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Short communication

MAREK DISEASE VIRUS IN SERBIA: DETECTION AND MOLECULAR CHARACTERIZATION (2015 – 2022)

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Out of 236 samples collected from clinically suspected farms and yards, the genome of Marek's disease virus was detected in 84 (35.59 %) samples. Results showed the significant prevalence of the virus in intensive production/commercial farms (92.86 % of total positive samples) and in the flocks of extensively raised poultry/small-scale farms (7.14 % of total positive samples). Nucleotide sequence analysis of the ICP4 gene revealed that Serbian strains were classified into A and C groups of serotypes 1, showing high similarity (>98 %) with very virulent plus (vv+MDV) pathotypes of the Hungarian strains. A retrospective analysis from 2015 to 2022 showed that the Marek disease virus continually circulates in Serbia. This study aimed to detect and molecularly characterize Marek's disease virus strain in Serbia.

Keywords: Marek's disease, molecular characterization, serotype 1, Serbia

INTRODUCTION

Marek's disease (MD) is a contagious, lymphoproliferative disease of poultry caused by the Gallid alphaherpesvirus 2, serotype 1, that belongs to the family of *Herpesviridae*, subfamily *Alphaherpesvirinae*, the genus *Mardivirus*, designated Marek's disease virus (MDV) (Nair, 2005). Based on its virulence, serotype 1 is separated into pathotypes: very virulent plus (vv+MDV), very virulent (vvMDV), virulent (vMDV), and mild MDV (mMDV) (Witter et al., 2005). Serotype 1 also includes some attenuated vaccine strains [1,2].

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The virus induces generalized nerve inflammation, immunosuppression, and T-cell lymphomas, and appears in the acute or chronic form [3,4]. Clinical signs depend on the strain virulence, poultry age, and previous health condition [4,5]. MD is one of the most commonly reported causes of mortality in small-scale flocks [6,7]. Vaccination as a preventive measure can protect flocks from disease development and increased mortality rate [8]. In Serbia, immunoprophylactic measures are usually carried out according to the vaccination programs in parent flocks and flocks of laying hens. However, there may be an increased need for additional MD protection according to the current vaccination protocol [9]. MD is not a notifiable disease in Serbia, so to obtain data about disease prevalence and its significance is difficult. There is little knowledge about the prevalence, distribution, pathological changes, and molecular characteristics of the Marek's disease virus in Serbia. As the virus continues to be a significant problem in poultry flocks, this study aims to investigate the molecular characterization of the detected Serbian MDV field strain.

MATERIALS AND METHODS

During 2015-2022, the Scientific Institute of Veterinary Medicine of Serbia received 236 samples from flocks clinically suspected to be infected with Marek's disease virus. Samples originated from non-vaccinated poultry showing clinical signs of MD kept in intensively raised farms (41 farms) and extensively (8 in total) and from different species – turkeys and chickens. The samples comprised tissue samples (liver, spleen, proventriculus, lungs, heart), blood, and feather follicles. In the cases when the carcasses were submitted, necropsy was performed on 79 carcasses. The tissue samples (liver, spleen, proventriculus, lungs, hearth) were subjected to molecular analysis after the necropsy of suspected animals. Collected samples were homogenized and prepared as a 10 % suspension in PBS. The suspensions were centrifuged for 10 min at 2,000 g and decanted supernatants were used for DNA extraction (IndiSpin Pathogen Kit, Indical, Germany). PCR was completed using a commercial kit, HotStarTaq Master Mix Kit (Qiagen, Germany). The reaction mix was composed of 2 μl template DNA, 10 μl 1x HotStar Master Mix, 0.6 μl of each primer (10 μM) previously published [10] and 6.8 μl Rnase-free water. A partial region of the ICP4 gene was sequenced using a commercial service Macrogen (Netherlands). The consensus sequences were constructed using the Staden package and submitted to NCBI GenBank under accession numbers OP791960-OP791972. The evolutionary history was inferred by using the Maximum Likelihood method and the Tamura-Nei model [11]. The tree with the highest log likelihood (-9529.43) is shown. The percentage of trees in which the associated taxa clustered together is shown below the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This

analysis involved 30 nucleotide sequences. There was a total of 6498 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 [11–13].

RESULTS AND DISCUSSION

Retrospective analysis of all suspected cases and received samples from farms that reported clinical disease shows that the Marek's disease virus continually circulates in Serbia. Out of a total of 236 tested, the presence of the Marek's disease virus genome was determined in 84 (35.59 %) samples (Table 1).

