AVIAN DISEASES 57:785–789, 2013

Research Note—

Use of Polymerase Chain Reaction in Detection of Marek's Disease and Reticuloendotheliosis Viruses in Formalin-Fixed, Paraffin-Embedded Tumorous Tissues

Weisheng Cao,[AB] Jody Mays,[A] John Dunn,[A] Richard Fulton,[C] Robert Silva,[A] and Aly Fadly[AD]

[A]United States Department of Agriculture, Agriculture Research Service, Avian Disease and Oncology Laboratory, East Lansing, MI 48823
[B]College of Veterinary Medicine, South China Agricultural University, Guangdong Province, 510642, P. R. China
[C]College of Veterinary Medicine, Michigan State University, East Lansing, MI 48824

Received 4 April 2013; Accepted 19 June 2013; Published ahead of print 19 June 2013

SUMMARY. A simple PCR method was developed for the detection of Marek's disease (MD) and reticuloendotheliosis (RE) in formalin-fixed paraffin-embedded (FFPE) tissues, and for the detection of MD in tissues only preserved in 10% neutral buffered formalin. MD virus (MDV) and RE virus proviral DNA were detected in FFPE tissues stored for over 20 yr. MDV was also detected in tissues only preserved in formalin for up to 6 mo. The data indicate that PCR of formalin-fixed and FFPE tissues is a simple and valuable tool that can be used to identify MD and RE infection. The method described in this paper is a good alternative to any biologic or immunohistochemical assay to confirm the detection of MD and RE, as it does not require shipping frozen tissues to the diagnostic laboratory.

RESUMEN. *Nota de Investigación*—Aplicación de la reacción en cadena de la polimerasa para la detección de los virus de la enfermedad de Marek y de la reticuloendoteliosis en tejidos tumorales fijados en formalina e incluidos en parafina.

Se desarrolló un método simple de PCR para la detección de la enfermedad de Marek (MD) y de la reticuloendoteliosis (ER) en tejidos fijados con formalina e incluidos en parafina (FFPE) y para la detección del virus de Marek únicamente en los tejidos conservados en formalina al 10% neutra y amortiguada. Se detectaron los virus de Marek y el ADN proviral del virus de la reticuloendoteliosis en tejidos fijados e incluidos en parafina que estaban almacenados durante más de 20 años. El virus de Marek también se detectó en los tejidos que estaban únicamente conservados en formol por hasta seis meses. Los datos indican que el método de PCR utilizando tejidos fijados con formalina e incluidos en parafina es una herramienta simple y valiosa que se puede utilizar para identificar al virus de la enfermedad de Marek y de la reticuloendoteliosis. El método descrito en este documento es una buena alternativa para cualquier ensayo biológico o inmunohistoquímico para confirmar la detección de estos dos virus, ya que no requiere del envío de tejidos congelados al laboratorio de diagnóstico.

Key words: Marek's disease virus, reticuloendotheliosis virus, formalin-fixed paraffin-embedded tissues, polymerase chain reaction, detection

Abbreviations: ADOL = Avian Disease and Oncology Laboratory; ALV = avian leukosis virus; FF = formalin-fixed; FFPE = formalin-fixed paraffin-embedded; GAPDH = glyceraldehyde 3-phosphate dehydrogenase; HVT = turkey herpesvirus; IBDV = infectious bursal disease virus; IHC = immunohistochemistry; LTR = long terminal repeat; MD = Marek's disease; MDV = Marek's disease virus; NDV = Newcastle disease virus; PBS = phosphate-buffered saline; qPCR = quantitative real-time polymerase chain reaction; RE = reticuloendotheliosis; REV = reticuloendotheliosis virus; RT-PCR = reverse-transcriptase polymerase chain reaction; SNV = spleen necrosis virus

Marek's disease (MD), a common lymphoproliferative disease of chickens, is caused by a highly transmissible cell-associated oncogenic alphaherpesvirus (2,13,18,24) designated MD virus (MDV). MD is characterized by mononuclear cellular infiltrates in peripheral nerves and various organs and tissues (20).

