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Detection of Pathogenic Marek's Disease Virus by Dot Blot Hybridization and PCR in Commercial Chickens in China

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Abstract: Marek's Disease Virus (MDV) is an oncogenic herpesvirus that causes various clinical syndromes in chickens which lead to huge economic losses for poultry industry. Compared with virulent MDV (vMDV) strains it was found that the vaccine strain CVI988 had a 5 bp deletion in the bi-directional promoter region in the genome of MDV. Based on the deletion, researchers developed a Polymerase Chain Reaction (PCR) for the rapid identification of vMDV strains from vaccine CVI988 strain. In the present study, the sensitivity and specificity of different methods including PCR, virus isolation and dot blot hybridization for detecting MDV were compared. The results showed that PCR and dot blot hybridization assays were comparable in sensitivity to virus isolation and there were good correlations in positive results among the three techniques. In addition, the PCR assay similar with virus isolation was specific enough to identify CVI988 vaccine strain from vMDV. In company with PCR, the dot blot hybridization with the probe of *pp38* gene labeled with digoxigenin were used to monitor the vMDV infection in commercial chicken flocks in China. The data demonstrated that MDV were widespread in commercial chickens in China and the combination of dot blot hybridization and PCR assay was useful for screening for wild vMDV strains infection in chickens.

Key words: Detection, Marek's disease virus, dot blot hybridization, PCR, China

INTRODUCTION

Marek's Disease Virus serotype 1 (MDV-1), a chicken alphaherpesvirus causes malignant T-cell lymphomas and neurological disorders (Biggs *et al*., 1965; Lampert *et al*., 1977; Witter, 1997). MDV-1 induced tumors could be effectively controlled by the use of live MDV vaccines of three serotypes including attenuated, avirulent MDV-1 (Gallid herpes virus 2/GaHV-2), the naturally occurring avirulent MDV-2 (Gallid herpes virus 3/GaHV-3) and the naturally occurring avirulent herpesvirus of Turkey (HVT or Meleagrid herpes virus 1/MeHV-1) (De Boer *et al*., 1986; Okazaki *et al*., 1970; Schat and Calnek, 1978). However, vaccination reduces but does not prevent the superinfection, replication and shedding of virulent MD challenge viruses (Davison and Nair, 2005; Gimeno, 2008). Therefore, in the field, chickens could be infected simultaneously with vaccine and vMDV strains.

Accurate differential measurement of the vaccine and virulent strains is important to investigate the mechanisms of vaccine protection and the prevalence of MDV. A traditional differential method of MD was based on identification of the serotypes of isolated viruses by monoclonal Antibodies (mAb) specificity for three different serotypes of MDV in chickens (Lee *et al*., 1983). The mAb BA4 can react with pathogenic and attenuated strains of MDV-1 and mAb H19 can recognize the epitope of all tested MDV-1 strains except CVI988. Therefore, BA4 and H19 can be used to distinguish vMDV strains from CVI988 by indirect Immunofluorescent Assay (IFA) (Lee *et al*., 1983) however the method of virus cultivation takes more than a week to perform. In the laboratory, researchers have developed the dot blot hybridization technique with nucleic acid probe for MDV surveillance by which samples from chickens infected with MDV could be detected within 24 h (Ji *et al*., 2002). The probe in the lab only reacts with various strains of MDV-1 and not reacts with strains of MDV-2 and 3. However, this probe could not differentiate vaccine strain CV1988 and wide-type vMDV strains. Therefore, there is a need for a detection method that is specific for vMDV strains and does not cross-react with vaccine strains for the surveillance of MDV. Furthermore, the repeat reports of infection and the long-term endemicity in poultry of MDV

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in China lend urgency to monitor their infection (Jiang *et al*., 2005; Qin *et al*., 2010; Zhang *et al*., 2010).

In the present study, eight MDV strains of different pathotypes were compared for their bi-directional promoter sequences. It was found that there was a 5-consecutive base pairs (bp) deletion in CVI988 compared to vMDV strains. And researchers aimed to use the absence in the bi-directional promoter sequence to establishment of a rapid and specific PCR assay to diagnose MDV infection from CVI988 vaccine challenge. Also, researchers used this PCR assay in company with dot blot hybridization to monitor the MDV infection in tumor disease related commercial chicken flocks in China. The data demonstrated that MDV were widespread in commercial chickens in China and researchers could combine dot blot hybridization and PCR assays to survey the MDV infection.

