Praca oryginalna *Original paper*

Comparison of different methods for the detection of Marek's disease virus

KATARZYNA KRÓL, ELŻBIETA SAMOREK-SALAMONOWICZ, WOJCIECH KOZDRUŃ, GRZEGORZ WOŹNIAKOWSKI

Department of Poultry Viral Diseases, National Veterinary Research Institute, Al. Partyzantów 57, 24-100 Puławy

Król K., Samorek-Salamonowicz E., Kozdruń W., Woźniakowski G.

Comparison of different methods for the detection of Marek's disease virus

Summary

The aim of this study was the comparison of accordance, sensitivity, and specificity of the radial agar gel immunodiffusion test (RID) and PCR for the detection of Marek's disease virus (MDV) infections. Feather tips, liver, and spleen sections collected during necropsy from 256 chickens gathered in 31 farms were used in the experiment. Characteristic for Marek's disease (MD) lesions were observed in 126 examined birds (49.2%). The viral antigen in feather follicles was detected in 145 chickens (56.6%). The results of necropsy showed 80.8% accordance with RID test. The organ sections were the source of total cellular DNA. Primers specific for 132 bp sequence of MDV genome and for viral gene meq were used for two different PCRs. The obtained results were identical in both molecular reactions. The presence of the viral DNA was confirmed in 181 samples (70.7%). The accordance of RID test and PCR results was valued at 85.9%. The percentage of positive results in RID was valued at 80.1% positive results for PCR. The specificity of PCR were 100%. The conducted experiment confirmed that the PCR is a valuable and efficient supplementary method of the classical diagnostic techniques of MD.

Keywords: Marek's disease, RID, gen meq, 132 bp sequence, PCR

Marek's disease (MD) is a viral disease of poultry that is clinically manifested by paralysis of legs and wings. During the post mortem examinations the presence of tumors in visceral organs can be observed. The etiological agent of the disease is a herpesvirus called Marek's disease virus (MDV) belonging to *Herpesviridae* family and *Alphaherpesvirinae* subfamily (5, 7, 9, 12).

On the basis of its serological features, the virus can be classified into three serotypes. The first serotype is represented by all the most virulent and oncogenic strains. The second serotype includes naturally nonpathogenic strains, while the third serotype is represented by nonpathogenic strains of turkeys. Inside the first serotype four pathotypes with different virulence and oncogenicity can be distinguished. Three genes, including 132 bp sequence, Meq and pp38 genes, are directly associated with oncogenicity of the first MDV serotype (5).

The diagnostics of MD is mainly based on anatomopathologic, histopatologic, virologic, and serologic examinations. The methods recommended by the World Organization of Animal Health (OIE) include a radial immunodiffusion test in agar gel, which is capable of detecting MDV in feather follicles, and a immunodiffusion assay in agar gel (AGID) for the detection of antibodies against Marek's disease virus antigens. These methods are rapid, cheap and routinely applied for the detection of the virus under survival conditions (10). Presently, in laboratory diagnostics the use of the polymerase chain reaction (PCR) and its several variants have become more common. The familiarity with MDV genome has allowed the application of PCR for the detection of the132 bp sequence and *meq* oncoprotein (1-3, 8).

The aim of the study was the comparison of the accordance, sensitivity, and specificity of anatomicopathologic changes with RID test and the recently developed PCR for the detection of Marek's disease virus in field samples.

Material and methods

Materials. Samples were collected during the necropsy of 256 chickens from 31 chicken farms suspected of Marek's disease virus infection. Feather follicles, and sections from the liver and spleen were taken.

Radial immunodiffusion assay (RID). The assay was conducted according to recommendations given by Marquardt et al. (11). The 1% solution of 1% agar in 8% sodium chloride was prepared. After heating the whole

mixture to 56°C, the solution of 20% standard positive serum against MDV with titer 8 was added. Petri dishes, 6 mm in diameter, were covered with a 3 mm agar layer. The feather tips were inserted into the agar at approximately 1 cm distance from each other. The dishes were incubated in a humid chamber at 37.2°C for 24-48 hours. The precipitate rings around the feather tips indicated the presence of the viral antigen.

DNA extraction. The extraction was performed with the A&A Biotechnology (DNA Gdańsk) commercial kit. The materials taken from the birds were homogenized. Two hundred microliters of the homogenisate were suspended in a mixture of 100 µl of TE buffer (10 mM TRIS, 1 mM EDTA, pH 8.0), 200 µl of LT buffer (phenol, chloroform, and isoamyl alcohol solution in 1 : 1 : 24 ratio) and 20 µl of proteinase K solution (20 mg of proteinase K in 1 ml of distilled water). The obtained suspension was incubated at 37°C until the total clarification of the solution was attained. After vigorous shaking for 20 seconds and incubation for 5 minutes in 75°C, the samples were centrifuged for 3 minutes at 500 × g. The volume of 300 µl of the supernatant was moved to the separation columns filled with silica bed in Eppendorf tubes and then centrifuged for 1 minute at 500 × g. Subsequently, 500 µl of A1 solution (96% ethanol with 5 M sodium chloride) was added and centrifuged again under the conditions previously used. The columns were moved to new tubes and 300 µl of A1 solution was added, after which all the tubes were centrifuged again. In the next step, TE buffer was heated to 75°C, and 200 µl of the buffer were added to each column. The silica bed was incubated for 5 minutes at room temperature and then centrifuged for 30 seconds at 500 × g. The extracted DNA was stored at –20°C.