Table 1. Sample origin and number of samples where the presence of the Marek disease virus genome was determined

Type of production	Species	Number of farms	Number of samples	Number of positive samples
Intensive	Chicken	34	221	73
Extensive	Chicken	7	7	5
Intensive	Turkey	7	7	5
Extensive	Turkey	1	1	1

The average percentage of PCR-positive samples at the annual level was 50.14 %, depending on the year it ranged from 6.25 to 100 %. According to the Medicines and Medical Devices Agency of Serbia, Serbia registered monovalent vaccines based on Rispens/CVI988 strain [14], RN 1250 strain [15], or vHVT013-69 strain [16], and bivalent vaccines based on the combination of two of those three stains. However, the vaccination against Marek's disease virus is not legally binding and in practice, only breeding farms are performing the vaccination against MDV. The continued evolution of the virus and its emergence as more virulent MDV pathotypes despite a large vaccination program for poultry are of important concern [17]. Results confirmed the significant presence of the virus in intensive production systems, with 78 positive samples (92.86 % of total positive samples), but also in the flocks of extensively raised poultry – small-scale farms, 8 positive samples (7.14 % of total positive samples). During the period 2021-2022, in most cases (66.66 %), positive samples detected by PCR were follicle epithelium samples, and it is assumed that this type of sample can be used to surveil poultry flocks for the presence of MDV [10].

A partial region of the ICP4 of 13 representative archival MDV-positive samples (4 positive samples of feathers and 9 positive tissue samples) collected from 2017 to 2021 was used for the phylogenetic analysis of the Marek disease virus. Analyzing nucleotide sequences of the ICP4 gene, 246 bp in length, by subjecting to BLAST

searches against reference genomes in the NCBI (table 2) it was confirmed that the samples contained a genome of Marek's disease virus belonging to serotype 1.

Table 2. Details of the MDV-1 strains, retrieved from GenBank, that were used for the phylogenetic analysis.

Strain	Year isolated	Accession No	Country	Virulence	Reference
ATE2539	2000	MF431493	Hungary	vv+	[15]
MD70/13	1970	MF431495	Hungary	m	[15]
HNLC503	2011	MG518371	China	-	-
HC/0803	2008	MW531728	China	m	[16]
LMS	2007	JQ314003	China	vv	[17]
CVI988	2007	DQ530348	Netherland	m	[18]
CU-2	2008	EU488381	USA	m	[19]
RB1B_JM102	2020	MT813453	Germany	v	[20]

Based on the partial ICP4 genome region, three distinctive MDV groups are divided into tree branches on the NJ tree. Serbian MDV strains (GenBank accession numbers: OP791962, OP791963, OP791965, OP791966, OP791967, OP791968, OP791969, OP791971, OP791972 were highly related (>98 %) with very virulent plus (vv+MDV) pathotype of Hungarian strain – GenBank accession number MF431493 [18] and clustered into A group. Serbian MDV strains (GenBank accession numbers: OP791960, OP791961, OP791964, and OP791970) were grouped with high similarity (>98 %) into the same clade with mild pathotype (mMDV) of the Hungarian strain – GenBank accession number MF431495 [18] within C groups (Figure 1). Due to the extensive trade exchange between Serbia and neighboring countries and that Serbia is on the way to the main routes of EU live animal transport, the virus may be introduced to the country through importation [19]. According to data, one of the most important trading partners for the import of fertilized eggs and day-old chicks is Hungary [20]. However, Serbian MDV strains from group C showed a high similarity of 98.78 % to the live attenuated vaccinal strain CVI988 (GenBank accession number DQ530348). Despite the great effectiveness of this vaccine, according to previous findings, poultry stays susceptible to superinfection to very virulent plus (vv+ MDV) field strains and shed the virus without clinically obvious symptoms [21].

