575_Can2	K---------	------H-E-	----Y---K-	---RGATS--	-P-
EF418576_Can4	----------	L----TK-E-	A--T----K-	---R-VTR--	-P-
EF418577_Can5	----------	--------G-	---T----K-	-----A-S-Q	-P-
EF418578_Can6	--------I-	--------G-	---T----K-	-----A-S-Q	-P-
EF418584_Can7	----------	--------G-	-RT-----K-	---R-ATS--	-A-
EF418579_Can8	----------	-----S--G-	----------	-----V-S--	-P-
EF418590_Can9	K---------	-------AG-	--------K-	------GR-E	-V-
C-105	K---------	------H-G-	--------K-	-F-R-EN---	---
C-112	----------	------C-G-	-R-T----K-	-F-G--DKEQ	---
C-117	K---------	------H-G-	--------K-	---R-EN---	---
C-120	K---------	--------G-	-R-T--I-K-	---G--N-Q-	RL-
C-126	K-----T---	------CAE-	-R------K-	-F-RQASKS-	RRS
C-129	K---------	--------I-	--------K-	-----DNS--	---
C-132	K-----T---	--------G-	-------VKV	---R-SKSDQ	---
C-134	--------I-	--------G-	-R-T----K-	---R-SNT-R	-V-
C-135	K-----A---	-----GR-E-	-R------K-	-L-R-EN---	---
C-137	K-----A---	-----GR-E-	-R------K-	-L-R-EN---	---
C-141	K---------	--------A-	--------K-	---R-SN---	H--
C-173	----------	------H-G-	--------K-	----RSG---	H--
C-196	K-----T---	------H-G-	--------K-	-FGREEKS--	---
C-178	K-----A-I-	-----GR-E-	-R------K-	-L-R-EN---	---
C-179	K-----A---	-----GC-E-	--------K-	-L-R-EN---	---
C-143	K---------	-----TH-G-	--------K-	---R-DNS--	-P-
C-144	K-----T-I-	------CAE-	--------K-	-F-RQASKS-	RRS
C-151	--------I-	--------G-	---T------	---R-SN--R	-V-
C-153	----------	------H-G-	---T----K-	-H-R-SNS--	H--
C-154	K---------	------H-G-	--------K-	-F-R-EN---	---
C-182	K-----T---	-------AG-	--------K-	--A-G---Q	R--
C-185	--------I-	------C-G-	-R-T----K-	-F-R--DK-Q	---
CRO-1_2002	--------I-	--------G-	--T-----K-	---R-SN--R	-V-
C-190	--------I-	--------G-	---T----K-	-F-R-NNS--	H--
C-192	K---------	--------R-	----------	---R-NDS--	---
C-197	----------	--------G-	----------	---R-SNS--	HV-
C-168	K---------	--------A-	----------	---R-SN---	H--

Fig. 3. Amino acid alignment of the partial gag protein sequence (aa 18-119) of Croatian EIAV isolates in comparison with American and Canadian isolates.

Predicted amino acid sequence is corresponding to part of Gag CTL epitope cluster EC1 (aa 1-35) and complete epitope cluster EC2 (aa 77-119). Dots indicate identical amino acids as that of prototype EIAV Wyoming (USA); dashes indicate deletions.

Discussion

To the best of our knowledge this is the first extensive genetic characterisation of Croatian EIAV isolates. All the obtained sequences were amplified by the set of primers described by NAGARAJAN and SIMARD (2007). It was a surprise that the protocol described by CAPPELLI et al. (2011), using primers based on consensus sequences of American Asian but also Italian strains, did not result in amplification of PCR specific fragments in all tested samples. Taking into account the geographic grouping of EIAV isolates, we expected a higher percentage of PCR positive samples using the sets of primers described by Italian authors. On the other hand, the Croatian sequences showed unexpected genetic diversity if we consider that the obtained sequences are part of the *gag* gene, a relatively conserved part of the viral genome. Most divergent sequences (C-126 and C-144) were only 77.5% identical on a nucleotide level compared with the rest of the Croatian isolates. The high diversity is a possible explanation for the differences in the obtained results