MATERIALS AND METHODS

MDV reference strains and monoclonal antibodies: Eight distinct MDV strains were used as templates to amplify the promoter regions: virulent strains (GX0101, GA and JM) (Eidson and Schmittle, 1968; Schat and Calnek, 1978; Zhang and Cui, 2005), very virulent strains (Md5, G2 and RB1B) (Witter *et al*., 1999; Schat *et al*., 1982), very virulent plus strain (648A) (Witter *et al*., 1999) and vaccine strain (CVI988) (Rispens *et al*., 1972a, b). These viruses were propagated in primary Chicken Embryo Fibroblast (CEF) cells. All the strains and MDV-specific mAb (H19 and BA4) (Lee *et al*., 1983) were kept in the laboratory.

PCR assay for distinguishing vMDV strains from CVI988: Sequence analysis of bi-directional promoter sequence among different strains of MDV. Total cellular DNA was extracted from CEF infected with the different MDV strains described above using a DNA isolation kit (TaKaRa, Dalian, China) and the bi-directional promoter sequences were amplified by PCR with primers Fpro: 5'-GTCGCCTAGCGTAGCGTTCCTT-3' (base No. -751 to -730 relative to pp38 initiation codon as in Fig. 1) and Rpro: 5'-CTCTTATCCTATACCGCCG-3' (base No. -341 to -321). PCR products were purified by DNA purification kit and then cloned into TA vector (TaKaRa, Dalian, China) for sequencing. All the data were then analyzed using the DNAStar program.

Differentiation of pathogenic and CVI988 vaccine strain of MDV using PCR: A 5-consecutive bp was found absent in CVI988 by sequence alignment. Based on the absence, two pairs of primers were designed and synthesized to differentiate CVI988 from other MDV strains. Primers F1 and R1 (F1: 5'-CTTTTATACACA AGAGCCG AG-3', R1: 5'-TTTATCGCGGTTGTGGGTCA TG-3') could amplify a 560 bp fragment from the MDV genome expect for the CVI988 strain. Primers F2 and R2 could only amplify a 560 bp fragment from the MDV CVI988 strain (F2: 5'-CTTTTATACACAAGAGCCGC-3', R2: 5'-TTTATCGCGGT TGTGGGTCATG-3'). The locations of F1, F2 were marked in Fig. 1 and R1 have the same sequence with R2. The genomic DNA extracted from eight reference MDV strains was used as templates to validate the two pair primers. Reactions were optimal when denatured at 95°C for 30 sec (5 min was used for the first cycle), annealed at 54°C for 30 sec and extended at 72°C for 75 sec for 30 cycles. Amplified products from the viruses were identified by sequencing.

Sensitivity and specificity of dot blot hybridization for detecting MDV: Total cellular DNA samples extracted from CEF infected with MDV were used as templates for the sensitivity assay of dot blot hybridization. The MDV-specific probe was *pp38* gene fragment of MDV which was labeled with Digoxigenin (DIG) using a DIG nucleic acid Labeling and Detection kit (Roche, Cat. No. 11093657910). About 1 μL of DNA solution from each sample was pipetted onto a piece of nitrocellulose study and denatured by 0.1 M NaOH solution then dried and place into an oven at 80°C for 2 h. Then, the hybridization and detection were conducted according to the instruction of the manufacturer. For dot blot hybridization sensitivity test, MDV DNA diluted with serial 10 fold with TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0) from 1-1000 pg μL^{-1} were used. For dot blot hybridization specificity test, DNA samples of Md5 (MDV-1), CVI988 (MDV-1), SB1 (MDV-2), FC126 (MDV-3), REV and ALV-J infected CEF were used.