Prior to the introduction of MD vaccines, MD was a major economic threat to the poultry industry with MD outbreaks sweeping through poultry flocks in the early 1960s (27). With the gradual introduction of MD vaccines including the first MD vaccine HPRS-16 (3) followed by a naturally avirulent turkey herpesvirus (HVT) (15,30), nonpathogenic serotype 2 MDV, SB-1 (19) or 301B/1 (26), and attenuated serotype 1 MDV CVI988 (16), massive economic losses as a result of MD-related condemnations and mortality were substantially reduced for breeders, layers, and broilers. However, there is evidence that MDV continues to evolve, with major virulence shifts observed following HVT and bivalent vaccination (28). In addition, several recent isolates obtained between 1998–2000 demonstrated higher pathogenicity in chickens vaccinated with CVI988 (28).

Other economically important neoplastic diseases of poultry include the avian retroviruses avian leukosis virus (ALV) and reticuloendotheliosis virus (REV). Diagnosis of tumorous diseases of poultry depends upon pathologic criteria, especially the distribution of lesions and histologic characteristics of the tumor cells (22). Because many of the avian tumor viruses sometimes induce a variety of neoplasms of similar pathologic lesions, classification and differentiation among MDV, ALV, and REV can be difficult (6). This dilemma is further confounded by the fact that avian tumor viruses are widespread and infection can occur without tumor formation (6). In the absence of pathognomonic gross and microscopic lesions, definitive diagnosis of virus-induced tumorous diseases of poultry requires further testing including biologic as well as molecular or immunohistochemistry (IHC) assays (or both) (6).

[D]Corresponding Author. E-mail: ALY.FADLY@ARS.USDA.GOV

Table 1. List of FFPE blocks of tissues collected from chickens inoculated with MDV and stored for various time intervals before processing for PCR tests.

Specimen[A]	Virus strain	Pathotype	Tissue[B]	Storage time
1	AC722	vv+	Ht, Gon, Kid, Liv	7 days
2	AC722	vv+	Spl	7 days
3	AC722	vv+	Ht, Spl, Kid, Liv	7 days
4	AC722	vv+	Ht, Vag	7 days
5	648A-8	vv+	Bur	7 days
6	648A-50	vv+	Spl	5 mo
7	648A-50	vv+	Liv	5 mo
8	648A-50	vv+	Kid, Lng, Gon	5 mo
9	648A-50	vv+	Lng, Kid, Spl	5 mo
10	648A-50	vv+	Ht, Kid	5 mo
11	rMD5-10	vv	Spl, Gon	2–3 yr
12	rMD5-REV-LTR	vv	Ht, Bur	2–3 yr
13	rMD5-40	vv	Spl, Ht, Vag	2–3 yr
14	rMD5-40	vv	Spl	2–3 yr
15	rMD5-10	vv	Prov	2–3 yr
16	rMD5-10	vv	Ht, Spl	2–3 yr
17	RB1B	vv	Liv, Spl	20 yr
18	RB1B	vv	Liv	20 yr
19	RB1B	vv	Liv, Vag	20 yr
20	RB1B	vv	Vag, Gon	20 yr
21	RB1B	vv	Liv, Vag	20 yr
22	None		Spl, Bur	20 yr
23	None		Kid, Spl, Thy	2 yr
24	None		Bur	2 yr
25	Trivalent MDV vaccine		Spl	7 days
26	Trivalent MDV vaccine		Spl	7 days
27	Trivalent MDV vaccine		Spl	7 days
28	REV-SNV		Lng	20 yr
29	REV-SNV		Liv, Lng	20 yr
30	REV-SNV		Liv, Kid, Ht	20 yr

[A]Specimens 22–27 are nontumorous tissues and were collected from uninoculated control chickens (22–24), and from chickens vaccinated with trivalent MDV vaccine (25–27). Specimens 28–30 had lesions of REV-induced lymphoma.

[B]Bur = bursa of Fabricius; Gon = gonads; Ht = heart; Kid = kidney; Liv = liver; Lng = lung; Prov = proventriculus; Spl = spleen; Thy = thymus; Vag = vagus nerve.