with the degenerated primers used by NAGARAJAN and SIMARD (2007) and the primers of CAPPELLI et al. (2011). When looking at the phylogenetic tree, it may be concluded that there are at least three gag gene subtypes circulating in Croatia, similar to the results of Slovenian studies (KUHAR et al., 2014; KUHAR and MALOVRH, 2015). Taking into account the limitations of the methods used in this study, the diversity of isolates may be at least partially explained by the specifics of EIA epizootiology in Croatia. There are a few main reasons for the enzootic character of EIA in Croatia. There are still parts of Croatia where horses are used as working animals. The dense population of horses and vectors at lodging sites support the circulation of the virus (PETROVIĆ et al., 1972; VELIĆ et al., 2011). The spread of the disease is also highly plausible in the free range horse population in the flood area of the River Sava. Traditionally, a number of horses are kept in a swamp area in Central Croatia, and that is exactly the area where most of the outbreaks have been described (PETROVIĆ et al., 1975; ŽUPANČIĆ and JUKIĆ, 1981; STAREŠINA, 2004). Most of the populations of horses at the lodging sites and from swamp areas are kept isolated from each other, so it is possible that viral strains evolve independently to some extent. On the other hand, the introduction of new viral strains through the horse trade cannot be excluded as a cause of at least some of the outbreaks in Croatia. Illegal animal trade is a well-known problem (STAREŠINA, 2004). The seroprevalence of EIA in countries surrounding Croatia is much higher, and the main source of working horses in Croatia is Bosnia and Herzegovina, with a high percentage of EIAV positive animals (VELIĆ et al., 2011). Trade of horse for meat production is a highly possible reason for the high identity of two Italian strains with the Croatian ones, as horses are traditionally exported to Italy.

The diversity of Croatian isolates has also highlighted the limitations of the PCR method currently used in diagnostic purposes. Since the AGID test was formally introduced to diagnostic of EIA in Croatia in 1972, disease prevalence has significantly fallen (STEVANOVIĆ, 2015). Nevertheless, EIA seroprevalence in the last five years has been relatively constant, ranging between 0.02 and 0.07%, and this is probably the best we can achieve using solely the AGID based system of disease control. To be able to lower the seroprevalence further new diagnostic methods should be introduced, PCR being one of possibilities, since it gives us the opportunity to find animals in the early course of infection, when there is active viral replication and the amount of antibodies produced is still below the threshold to give a positive AGID result. In this paper, we found such an example. Samples C-135 and C-137 are sera samples from AGID positive horses from one horse farm, taken in 2013. The remaining horses at that farm were tested twice in a six-month period and gave AGID negative results, but in 2014 two more AGID positive animals were found (C-178 and C-179). Sequence analysis showed the same source of infection for all the animals, so the logical conclusion is that AGID was false negative for the animals C-178 and C-179. Apart from acute infection as one of the reasons for AGID negative results, it is also possible that those two horses needed a longer time to

produce enough anti p26 antibodies to be detected by immunoprecipitation. Even though we tried two described nested PCR protocols, the PCR product amplified from viral RNA was detected in less than one fourth of tested sera samples, which is clearly insufficient in terms of diagnostics. In contrast to the results of other authors we had much less success when proviral DNA was used for amplification (CAPPELLI et al., 2011; COOK et al., 2013).

Interestingly, the identity of the nucleotide sequences of four isolates from the same horse farm was as low as 97.4% and, at aa level, 94.1%, especially when we consider that the amplified sequences correspond to epitopes in EC1 and EC2 clusters that were considered conserved (CHUNG et al. 2005). All four isolates from the same horse farm had amino acid substitution at all seven observed epitopes (one EC1 and six EC2). Croatian isolates displayed substantial variations in all six epitopes of the EC2 cluster with deletions in four of the Croatian strains (C-112, C-120, C-182 and C-185). Interestingly, three of four strains with deletions had deletion of threonine at position 112, as in the published Idaho strain (NAGARAJAN and SIMARD, 2007). Moreover, the Canadian and Idaho strains with duplications and deletions, as well as all the Croatian strains with deletions, had changes in only seven amino acid positions, 109-115.

Comprehensive studies of EIAV genome variations are necessity for a better understanding of the evolution of EIAV and lentiviruses in general, and their ability to escape host immune response. What once was considered a conserved part of the genome has now been proven to be variable. Broader knowledge of the evolution of EIAV could give us valuable information about the human counterpart, HIV, but also a useful tool for EIA control. Disease control systems based on serology alone have proven to be inadequate for EIAV. Since isolation of EIAV has many difficulties, direct detection of the virus is moving towards PCR methods. As shown in this study, even geographically closely related strains display variations in parts of the genome that were considered conserved by other authors. In general we need to have more of the available sequences in order to identify parts of the viral genome that could be used for detection of EIAV using PCR. We believe that the sequences revealed in this study will contribute to this goal.