Comparison of different methods for detecting MDV Experiment 1: To compare the methods for detecting MDV in different tissues of infected chickens, 1 day old SPF chickens were randomly divided into two groups and kept in two isolators under positive filtered air. In this experiment, 40 birds were inoculated intraperitoneally with 1000 Plaque Forming Units (PFU) of Md5. A control group of 10 birds was inoculated with uninfected CEF. About 5 chickens infected with Md5 were chosen randomly for sampling on days 7, 14, 21 and 28 post-inoculation (p.i.), respectively. At days 28 p.i, five control chickens were also sampled randomly. Peripheral blood was taken for virus isolation as follows. Briefly, blood samples in anticoagulants were collected from five randomly selected chickens from each group at each time point and lymphocytes from the buffy-coats were counted and

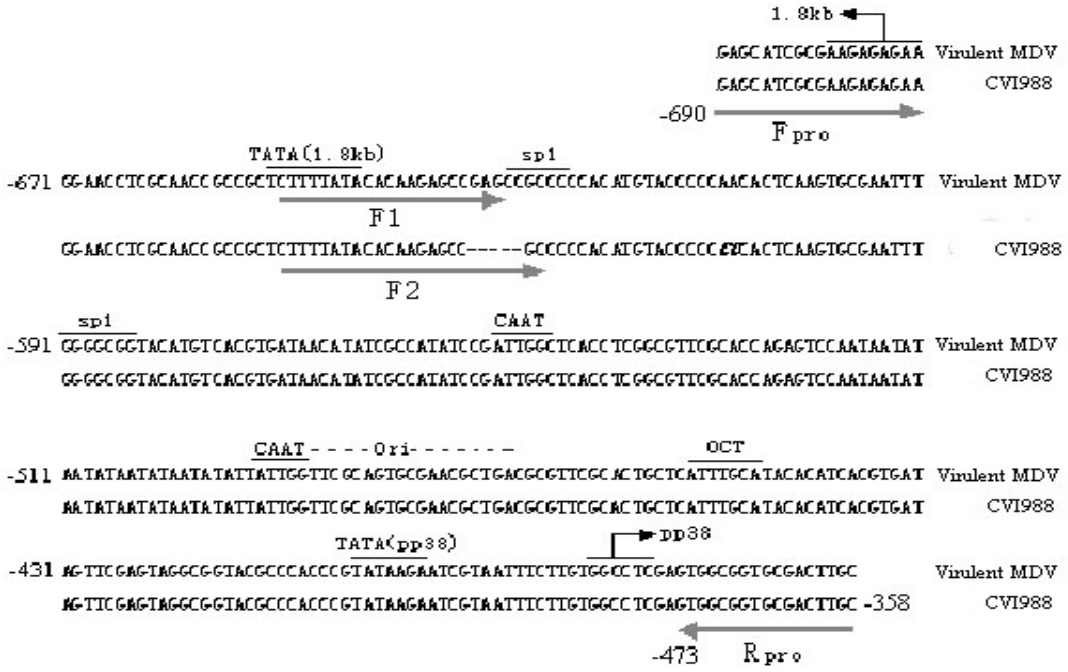

Fig. 1: Nucleotide sequence comparison of the bi-directional promoter region of two strains: vMDV and CVI988. There is a 5 bp GAGCC repeat deletion near/in a sp1 site (from -628 to -632) in CVI988 compared to vMDV strain, the transcriptional start sites and TATA boxes for both pp 38 and 1.8 kb mRNA transcript in two opposite directions and other enhancer motifs were indicated

duplicated 35 mm plates of freshly seeded CEF monolayers. Visible viral plaques were confirmed by IFA as described earlier (Lee *et al*., 1983) and the mAb, H19 or BA4 was used at a working dilution of 1:300. In addition, all lymphocyte, spleen and feather samples were used for DNA preparation for detection of MDV by dot blot hybridization and PCR assays. The DNA of the samples was extracted as described earlier (Murata *et al*., 2007).

Experiment 2: Two groups of 1 day old SPF chickens (10 chickens per group) were inoculated intraperitoneally with either 1,000 PFU of Md5 or CVI988, respectively. In addition, another group (10 chickens) was co-infected with 1,000 PFU of Md5 and CVI988. Feather tips collected from wings of chickens infected with MDV once per week after inoculation were used by dot blot hybridization and PCR assays for 6 weeks.