Witter and colleagues (8,29) developed a useful strategy toward differential diagnosis of viral lymphomas in chickens based on quantitative real-time PCR (qPCR) and IHC. However, both procedures require fresh or frozen tissue. Although qPCR has been used in the diagnosis of MD in tumor imprints, feather pulp, or blood collected in FTA cards (4), most diagnostic laboratories routinely process affected tissues for microscopic examination to confirm diagnosis of avian tumors and, thus, there is a need to determine whether DNA extracted from formalin-fixed paraffin-embedded (FFPE) and formalin-fixed (FF) tissues can be used for detection of these two important avian tumor viruses, namely MDV and REV. It is also recognized that fresh or frozen tissues are not always readily available, and shipping restrictions for shipping frozen tissues on dry ice further limit the availability of tissue samples for analysis.

Preservation of tumor tissues in FFPE blocks is a useful method to preserve the samples for microscopic diagnosis. Techniques for processing FFPE tissues for microscopic evaluation are available in most diagnostic laboratories. Nucleic acid has been successfully extracted from archived FFPE tissues and analyzed by reverse-transcriptase PCR (RT-PCR) or qPCR (or both) for the identification of human or fish diseases (7,9,12,14,21,23). Other avian viruses, Newcastle disease virus (NDV) (25) and infectious bursal disease virus (IBDV) (10), have been detected in FFPE tissues by RT-PCR and IHC; also, IHC and *in situ* hybridization have been used successfully in demonstrating REV in FFPE tissues (17). The specificity and

Table 2. Primers used for detection of MDV and REV in FFPE or formalin-fixed tissues.

Primer name RE (reference no.)	Sequence	Product length (bp)/target gene
GAPDH-AB (31)		(123)/GAPDH
Forward	5′-TGCCATCACAGCCACACAGAAG-3′	
Reverse	5′-ACTTTCCCCACAGCCTTAGCAG-3′	
MdCv-F/R (5)		(225)/MDV pp38
Forward	5′-GTGATGGGAAGGCGATAGAA-3′	
Reverse	5′-TCCGCATATGTTCCTCCTTC-3′	
SNV-LTR-F/R (11)		(200)/REV-LTR
Forward	5′-AATGGTTGTAAAGGGCAGAT-3′	
Reverse	5′-CTCCTCTCACTGCCAATCT-3′	

Table 3. Detection of MDV by PCR in FFPE tissues that had been stored for various time intervals.

Infection status	Storage time	No. of PCR+/no. tested	
		pp38	GAPDH
MDV infected	1 wk	5/5	5/5
	5 mo	5/5	5/5
	2–3 yr	7/7	7/7
	20 yr	4/4	4/4
Uninfected	20 yr	0/3	3/3
Trivalent MD vaccine	1 wk	0/3	3/3
REV infected	20+ yr	0/3	3/3

Table 4. Detection of REV by PCR in archived FFPE tissues.

Infection status	Storage time	No. of PCR+/no. tested	
		REV-LTR	GAPDH
REV infected	20 yr	13/19	19/19
Uninfected	5 months	0/1	1/1
	2–3 yr	0/2	2/2
	20 yr	0/1	1/1
MDV infected	2–3 yr	0/1	1/1
	20 yr	0/1	1/1

sensitivity of PCR using FFPE or FF tissues in the detection of MDV and REV is not known and need to be determined.

We have developed a simple and effective PCR method for the detection of MDV and REV proviral DNA in FFPE tissues as well as for MDV in tissues only preserved in 10% neutral buffered formalin.

MATERIALS AND METHODS

Tissue samples and inoculation. Archived FFPE tissues from chickens experimentally infected with MDV and REV were used in this study. For MDV detection, archived tissues were obtained from a total of 21 FFPE blocks of various tissues from MDV-infected chickens stored over a span of 20 yr (Table 1). All embedded tissues were from tumors collected fresh and immediately fixed in 10% neutral buffered formalin (average period of fixation was 5–7 days at room temperature). Fixed tissues were routinely processed through dehydration in graded ethanol and then cleared in xylene and embedded in paraffin blocks. A few FFPE tissue blocks were stored for 7 days; however, the majority of FFPE tissue blocks were stored at room temperature for a period that ranged from 5 to 243 mo.

For REV detection a total of 19 archived FFPE blocks were from chickens experimentally infected with strain spleen necrosis virus (SNV) of REV only, or SNV + HVT, or SNV + strain 301B of serotype 2 MDV (1). At the time of PCR analysis, all REV FFPE tissue blocks had been stored at room temperature for a period of 240 to 245 mo.