Primers. The primers applied for the amplification were specific for 132 bp sequence and meq of Marek's disease virus. The primers' sequences for the 132 bp region were: 132F 5' TAC TTC TAT ATA GAT TGA GAC GT3' i 132R 5' GAG ATC CTC GTA AGG TGT AAT ATA 3'. The primers sequences specific for meq gene were: Meq1 5' GCA CTC TAG AGG TGT AAA GAG ATG TCT CAG3', Meq2 5'TAA CTC GAG GAG AAG AAA CAT GGG GCA TAG3'.

PCR conditions. Total volume of the PCR mixture was 50 µl and contained 5 µl of PCR buffer, 2 µl of dNTP mixture (0.2 mM), 2 µl of 5 mM magnesium chloride (not applied for meq gene amplification), 2 µl of each primer, 4 µl of template DNA, 1 µl of thermostabile DNA polymerase, and 32 µl of deionized water.

Thermal conditions. The following thermal conditions were applied for the amplification of 132 bp repeats and meq gene: 96°C for 2 minutes (initial detaturation); 94°C for 1 minute (exact denaturaion); 56°C for 1 minute (primers anealing); 72°C for 1 minute (chain prolongation); 72°C – 10 minutes (final prolongation of chain).

PCR products analysis. The PCR products were separated in 2% agarose gel in a TBE buffer (TRIS Base – 10.8 g; boric acid – 5.5 g; 4 ml of 0.5 M; EDTA at pH 8.0) for 1 hour under 120 V. Into each well 5 µl of reaction mixture and 2 µl of loading buffer (0.25% bromophenol blue; 40% water solution of sucrose) was loaded. The molecular weight of the obtained products was determined on the basis of a molecular weight marker, which was pUC19 plasmid DNA digested with HaeIII and TaqI (DNA Gdańsk). After finishing the separation, the gel was stained in ethidium bromide (1 µg/ml) for a half an hour.

Estimation of the accordance. Comparison of the necropsy, RID test, and PCR results was conducted out according to the given method (Fairchild et al.) (6).

Results and discussion

Marek's disease was a serious economic threat to the poultry industry until an effective vaccine was developed in 1969. In spite of regular immunoprophylactics, Marek's disease is still often diagnosed in chicken farms. The presence and intensity of characteristic lesions are conditioned by many different factors, like age of birds, breed, conditions of raising, and immunological status.

The percentage of birds with specific lesions reached about 20%-60% in the flocks. The enlargement of the liver and spleen with a number of necrotic and tumorous changes were observed in 126 examined birds (49.2%). The viral antigen in feather follicles was detected in of 145 birds using RID test (56.6%) (tab. 1).

Tab. 1. Summary of conducted experiments

Number of flocks	Number of samples	Number of birds with lesions	Number of positive results	
			RID	PCR
31	256	126 (49.2%)	145 (56.6%)	181 (70.7%)

In the next step of the trial, the PCR for detection of meq gene and 132 bp sequence, characteristic for strains of serotype 1 MDV, was applied. In the case of virulent MDV strains one specific product for meq gene – s-meq (1062 bp) was detected on the gel. In case of vaccine strains an additional band of size 1240 bp (l-meq) was visible. The second PCR product is an effect of attenuation, which leads to the insertion within one of two copies of the meq gene in MDV genome (4). Based on PCR for 132 bp sequence, only one product of size 434 bp was obtained for virulent strains, and for vaccine strains a characteristic „ladder" of PCR products (302 bp, 434 bp, 506 bp) was detectable. The ladder is a consequence of viral attenuation that causes duplication of 132 bp sequence in MDV genome (13). The results of both PCRs were identical, and the presence of MDV genome was confirmed in 181 examined samples (70.7%).

Subsequently, the accordance, sensitivity, and specificity of necropsy, RID test and PCRs results were confronted.

The MDV antigen was detected in feather follicles of 111 birds with lesions. The comparison of necropsy and RID test results (tab. 2) revealed 80.8% of their accordance. In the case of 34 animals in which no lesions were found, viral antigens were detected in 15 birds. On the other hand, the presence of an antigen was not confirmed in 15 birds with MD lesions.