Detection of MDV from feather tips in commercial chickens by dot blots hybridization and PCR: To investigate the prevalence of MDV in commercial chickens from November 2005 to May 2009, 1250 feather tip samples were collected from 44 poultry farms experiencing severe tumor disease in China. Researchers marked 1-1250 of these samples for convenient to select before performing the study. Total cellular DNA of these samples were extracted as described earlier and analyzed by dot blots hybridization and PCR.

RESULTS AND DISCUSSION

Differentiation of pathogenic and CVI988 vaccine strain of MDV using PCR: As a result of sequencing and alignment, it was found that a 5-consecutive bp (5'-GAG CC-3') was deleted only in vaccine strain CVI988 (Fig. 1). This deletion was used as the target sequence for designing primers to identify CVI988 from other MDV strains. By PCR with the primer pairs F1 and R1, 7 out of 8 MDV reference strains except CVI988 were positive and only CVI988 was positive with the primer pair F2 and R2 in the 8 samples (Fig. 2) which was consistent with the results of virus isolation (Fig. 3).

Sensitivity and specificity of dot blot hybridization for the detection of MDV: As shown in Fig. 4a, the results of sensitivity assay suggested that 1-10 pg MDV DNA could be accurately detected by dot blot hybridization and the control sample did not be detected. The data of the specificity assay indicated that the probe (*pp38* gene of MDV which was labeled with digoxigeni) reacts only with MDV-1 (vMDV and CVI988 vaccine strain) and did

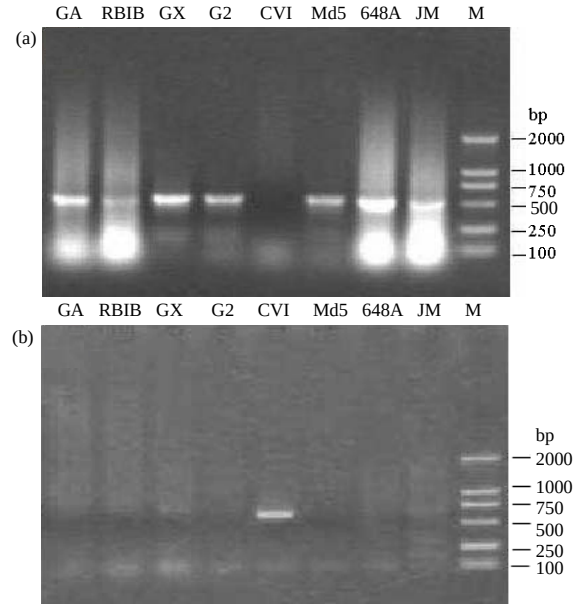

Fig. 2: PCR analysis of cells infected with different MDV strains. DNA samples infected with 1000 PFU different MDV strains were detected by PCR with the primer pairs F1 and R. Seven out of eifgt MDV reference strains except CVI988 were positive and only CVI988 was positive with the primer pair F2 and R2 in the 8 samples; a) PCR with primers F1 and R1and b) PCR with primers F2 and R2; Abbreviations: GX, GX0101; CVI, CVI988; M, Molecular size marker (2,000 bp DNA ladder)

not react with MDV-2, MDV-3, REV and ALV-J in dot blot hybridization test (Fig. 4b). These results suggested that the probe of dot blot hybridization was sensitive and specific for MDV-1 strains.

Comparison of different techniques for detecting MDV: The data obtained from the different tissues using three techniques (virus isolation, dot blot hybridization and PCR) were shown in Table 1. For each MDV detection technique, different tissues from individual chickens were compared. For virus isolation, the rates of positive detection were lower than that of dot blot hybridization and PCR on day 7 p.i. however there were no differences among the three methods on days 14, 21 and 28 p.i. For dot blot hybridization and PCR there were high correlations between spleen and feather tips.