In order to evaluate the ability of PCR to detect pp38, an MDV gene in chickens vaccinated with attenuated serotype 1 MDV vaccine, 30 1-day-old chicks from the Avian Disease and Oncology Laboratory (ADOL) line $6_3 \times 7_2$ were vaccinated via the intra-abdominal route with 2000 plaque-forming units (pfu) of strain CVI988 of MDV vaccine. At 1, 3, 5, and 7 wk postinfection, spleen samples were taken from five randomly selected chickens. The spleen tissues were fixed in 10% buffered Formalde-Fresh® formalin (Fisher Chemicals, Pittsburgh, PA) containing 4% w/v formaldehyde, 0.075 M sodium phosphate buffer, and 1.5% w/v methanol. At 7 days postfixation, 0.01 g tissue was washed twice in sterile phosphate-buffered-saline (PBS) and homogenized in 50 µl sterile PBS using a Kontes Pellet Pestle® and Motor (Daigger®, Vernon Hills, IL). The pestle was washed with 1 ml sterile PBS and the homogenate was centrifuged for 5 min at 3000 rpm (Beckman J6-HC Centrifuge, Beckman Instruments, Inc., Pasadena, CA) before DNA extraction.

In order to evaluate the effect of fixation time on quantity and quality of DNA extracted from fixed tissues, 18 fresh tumorous tissues (spleen, heart, liver, and gonad) were collected from MDV-infected, maternal antibody-positive ADOL $15I_5 \times 7_1$ chickens and placed immediately in 10% neutral buffered formalin. At 0, 7, 14, 21, 28, 35, 49, 70, 90, 120, 150, and 180 days postfixation, 0.01 g of tissue was processed and homogenized as above for DNA extraction and PCR analysis.

DNA extraction. The DNA extraction procedure was based upon heat-induced extraction in alkaline solution as previously described by Shi *et al.* (21) with some modification. Briefly, two 10-µm thick sections were cut from each paraffin block by a Leica RM2125 rotary microtome (Leica Microsystems Inc., Richmond, IL). Sections from FFPE blocks and homogenized tissue pellets from tissues only fixed in formalin were suspended in 600 µl of 0.1 M NaOH and autoclaved at 120 C for 20 min. After cooling to room temperature for about 5 min, DNA was extracted using phenol/chloroform extraction and precipitated with 0.1 vol 3 M Na acetate and 1.0 vol isopropanol. The pelleted DNA was dissolved in 50 µl double-distilled DNAse- and RNAse-free water and stored at −20 C until further analysis by PCR.

PCR analysis. Each DNA extract was used as a template for PCR amplification using primers specific for chicken GAPDH, MDV pp38, and REV long terminal repeat (LTR; Table 2). In a total reaction volume of 25 µl, 50 ng of template DNA in a 2-µl volume was mixed with 25 µM of each primer and 12.5 µl GoTaq® Green Master Mix 2X (Promega, Madison, WI) containing dNTP. The thermal cycling was