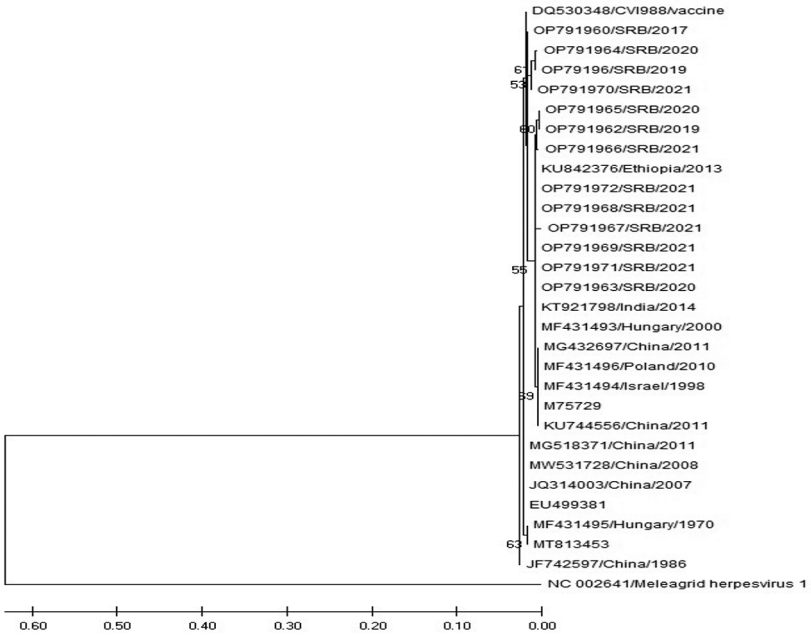

Figure 1. The phylogenetic tree was inferred using the Neighbor-Joining method and the Tamura-Nei model. The tree with the highest log likelihood (-9529.43) is shown.

This study assumes that the type of production (intensive/extensive) and poultry species are unrelated to the virus group (Table 3).

Table 3. Analyzed Serbian MDV strains, with NCBI accession numbers and qualification of virus group.

Year of sample collection	Type of production and species	NCBI accession numbers	Virus group
2017	Intensive/chicken	OP791960	C
2019	Intensive/chicken	OP791961	C
2019	Extensive/turkey	OP791962	A
2020	Intensive/turkey	OP791963	A
2020	Extensive/chicken	OP791964	C
2020	Intensive/chicken	OP791965	A
2021	Intensive/chicken	OP791966	A
2021	Intensive/chicken	OP791967	A
2021	Intensive/chicken	OP791968	A
2021	Intensive/chicken	OP791969	A
2021	Extensive/chicken	OP791970	C
2021	Extensive/chicken	OP791971	A
2021	Intensive/chicken	OP791972	A

At necropsy, various pathological lesions were observed resembling Marek's disease lesions: liver and spleen enlargement, tumor nodules in the liver, and enlargement of the glandular part of the ventriculus with erosions and ulcerations on the mucosal surface. Additional lesions were observed also such as cachexia, degeneration, necrotic foci in the liver, cloacal prolapse, gizzard erosions, uricosis, and hemorrhages in the cecal tonsils. Recorded lesions are consistent with previously published reports by other authors [7,17,22-24] who reported pathological changes in certain internal organs.

The limitation of the study lies in the fact that the ICP4 fragment is short and highly conserved, which prevents the construction of a high-resolution phylogenetic tree. However, the objective is to present preliminary results, with plans to sequence and analyze the entire genome or major genes in the future.

CONCLUSION

In the present study, sequence analysis revealed that all strains circulating in intensive and extensively raised poultry productions in Serbia had high similarity with vv+MDV pathotypes. It could be expected occurrence of the same or similar pathotypes is due to the extensive trade exchange between Serbia and neighboring countries. It is also revealed that detected strains belong to the A and C virus groups. The prevalence monitoring and molecular characterization of MDV-isolated strains are necessary for large-scale outbreak prevention.

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Authors' contributions

JM and VM carried out the experimental work, made substantial contributions to the acquisition, analysis, interpretation of data and participated in manuscript writing. BK and BM have been involved in drafting and manuscript writing. LJS and NJ supervised the entire work. JM, VM, JK, BK, NJ and BM carried out laboratory diagnostics. All authors provided critical feedback and helped to shape the research and final paper.

Informed consent

Informed consent has been obtained for client-owned animals included in this study.

Ethical approval

Ethical approval is not applied as it is a retrospective analysis of historical samples.

Declaration of interest statement

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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VIRUS MAREKOVE BOLESTI U SRBIJI: DETEKCIJA I MOLEKULARNA KARAKTERIZACIJA (2015 – 2022)

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U okviru ovog istraživanja, prikupljeno je 236 uzoraka poreklom od živine iz intenzivnog i ekstenzivnog načina uzgoja koja su bila klinički suspektna na prisustvo virusa Marekove bolesti. Genom virusa Marekove bolesti je utvrđen u 84 (35,59 %) uzorka. Dobijeni rezultati ukazuju na značajnu prevalenciju virusa kod živine iz intenzivnog načina uzgoja (92,86 % ukupnih pozitivnih uzoraka) i u jatima ekstenzivno gajene živine (7,14 % ukupnih pozitivnih uzoraka). Analiza nukleotidne sekvence ICP4 gena pokazala je da su srpski sojevi klasifikovani u A i C grupe serotip 1, pokazujući visoku sličnost (>98 %) sa veoma virulentnim plus (vv+MDV) patotipovima mađarskih sojeva. Retrospektivna analiza od 2015. do 2022. godine pokazala je da virus Marekove bolesti kontinuirano cirkuliše u Srbiji. Cilj ovog istraživanja je bila detekcija i molekularna karakterizacija sojeva virusa Marekove bolesti u Srbiji.