The results in Table 2 showed that the bi-directional promoter sequence was detected in feather tips DNA of chickens either infected or co-infected with virulent and vaccine MDV strains, the positive results of chicken samples infected with either virulent or vaccine

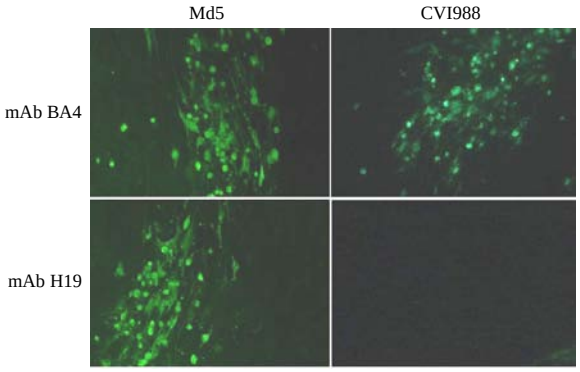

Fig. 3: Immunofluorescence analysis of CEF cells infected with different MDV strain. The mAb BA4 can react with pathogenic (Md5) and attenuated strains of MDV-1(CVI988) and mAb H19 can recognize the epitope of all tested MDV-1 strains except CVI988

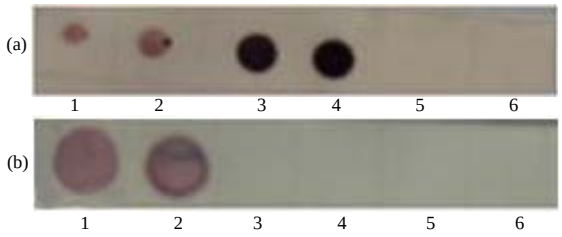

Fig. 4: The sensitivity and specificity of dot blot hybridization for detecting MDV. For dot blot hybridization sensitivity test; a) MDV DNA diluted with serial 10 fold with TE from 1-1000 pg μL^{-1} were used and one pg MDV DNA could be accurately detected by dot blot hybridization. 1: 1 pg DNA; 2: 10 pg DNA; 3: 100 pg DNA; 4: 1000 pg DNA; 5 and 6: Negative control. For dot blot hybridization specificity test; b) DNA samples of MDV strains were used. 1: Md5 (MDV-1); 2: CVI988 (MDV-1); 3: SB1 (MDV-2); 4: FC126 (MDV-3); 5: REV; 6: ALV-J. And the probe reacts only with MDV-1 (vMDV and CVI988 vaccine strain) and did not react with MDV-2, MDV-3, REV and ALV-J in dot blot hybridization test

MDV strains had a higher correlation between dot blot hybridization and PCR and the vMDV can be specifically detected and differentiated in co-infected chickens by the PCR Method targeting the bi-directional promoter sequence. Interestingly, the Md5 or CVI988 positive number in co-infected group was decreased compared with Md5 or CVI988 infected group.

Table 1: The detection of Md5 in different samples by virus isolation, dot blot hybridization and PCR

		Days post-inoculation				
Tissues	Methods	7	14	21	28	Control
Lymphocyte	Virus isolation	3/5[a]	4/5	5/5	5/5	0/5
	Dot blot hybridization	3/5	5/5	5/5	5/5	0/5
	PCR	4/5	5/5	5/5	5/5	0/5
Spleen	Dot blot hybridization	4/5	5/5	5/5	5/5	0/5
	PCR	4/5	5/5	5/5	5/5	0/5
Feather	Dot blot hybridization	4/5	5/5	5/5	5/5	0/5
	PCR	4/5	5/5	5/5	5/5	0/5

[a]Results of virus isolation, dot blot and PCR were given as the number of positive chickens

Table 2: Comparison of the diagnostic sensitivity of PCR and dot blot hybridization for the detection of MDV in feather tip-DNA samples

		Weeks post-inoculation					
Methods	Virus strain	1	2	3	4	5	6
PCR	Md5 (Primer # 1)	4/5	5/5	5/5	5/5	4/5	5/5
	CVI988 (Primer # 2)	3/5	5/5	5/5	5/5	5/5	5/5
	Md5+CVI98 (Primer # 1)	2/5	3/5	4/5	4/5	3/5	4/5
	Md5+CVI98 (Primer # 2)	3/5	2/5	1/5	1/5	2/5	2/5
Dot blot	Md5	3/5	5/5	5/5	5/5	5/5	5/5
hybridization	CVI988	3/5	5/5	5/5	5/5	5/5	5/5
	Md5+CVI988	3/5	3/5	5/5	4/5	3/5	4/5