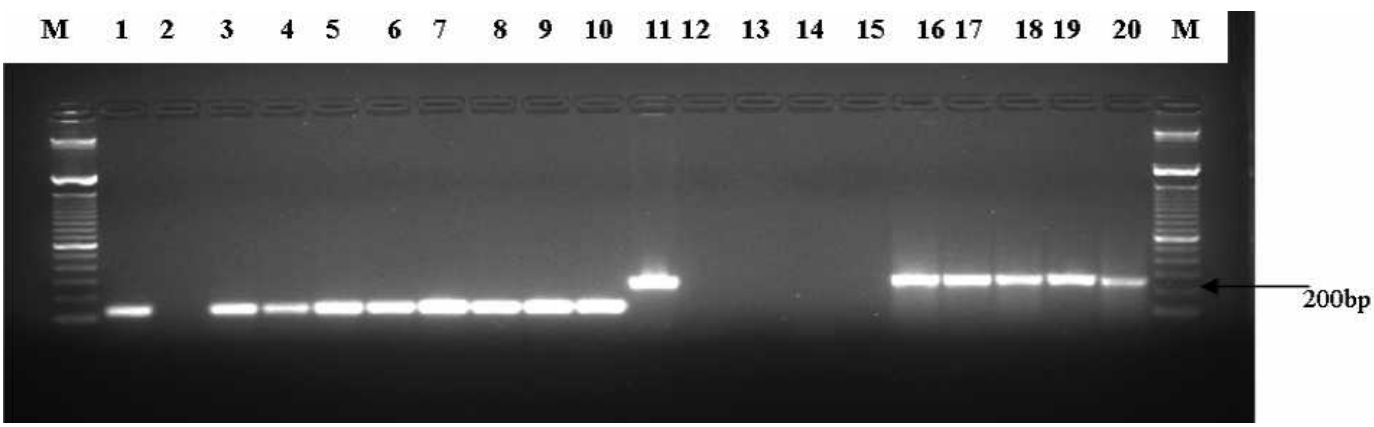

Fig. 1. PCR detection of MDV pp38 in archived FFPE tissues. Lane M: DNA molecular weight markers; Lanes 1–10: GAPDH primers; Lane 1: CEF; Lane 2: H_2O; Lane 3: uninfected; Lane 4: CVI988 vaccinated (74 days PV); Lane 5: RE FFPE; Lanes 6–10: MD FFPE samples; Lanes 11–20: pp38 primers; Lane 11: strain 648A of vv+ MDV (passage 50); Lane 12: H_2O; Lane 13: uninfected; Lane 14: CVI988 vaccinated (74 days PV); Lane 15: RE FFPE sample; Lanes 16–20: MD FFPE samples.

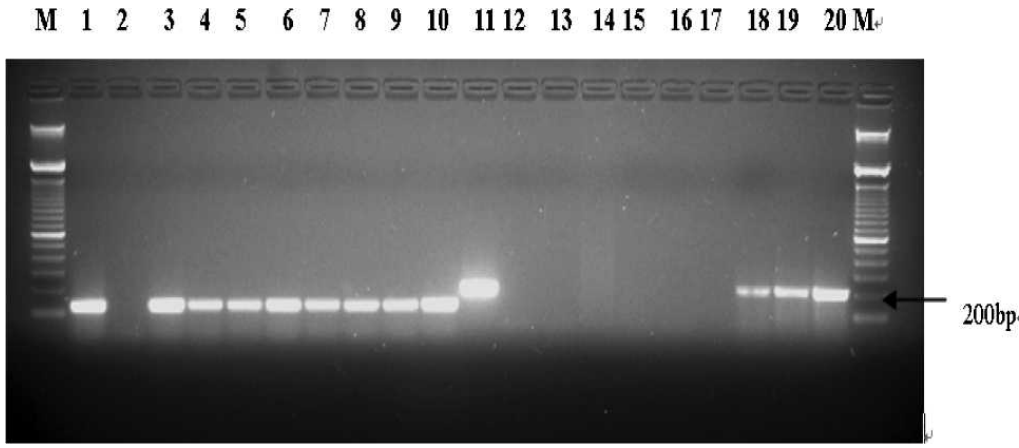

Fig. 2. PCR detection of REV LTR in archived FFPE tissues. Lane M: DNA molecular weight markers; Lanes 1–10: GAPDH primers; Lane 1: CEF; Lane 2: H_2O; Lanes 3–4: uninfected; Lanes 5–6: MDV FFPE; Lanes 7–10: RE FFPE; Lanes 11–20: REV LTR primers; Lane 11: SNV; Lane 12: H_2O; Lanes 13–14: uninfected; Lanes 15–16: MDV FFPE samples; Lanes 17–20: RE FFPE samples.

carried out with an initial 3-min denaturation step at 95 C coupled with repeated cycle parameters of 40 sec at 95 C (denaturation), 40 sec at 57 C (annealing), and 30 sec at 72 C (extension) for 36 cycles, followed by an 8-min completion step at 72 C and then cooled down to 4 C. All PCR reactions were carried out using the PTC-100® Peltier Thermal cycler (Bio-Rad, Hercules, CA). The PCR products were visualized and photographed in a 1.0% agarose gel containing 0.5 μg/ml ethidium bromide using the AlphaImager™ (Alpha Innotech Inc., San Leandro, CA).