Tab. 2. Comparison of necropsy and RID test results

Necropsy changes	RID		In general
	+	–	
+	111	15	126
–	34	96	130
Total	145	111	256

Accordance: $\frac{111 + 96}{256} \times 100\% = 80.8\%$;

Sensivity: $\frac{111}{145} \times 100\% = 76.5\%$;

Specificity: $\frac{96}{111} \times 100\% = 86.4\%$

Tab. 3. Comparison of necropsy and PCR results

Lesions	PCR		In general
	+	–	
+	126	0	126
–	55	75	111
Total	181	75	256

Accordance: $\frac{126 + 75}{256} \times 100\% = 78.5\%$;

Sensivity: $\frac{126}{181} \times 100\% = 69.6\%$;

Specificity: $\frac{75}{75} \times 100\% = 100\%$

Tab. 4. Comparison of RID test and PCR results

RID	PCR		In general
	+	–	
+	145	0	145
–	36	75	111
Total	181	75	256

Accordance: $\frac{145 + 75}{256} \times 100\% = 85.9\%$;

Sensivity: $\frac{145}{181} \times 100\% = 80.1\%$;

Specificity: $\frac{75}{75} \times 100\% = 100\%$

The sensitivity of RID test was estimated for 76.5%, and the specificity for 86.4%. The detectability of infected birds using RID test was higher as compared to necropsy.

The comparison of necropsy and PCR results showed that the characteristic amplification products for meq gene and 132 bp sequence was detected in 181 DNA samples and the tumors were observed in 126 birds (tab. 3). The accordance of both methods was 78.5%. Necropsy revealed the disease symptoms only in 69.6% of the examined birds in which the presence of MDV genome was confirmed. The specificity of PCR was evaluated for 100%.

In comparison to 181 positive samples in PCR, RID test disclosed viral antigen in 126 birds. The calculated accordance and sensitivity of both tests were in line in 85.9%, and 80.1%, respectively. The specificity of PCR was estimated at 100%, like previously.

Additionally, in 44 samples examined with PCR, the presence of products specific for MDV1 vaccine strain Rispens CVI988 was detected.

The elaboration of appropriate diagnostic methods is crucial for quick and reliable disease recognition and the implementation of administrative procedures. Nowadays, molecular methods, like PCR, are commonly applied for fast and efficient detection of viruses that cause difficulties with *in vitro* cultivation. The PCR method enables the identification of an etiological factor even before the lesions appear.

The conducted experiment confirmed that also in the case of MDV infections the PCR is a valuable and efficient supplementary method in the routine diagnostic techniques of MD.

References

1. *Baigent S. J., Smith L. P., Currie R. J., Venugopol N.*: Replication kinetics of Marek's disease vaccine virus in feathers and lymphoid tissues using PCR and virus isolation. J. Gen. Virol. 2005, 86, 2989-2998.
2. *Becker Y., Asher Y., Tabor E., Davidson I., Malkinson M., Weisman Y.*: Polymerase chain reaction for differentiation between pathogenic and non-pathogenic serotype 1 Marek's disease virus (MDV) and vaccine viruses of MDV – serotype 2 and 3. J. Virol. Methods. 1992, 40, 307-322.
3. *Becker Y., Tabor E., Asher Y., Davidson I., Malkinson M., Witter R. L.*: PCR detection of amplified 132 bp repeats in Marek's disease virus type 1 (MDV-1) DNA can serve as an indicator for critical genomic rearrangement leading to the attenuation of virus virulence. Virus Genes. 1992, 3, 277-287.
4. *Chang K. S., Ohashi K., Onuma M.*: Diversity (Polymorphism) of the meq gene in the attenuated Marek's disease virus (MDV) serotype 1 and MDV T-transformed cell lines. J. Vet. Med. Sci. 2002, 64, 1097-1101.
5. *Davidson I., Borenstein R.*: Multiple infection of chickens and turkeys with avian oncogenic viruses: prevalence and molecular analysis. Acta Virol. 1999, 43, 136-142.
6. *Fairchild G. A., Satz J., Cohen D.*: Charcoal agglutination test for the detection of antibodies to ICHV. Comparison of agglutination, complement – fixation and serum – neutralization tests. Am. J. Vet. Res. 1968, 29, 1259--1263.
7. *Kozdruń W., Samorek-Salamonowicz E., Czekaj H.*: Budowa molekularna wirusa choroby Mareka. Medycyna Wet. 2004, 60, 1164-1167.
8. *Kozdruń W., Samorek-Salamonowicz S., Czekaj H., Król K.*: Opracowanie multi PCR do wykrywania szczepów wirusa choroby Mareka. Medycyna Wet. 2005, 61, 711-714.
9. *Król K., Samorek-Salamonowicz E., Kozdruń W.*: Molekularna charakterystyka genów i białek wirusa choroby Mareka. Post. Mikrobiol. 2005, 44, 351-356.
10. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. Chapter 2.7.2 – Marek's disease. OIE, Paris 2006.
11. *Marquardt W. M.*: Avian complements – fixation. Avian Dis. 1973, 17, 236--239.
12. *Nazerian K., Solomon J. J., Witter R. L., Burmester B. R.*: Studies on the etiology of Marek's disease. II. Finding of a herpesvirus in cell culture. Proc. Soc. Exp. Biol. Med. 1968, 127, 177-182.
13. *Reddy S. M., Witter R. L., Gimeno I.*: Development of a quantitative – competitive polymerase chain reaction assay for serotype 1 of Marek's disease virus. Avian Dis. 2000, 44, 770-775.

Author's address: dr Katarzyna Król, Al. Partyzantów 57, 24-100 Puławy; e-mail: katarzyna.krol@piwet.pulawy.pl