References

BAKHANASHVILI, M., A. HIZI (1993): Fidelity of DNA synthesis exhibited *in vitro* by the reverse transcriptase of the lentivirus equine infectious anemia virus. Biochemistry 32, 7559-7567.

BICOUT, D. J., R. CARVALHO, K. CHALVET-MONFRAY, P. SABATIER (2006): Distribution of equine infectious anemia in horses in the north of Minas Gerais State, Brazil. J. Vet. Diagn. Invest. 18, 479-482.

BOSNIĆ, L. (1936): Zarazna anemija kopitara (Anaemia infectiosa equorum). Vet. Arhiv 6, 417-527.

CAPPELLI, K., S. CAPOMACCIO, F. R. COOK, M. FELICETTI, M. L. MARENZONI, G. COPPOLA, A. VERINI-SUPPLIZI, M. COLETTI, F. PASSAMONTI (2011): Molecular detection, epidemiology, and genetic characterization of novel European field isolates of equine infectious anemia virus. J. Clin. Microbiol. 49, 27-33.

CHUNG, C., R. H. MEALEY, T. C. McGUIRE (2005): Evaluation of high functional avidity CTL to Gag epitope clusters in EIAV carrier horses. Virology 342, 228-239.

CHEEVERS, W. P., T. C. McGUIRE (1985): Equine infectious anemia virus: immunopathogenesis and persistence. Rev. Infect. Dis. 7, 83-88.

COGGINS, L., N. L. NORCROSS, S. R. NUSBAUM (1972): Diagnosis of equine infectious anemia by immunodiffusion test. Am. J. Vet. Res. 33, 11-18.

COOK, R. F., S. J. COOK, F. LI, R. C. MONTELARO, C. J. ISSEL (2002): Development of a multiplex real-time reverse transcriptase-polymerase chain reaction for equine infectious anemia virus (EIAV). J. Virol. Methods 105, 171-179.

COOK, R. F., C. LEROUX, S. J. COOK, S. L. BERGER, D. L. LICHTENSETEIN, N. N. GHABRIAL, R. C. MONTELARO, C. J. ISSEL (1998): Development and characterization of an *in vivo* pathogenic molecular clone of equine infectious anemia virus. J. Virol. 72, 1383-1393.

COOK, R. F., C. LEROUX, C. J. ISSEL (2013): Equine infectious anemia and equine infectious anemia virus in 2013: a review. Vet. Microbiol. 167, 181-204.

CULLINANE, A., M. QUINLIVAN, M. NELLY, H. PATTERSON, R. KENNA, M. GARVEY, S. GILDEA, P. LYONS, M. FLYNN, P. GALVIN, M. NEYLON, K. JANKOWSKA (2007): Diagnosis of equine infectious anaemia during the 2006 out-break in Ireland. Vet. Rec. 161, 647-652.

DONG, J. B., W. ZHU, F. R. COOK, Y. GOTO, Y. HORII, T. HAGA (2012): Development of a nested PCR assay to detect equine infectious anemia proviral DNA from peripheral blood of naturally infected horses. Arch. Virol. 157, 2105-2111.

HARROLD, S. M., S. J. COOK, R. F. COOK, K. E. RUSHLOW, C. J. ISSEL, R. C. MONTELARO (2000): Tissue sites of persistent infection and active replication of equine infectious anemia virus during acute disease and asymptomatic infection in experimentally infected equids. J. Virol. 74, 3112-3121.

ISSEL, C. J., J. M. MCMANUS, S. D. HAGIUS, L. D. FOIL, W. V. ADAMS Jr., R. C. MONTELARO (1990): Equine infectious anemia: prospects for control. Dev. Biol. Stand. 72, 49-57.

ISSEL, C. J., P. M. RWAMBO, R. C. MONTELARO (1987): Evolution of equine infectious anemia diagnostic tests: recognition of a need for detection of anti-EIAV glycoprotein antibodies. Equine Infectious Diseases V. Proceedings of the fifth international Conference, 7-10 October, Lexington, Kentucky. pp. 196-200.

ISSEL, C. J., W. V. Jr. ADAMS, L. MEEK, R. OCHOA (1982): Transmission of equine infectious anemia virus from horses without clinical signs of disease. J. Am. Vet. Med. Assoc. 180, 272-275.