RESULTS AND DISCUSSION

The availability of FFPE tissue blocks represents an invaluable and largely untapped resource for the detection and diagnosis of avian neoplastic diseases. In our study, storage time of FFPE tissues did not affect the ability to detect MDV pp38 in archived FFPE tissue stored for over 20 yr (Table 3; Fig. 1). The MDV pp38 primers were specific for MDV-infected tissues and did not amplify product from uninfected tissues, REV-infected tissues, or from tissues collected from chickens vaccinated with trivalent MDV vaccine. Also, PCR analysis of DNA extracted from archived (20+ yr) REV-infected FFPE tissues indicated that REV LTR was detected in 13/19 (68%) of REV-infected tissues examined (Table 4). The REV LTR PCR analysis was equally specific in that no PCR product was amplified in MDV-positive samples used as controls (Fig. 2).

Fixation of tissue samples in formaldehyde leads to extensive cross-linking of all tissue components, resulting in fragmentation of nucleic acids recovered from FFPE tissues (14). Many modifications in DNA and RNA extraction methods from FFPE tissues have been implemented in order to optimize nucleic acid yields and size; however, the PCR amplification of products over 300–400 bp is limited (9,14). In the present study we were able to achieve efficient PCR amplification of products less than 250 bp.

Fixation of MDV-infected tissues in 10% buffered formalin did not affect the ability to amplify PCR product when tissues were stored for less than 150 days (Table 5). However, only 4/5 (80%) and 2/5 (40%) of known MDV-infected tissues tested positive for MDV pp38 by PCR at 150 days and 180 days postfixation, respectively.

In this paper we report the detection of MDV and REV by PCR in archived FFPE tissues. Other avian viruses, NDV (25) and IBDV (10), have been detected in FFPE tissues by RT-PCR and IHC; also, IHC and *in situ* hybridization have been used successfully in demonstrating REV in FFPE tissues (17). Results from this study suggest that in most cases of MDV-specific tumors, the presence of CVI988 vaccination will not interfere with detection of MDV infection. Only 1/5 (20%) and 0/5 (0%) of spleens tested positive by PCR for CVI988 pp38 at 35 and 49 days postvaccination, respectively (Table 6), suggesting that CVI988 vaccination will not lead to false-positive results in analysis of FFPE or FF tissues when MDV tumors are collected from chickens 7 wk of age or older.

We have described a method for extracting DNA from FFPE tissues and the use of PCR to demonstrate MDV or REV in tumorous tissues. Use of FFPE tumorous tissues is a useful alternative with which to identify MDV and REV infection due to the relative ease of shipping FFPE tissues to diagnostic laboratories.

Table 5. PCR detection of MDV-infected tissue after being fixed in 10% buffered formalin for various time intervals.

MDV infected	Fixation time in days	No. of PCR+/no. tested	
		MDV pp38	GAPDH
+	0	11/11	11/11
+	7	18/18	18/18
+	14	18/18	18/18
+	21	18/18	18/18
+	28	18/18	18/18
+	35	18/18	18/18
+	49	17/17	17/17
+	70	5/5	5/5
+	90	5/5	5/5
+	120	5/5	5/5
+	150	4/5	5/5
+	180	2/5	5/5
−	0	0/2	2/2
−	7	0/2	2/2
−	21	0/2	2/2
−	35	0/2	2/2

Table 6. PCR detection of MDV pp38 in spleen tissues from chickens at various times following vaccination with Rispens strain of MDV at hatch.

Weeks postvaccination	No. of PCR+/ no. tested	
	MDV pp38	GAPDH
1	5/5	5/5
3	3/5	5/5
5	1/5	5/5
7	0/5	5/5

Finally, additional studies are required to determine if the methods described in this paper, namely use of DNA extracted from FFPE and FF tumorous tissues, are suitable for diagnosis of MD tumors by quantification of MDV DNA, as described elsewhere (4).

REFERENCES

1. Aly, M. M., R. L. Witter, and A. M. Fadly. Enhancement of reticuloendotheliosis virus-induced bursal lymphomas by serotype 2 Marek's disease virus. Avian Pathol. 25:81–94. 1996.