Prevalence of MDV in commercial chickens: Systematic surveillance of tumor related commercial chickens from 2005-2009 resulted in 502 MD positive isolates from 1250 feather tip samples (total isolation rate, 40.2%) by PCR. In the 502 MD positive isolates, the number of CVI988 was 33 (2.6%) which were collected from the same poultry farms, the number of vMDV was 469 (37.5%) and no positive samples co-infected with CVI988 and vMDV were found. The numbers of positive MDV detected by dot blot hybridization were 495 (39.6%) in which 30 were CVI988 positive samples and 465 were vMDV positive samples. Partial results of feather tip samples isolated from 24 poultry farms by PCR and dot bolt hybridization were shown in Fig. 5. All positive and negative samples by PCR assay also showed positive and negative in dot bolt hybridization and these results suggested that the detection results by PCR were consistent with that by dot blot hybridization.

MDV is an oncogenic herpesvirus that causes various clinical syndromes in chickens and MDV strains were frequently isolated in domestic poultry all over the world which lead to huge economic losses for poultry industry (Cui *et al*., 2010; De Laney *et al*., 1995; Imai *et al*., 1992; Kang *et al*., 2007; Murata *et al*., 2007; Renz *et al*., 2006). More importantly, there were still repeated reports of MD outbreaks caused by vMDV from vaccinated flocks (De Laney *et al*., 1995; Witter *et al*., 1980). In recent years, researchers have been monitoring the tumor-related diseases of domestic poultry by dot blot hybridization and researchersfound that the MDV, especially co-infection with other oncogenic-related virus such as ALV or REV have been prevalent in China. Therefore, the development of a rapid, convenient and inexpensive method to determine the prevalence of oncogenic MDV in the field is necessary.

The probe used for the diagnosis of MD has many advantages. In addition to the distinguish MDV-1 from avirulent MDV-2 and 3, the hybridization probe can detect 1-10 pg MDV DNA moreover probes can be reused with good repeatability which significantly reduced the test cost and also it does not require much equipment (Ji *et al*., 2002). However, the dot-blot hybridization could not differentiate of oncogenic from CVI988 strain which was widely used in the poultry industry to prevent vMDV. In the present study, researchers developed a PCR Method which allows distinction between virulent and attenuated MDV-1 strains by amplifying the bi-directional promoter sequence of the MDV genome.

To verify the sensitivity of PCR assay, other assays (virus isolation and dot blot hybridization) were used and the detection results showed that the established PCR assay was sensitive enough to identify MDV infection and identify CVI988 from vMDV. However, as researchers all known, it is under high costs and heavy workload using PCR to analyze a large number of samples and dot blot hybridization has its unique advantage when monitoring thousands of samples (Ji *et al*., 2002). Therefore, researchers combine the two methods conducting the primary detection by dot blot hybridization and then differentiation the positive samples by PCR which could play a good effect in practice. Further, studies will focus on a new dot-blot hybridization which could differentiate vaccine strain CV1988 and vMDV strains.

Feather tips are easy to collect and feather follicle epithelium is known to be the only site of productive replication of cell-free MDV (Calnek *et al*., 1970; Davidson *et al*., 1986). The experimental trials in chickens showed that for dot blot hybridization and PCR, there was a high correlation between spleen and feather tips, the results suggested that the feather tip-derived DNA from MDV infected chickens can be used for monitoring MDV infection. In the present study, researchers detected 1250 feather tip samples from commercial chickens suspected to be infected with oncogenic viruses in China by PCR and dot blot hybridization. About 502 MD positive isolates were detected from 1250 feather tip samples (total isolation rate, 40.2%) by PCR. In the 502 MD positive isolates, the number of CVI988 was 33 (2.6%) which were collected from the same poultry farms, the number of vMDV was 469 (37.5%).