KUHAR, U., J. ZAVRŠNIK, I. TOPLAK, T. MALOVRH (2014). Detection and molecular characterisation of equine infectious anaemia virus from field outbreaks in Slovenia. Equine Vet. J. 46, 386-391.

KUHAR, U., T. MALOVRH (2015): High genetic diversity of equine infectious anaemia virus strains from Slovenia revealed upon phylogenetic analysis of the p15 gag gene region. Equine Vet. J., doi: 10.1111/evj.12404

LEROUX, C., J. L. CADORE, R. C. MONTELARO (2004): Equine Infectious Anemia Virus (EIAV): what has HIV's country cousin got to tell us? Vet. Res. 35, 485-512.

MARESCA, C., E. SCOCCIA, L. FACCENDA, J. ZEMA, S. COSTARELLI (2012): Equine infectious anemia: active surveillance in central Italy 2007-2009. J. Equine Vet. Sci. 32, 596-598.

MATHEKA, H. D., L. COGGINS, J. N. SHIVELY, N. L. NORCROSS (1976): Purification and characterization of equine infectious anemia virus. Arch. Virol. 51, 107-114.

MATSUSHITA, T., L. K. HESTERBERG, J. P. PORTER, B. J. SMITH, L. E. NEWMAN (1989): Comparison of diagnostic tests for the detection of equine infectious anemia antibody. J. Vet. Diagn. Invest. 1, 50-52.

MORE, S. J., I. AZNAR, D. C. BAILEY, J. F. LARKIN, D. P. LEADON, P. LENIHAN, B. FLAHERTY, U. FOGARTY, P. BRANGAN (2008): An outbreak of equine infectious anaemia in Ireland during 2006: investigation methodology, initial source of infection, diagnosis and clinical presentation, modes of transmission and spread in the Meath cluster. Equine Vet. J. 40, 706-708.

NAGARAJAN, M. M., C. SIMARD (2007): Gag genetic heterogeneity of equine infectious anemia virus (EIAV) in naturally infected horses in Canada. Virus Res. 129, 228-235.

PERRY, S. T., M. T. FLAHERTY, M. J. KELLEY, D. L. CLABOUGH, S. R. TRONICK, L. COGGINS, L. WHETTER, C. R. LENGEL, F. FULLER (1992): The surface envelope protein gene region of equine infectious anemia virus is not an important determinant of tropism *in vitro*. J. Virol. 66, 4085-4097.

PETROVIĆ, D., B. MIKLAUŠIĆ, M. VULINEC, Ž. ŽUPANČIĆ, B. JUKIĆ (1972): Equine infectious anemia in Croatia from 1934 till the end of 1971. Vet. Arhiv 42, 233-239.

PETROVIĆ, D., Ž. ŽUPANČIĆ, B. JUKIĆ (1975): Incidence of equine infectious anemia in Yugoslavia, its determination by agar-gel diffusion test from May 1971 to May 1975, and results of its systematic control in same period. Vet. Arhiv 45, 103-115.

POSADA, D.: (2008): jModelTest: phylogenetic model averaging. Mol. Biol. Evol. 25, 1253-1256.

QUINLIVAN, M., F. COOK, R. KENNA, J. J. CALLINAN, A. CULLINANE (2013): Genetic characterization by composite sequence analysis of a new pathogenic field strain of equine infectious anemia virus from the 2006 outbreak in Ireland. J. Gen. Virol. 94, 612-622.

QUINLIVAN, M., R. F. COOK, A. CULLINANE (2007): Real-time quantitative RT-PCR and PCR assays for a novel European field isolate of equine infectious anaemia virus based on sequence determination of the gag gene. Vet. Rec. 160, 611-618.

SALINOVICH, O., S. L. PAYNE, R. C. MONTELARO, K. A. HUSSAIN, C. J. ISSEL, K. L. SCHNORR (1986): Rapid emergence of novel antigenic and genetic variants of equine infectious anemia virus during persistent infection. J. Virol. 57, 71-80.

STAREŠINA, V. (2004): Characterisation of equine infectious anemia virus isolated during enzooty in 2002 in Croatia. PhD Thesis, University of Zagreb. Zagreb, Croatia.

STEIN, C. D., J. C. LOTZE, L. O. MOTT (1942): Transmission of equine infectious anemia by the stable fly, *Stomoxys calcitrans*, the horsefly, *Tabanus sulcifrons (Macquart)*, and by injection of minute amounts of virus. Am. J. Vet. Res. 3, 183-193.