2. Buckmaster, A. E., S. D. Scott, M. J. Sanderson, M. E. G. Boursnell, L. J. N. Ross, and M. M. Binns. Gene sequence and mapping data from Marek's disease virus and herpesvirus of turkeys: implications for herpesvirus classification. J. Gen. Virol. 69:2033–2042. 1988.

3. Churchill, A. E., L. N. Payne, and R. C. Chubb. Immunization against Marek's disease using live attenuated virus. Nature 221:744–747. 1969.

4. Cortes, A. L., E. R. Montiel, S. Lemiere, and I. M. Gimeno. Comparison of blood and feather pulp samples for the diagnosis of Marek's disease and for monitoring Marek's disease vaccination by real time-PCR. Avian Dis. 55:302–310. 2011.

5. Dunn, J. R., R. L. Witter, R. F. Silva, L. F. Lee, J. Finlay, B. A. Marker, J. B. Kaneene, R. M. Fulton, and S. D. Fitzgerald. The effect of the time interval between exposures on the susceptibility of chickens to superinfection with Marek's disease virus. Avian Dis. 54:1038–1049. 2010.

6. Fadly, A. M. Neoplastic diseases. In: Diseases of poultry, 12th ed. Y. M. Saif, A. M. Fadly, J. R. Glisson, L. R. McDougald, L. K. Nolan, and D. E. Swayne, eds. Blackwell Publishing, Ames, IA. p. 449. 2008.

7. Fringuelli, E., A. W. Gordon, H. Rodger, M. D. Welsh, and D. A. Graham. Detection of *Neoparamoeba perurans* by duplex quantitative TaqMan real-time PCR in formalin-fixed, paraffin-embedded Atlantic salmonid gill tissues. J. Fish Dis. 35:711–724. 2012.

8. Gimeno, I. M., R. L. Witter, A. M. Fadly, and R. F. Silva. Novel criteria for the diagnosis of Marek's disease virus-induced lymphomas. Avian Pathol. 34:332–340. 2005.

9. Godfrey, T. E., S.-H. Kim, M. Chavira, D. W. Ruff, R. S. Warren, J. W. Fray, and R. H. Jensen. Quantitative mRNA expression analysis from formalin-fixed, paraffin-embedded tissues using 5′ nuclease quantitative reverse transcription-polymerase chain reaction. J. Mol. Diagn. 2:84–91. 2000.

10. Hamoud, M. M., P. Villegas, and S. M. Williams. Detection of infectious bursal disease virus from formalin-fixed paraffin-embedded tissue by immunohistochemistry and real-time reverse transcription-polymerase chain reaction. J. Vet. Diagn. Invest. 19:35–42. 2007.

11. Kim, T., J. Mays, A. Fadly, and R. F. Silva. Artificially inserting a reticuloendotheliosis virus long terminal repeat into a bacterial artificial chromosome clone of Marek's disease virus (MDV) alters expression of nearby MDV genes. Virus Genes 42:369–376. 2011.

12. Koepsell, S. A., S. H. Hinrichs, and P. C. Iwen. Applying a real-time PCR assay for *Histoplasma capsulatum* to clinically relevant formalin-fixed paraffin-embedded human tissue. J. Clin. Microbiol. 50:3395–3397. 2012.

13. Lee, L. F., P. Wu, D. Sui, D. Ren, J. Kamil, H. J. Kung, and R. L. Witter. The complete unique long sequence and the overall genomic organization of the GA strain of Marek's disease virus. Proc. Natl. Acad. Sci. U. S. A. 97:6091–6096. 2000.

14. Lehmann, U., and H. Kreipe. Real-time PCR analysis of DNA and RNA extracted from formalin-fixed and paraffin-embedded biopsies. Methods 25:409–418. 2001.

15. Okazaki, A. W., H. G. Purchase, and B. R. Burmester. Protection against Marek's disease by vaccination with herpesvirus of turkeys. Avian Dis. 14:413–429. 1970.

16. Rispens, B. H., H. Van Vloten, N. Mastenbroek, H. J. L. Maas, and K. Schat. Control of Marek's disease in the Netherlands, Part 2. Field trials on vaccination with an avirulent strain CVI998 of Marek's disease virus. Avian Dis. 16:126–138. 1972.