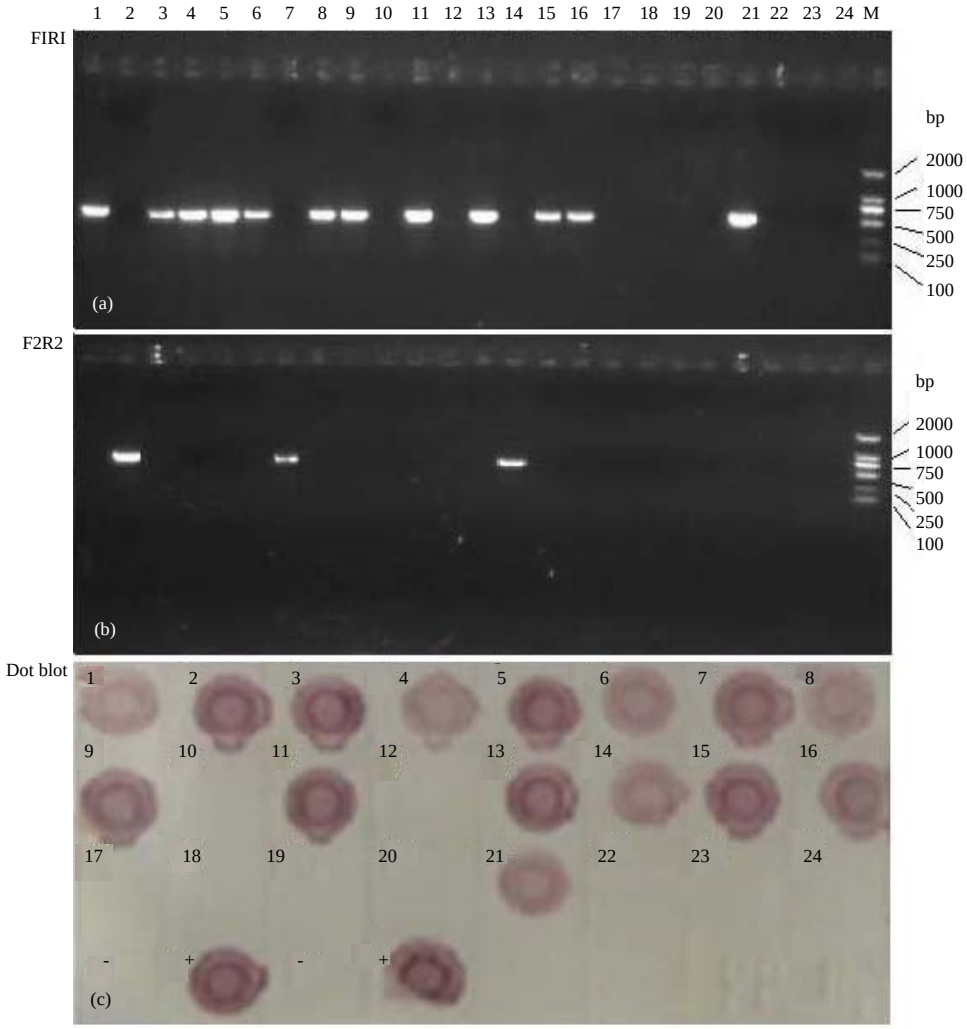

Fig. 5: Detection of MDV from feather tips in commercial chickens by PCR and dot blot hybridization. Systematic surveillance of tumor related commercial chickens from 2005-2009 by PCR; a) and dot blot hybiridization; b) 1-24. The partial detection results of feather tip samples from different poultry farms; M: Molecular size marker (2,000 bp DNA ladder). + : Positive control; - : Negative control

CONCLUSION

These results indicated that MDV infections were very prevalent in chicken flocks with tumors in China. Therefore, it is imperative that particular attention be paid to the further surveillance for the MDV infection in order to avert future greater losses for poultry industry. More interestingly, researchers did not find co-infection of CVI988 with vMDV from the same sample in this study and researchers speculate that the chances of vMDV infection were low in the vaccinated chicken flocks, though superinfection with pathogenic strains of MDV has been observed in vaccinated chickens (Biggs *et al*., 1970; Purchase and Okazaki, 1971). These findings further suggested that researchers should combine the two methods to monitor the MDV infection in practice in future.

ACKNOWLEDGEMENTS

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