STEVANOVIĆ, V. (2015): Implementation and efficacy of a three-tiered strategy in diagnosis of equine infectious anemia in Croatia. PhD Thesis, University of Zagreb. Zagreb, Croatia.

TAMURA, K., G. STECHER, D. PETERSON, A. FILIPSKI, S. KUMAR (2013): MEGA6: molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30, 2725-2729.

TU, Y. B., T. ZHOU, X. F. YUAN, H. J. QIU, F. XUE, C. Q. SUN, L. WANG, D. L. WU, J. M. PENG, X. G. KONG, G. Z. TONG (2007): Long terminal repeats are not the sole determinants of virulence for equine infectious anemia virus. Arch. Virol. 152, 209-218.

VELIĆ, R., L. VELIĆ, B. DUKIĆ, H. BEŠIROVIĆ, A. ALIĆ, A. ZUKO, J. OMERAGIĆ, A. GUŠIĆ (2011): Equine infectious anemia in Bosnia and Herzegovina in year 2010. Vet. stanica 323-326.

WEILAND, F., H. D. MATHEKA, L. COGGINS, D. HARTNER (1977): Electron microscopic studies on equine infectious anemia virus (EIAV). Arch. Virol. 55, 335-340.

ZHENG, Y. H., T. NAKAYA, H. SENTSUI, M. KAMEOKA, M. KISHI, K. HAGIWARA, H. TAKAHASHI, Y. KONO, K. IKUTA (1997): Insertions, duplications and substitutions in restricted gp90 regions of equine infectious anaemia virus during febrile episodes in an experimentally infected horse. J. Gen. Virol. 78, 807-820.

ŽUPANČIĆ, Ž., B. JUKIĆ (1981): Equine infectious anemia in Croatia and other parts of Yugoslavia from 1977 till 1979. Vet. stanica 5, 41-61.

Received: 7 July 2015
Accepted: 10 December 2015

STEVANOVIĆ, V., I. LOJKIĆ, LJ. BARBIĆ, S. KOVAČ, V. MOJČEC-PERKO, A. AMBRIOVIĆ-RISTOV, S. HAĐINA, L. VELIĆ, M. PERHARIĆ, V. STAREŠINA: Genetska raznolikost terenskih izolata virusa infekciozne anemije kopitara na području Republike Hrvatske. Vet. arhiv 86, 623-640, 2016.

SAŽETAK

Infekciozna anemija kopitara (IAK) je zarazna bolest kopitara koju uzrokuje lentivirus. Nakon infekcije virusom IAK provirusna DNK se ugrađuje u stanični genom domaćina i infekcija postaje doživotna. Zbog činjenice da se u većini država svijeta inficirani kopitari moraju uputiti na klanje ovo je jedna od najznačajnijih zaraznih bolesti kopitara uopće. Nadzor IAK se temelji na dokazu protutijela, ali se u novije vrijeme sve više truda ulaže u uvođenje metoda koje se temelje na lančanoj reakciji polimerazom (PCR). Za potrebe ovog istraživanja pretraženo je ukupno 98 uzoraka seruma, tri uzorka slezene serološki pozitivnih kopitara te uzorak stanične kulture inficirane izolatom virusa IAK. Kao dokaz infekcije pokušalo se umnožiti odsječak virusne RNK odnosno provirusne DNK koristeći dva opisana protokola za ugniježđenu PCR reakciju. Od ukupnog broja pretraženih uzoraka dio *gag* gena virusne RNK je umnožen u njih 27. Hrvatski su izolati pokazali izrazitu heterogenost unutar skupine dostupnih nukleotidnih sljedova virusa IAK s područja drugih europskih država.

Filogenetska analiza dobivenih izolata pokazala je da postoje najmanje tri podtipa *gag* gena u virusa koji kruže na području Republike Hrvatske. Dobiveni rezultati pokazuju ograničenu učinkovitost opisanih metoda PCR u dijagnostici IAK. Početnice korištene u ovom istraživanju umnažaju dio *gag* gena koji je do nedavno smatran genetički konzerviranim. Izrazita raznolikost dobivenih izolata ponovno je ukazala na potrebu za većim brojem dostupnih nukleotidnih sljedova virusa IAK u cilju otkrivanja više konzerviranih dijelova virusnog genoma pogodnih za umnažanje univerzalnim početnicama.

Ključne riječi: infekciozna anemija kopitara, dijagnostika, terenski sojevi, filogenetska analiza, Hrvatska