17. Santos, V. L. S. L., S. M. Williams, G. Zavala, T. Barbosa, J. Zhang, S. Cheng, H. L. Shivaprasad, S. Hafner, A. Fadly, R. L. Santos, and C. C. Brown. Detection of reticuloendotheliosis virus by immunohistochemistry and *in situ* hybridization in experimentally infected Japanese quail embryos and archived formalin-fixed and paraffin-embedded tumours. Avian Pathol. 37:451–456. 2008.

18. Schat, K. A. Characteristics of the virus. In: Marek's disease, scientific basis and methods of control. L. N. Payne, ed. Martinus Nijhoff, Dordrecht, the Netherlands. pp. 77–112. 1985.

19. Schat, K. A., and B. W. Calnek. Characterization of an apparently nononcogenic Marek's disease virus. J. Natl. Cancer Inst. 60:1075–1082. 1978.

20. Schat, K. A., and V. Nair. Marek's disease. In: Diseases of poultry, 12th ed. Y. M. Saif, A. M. Fadly, J. R. Glisson, L. R. McDougald, L. K. Nolan, and D. E. Swayne, eds. Blackwell Publishing, Ames, IA. pp. 452–514. 2008.

21. Shi, S.-R., R. Datar, C. Liu, L. Wu, Z. Zhang, R. J. Cote, and C. R. Taylor. DNA extraction from archival formalin-fixed, paraffin-embedded tissues: heat-induced retrieval in alkaline solution. Histochem. Cell Biol. 122:211–218. 2004.

22. Siccardi, F. J., and B. R. Burmester. The differential diagnosis of lymphoid leukosis and Marek's disease. USDA Technical Bulletin 1412:1–24. 1970.

23. Sinicropi, D., K. Qu, F. Collin, M. Crager, M.-L. Liu, R. J. Pelham, M. Pho, A. D. Rossi, J. Jeong, A. Scott, R. Ambannavar, C. Zheng, R. Mena, J. Esteban, J. Stephans, J. Morlan, and J. Baker. Whole transcriptome RNA-Seq analysis of breast cancer recurrence risk using formalin-fixed paraffin-embedded tumor tissue. PLoS One 7:e40092. 2012.

24. Tulman, E. R., C. L. Afonso, Z. Lu, L. Zsak, D. L. Rock, and G. F. Kutish. The genome of a very virulent Marek's disease virus. J. Virol. 74:7980–7988. 2000.

25. Wakamatsu, N., D. J. King, B. S. Seal, and C. C. Brown. Detection of Newcastle disease virus RNA by reverse transcription-polymerase chain reaction using formalin-fixed, paraffin-embedded tissue and comparison with immunohistochemistry and *in situ* hybridization. J. Vet. Diagn. Invest. 19:396–399. 2007.

26. Witter, R. L. New serotype 2 and attenuated serotype 1 Marek's disease vaccine viruses: comparative efficacy. Avian Dis. 31:752–765. 1987.

27. Witter, R. L. Increased virulence of Marek's disease virus field isolates. Avian Dis. 41:149–163. 1997.

28. Witter, R. L. Marek's disease vaccines—past, present, and future. In: Current progress on Marek's disease research. Proc. 6th International Symposium on Marek's disease, American Association of Avian Pathologists, Kennett Square, PA. pp. 1–9. 2001.

29. Witter, R. L., I. M. Gimeno, and A. M. Fadly. Differential diagnosis of lymphoid and myeloid tumors in the chicken. AAAP slide study set. American Association of Avian Pathologists, Athens, GA 27:1–49. 2005.

30. Witter, R. L., and K. Nazerian. Isolation from turkeys of a cell associated herpesvirus antigenically related to Marek's disease virus. Amer. J. Vet. Res. 31:525–538. 1970.

31. Xu, M., H. Zhang, L. Lee, H. Gao, S. Sharif, R. F. Silva, and M. Heidari. Gene expression profiling in rMd5- and rMd5Δmeq-infected chickens. Avian Dis. 55:358–367. 2011.

ACKNOWLEDGMENTS

We thank Ms. Melanie Flesberg and Mr. Lonnie Milam for their excellent technical assistance.