

## Very Virulent Marek's Disease Virus (MDV) in Commercial Vaccinated Flocks in India

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### Abstract

Marek's disease (MD) is a complex and highly infectious lymphoproliferative disease of chickens and is characterized by neurological signs due to the affliction of peripheral nerves, neoplasms of lymphocytes and visceral organs. A total of 307 samples from commercial vaccinated farms from different parts of South India were tested for detecting very virulent MD virus (vvMDV). The samples comprised of heparinized blood, spleen and other organ tissues that exhibited neoplasms and/or lesions suggesting neoplasm. Agar Gel Immuno Diffusion Test (AGID) was employed for initial screening for vvMDV and in turn, the technical replicates of the samples were subjected to Dot- Enzyme-Linked Immunosorbent Assay (Dot-ELISA), Latex Agglutination Test (LAT) and Polymerase Chain Reaction (PCR) for detecting the vvMDV antigens. Two sets of primers were used; one for very virulent MDV-1 serotype, the glycoprotein antigen A gene of MDV1 and the other for the 132 bp tandem repeat in the Bam HI-H region. A product of 434 bp tandem repeat sequence using the 132 bp tandem repeat primer sequence which is characteristic to pathogenic MDV-1 serotype and, a 314 bp product, specific to vvMDV using the glycoprotein antigen A gene were detected using PCR. We observed that LAT, Dot-ELISA and PCR detection methods gave similar detection rates with slightly higher detection rates by PCR, however, the differences were not significant. LAT, Dot-ELISA and PCR were found to be sensitive detection techniques compared to AGID for vvMDV.

**Keywords:** Marek's Disease Virus; AGID; Dot-ELISA; LAT; Polymerase Chain Reaction

### Introduction

Marek's disease (MD) in chickens is caused by a *gallid herpesvirus 2* (GaHV-2), a member of the *Mardivirus* genus in the subfamily of *Alphaherpesvirinae*, which is commonly referred to as MD virus (MDV). MDV is a highly infectious lymphoproliferative disease of chickens [1] which is characterized by visceral and neural lymphomatous lesions. Affected chickens exhibit neurological signs, immunosuppression, and neoplastic transformation of T lymphocytes, swelling of peripheral nerves and the visceral organs and the disease is predominantly reported in domestic fowl, turkeys and quails [2-4].

The development of vaccines for the control of MD was a significant landmark and any vaccination failure following vaccination hinders the development of adequate antibody titer levels and makes the population susceptible for disease outbreak [5, 6]. MDV contains the repeat sequences in the ULA gene which is considered to be responsible for lymphomagenesis [7] and the lymphomagenesis gene is characterised by the presence of tandem-arranged direct repeats each measuring up to 132 base pairs (bp) in length [8,9].

It was identified that the gene for the phosphoprotein pp 38 within the MDV-1 Bam HI-H DNA fragment to be consistently associated with the transformation [8, 9]. Thus, the amplification and/or duplication of the 132 bp repeats within this region was thought to lead to a change in the expression of pp38 gene and/or genes resulting in viral transformation. Three serotypes of naturally occurring MD viruses had been isolated to date that include Pathogenic MDV-1 [10], apathogenic MDV-1 [11] and herpes virus of turkeys (HVT) designated as MDV serotype 3 [11,12]. These three serotypes have been used as vaccine strains to protect chickens from virulent MDV-1 viruses widely by attenuating the pathogenicity through serial passages in chicken embryo fibroblast (CEF) *in vitro* [6].

It was reported that the DNA of the oncogenic MDV1 serotype contains three units of tandem direct repeats (with 132 bp repeats) within the inverted repeats of the long regions of the MDV1 genome and the attenuated, nononcogenic viral genome contains multiple units of tandem direct repeats [12,13]. It was reported that, specific nucleotide primers from the nucleotides of glycoprotein A gene of MDV-1 with little or no homology to the nucleotide sequence of antigen A gene of MDV-3 (HVT) enabled the differentiation between pathogenic and non-pathogenic MDV-1 serotypes and vaccine viruses of MDV-2 and MDV-3 (HVT) [7].

In this study we compared agar gel immunodiffusion test (AGID), latex agglutination test, Dot-ELISA and PCR to detect MDV in commercially vaccinated flocks from different parts of South India. PCR tests were employed to differentiate and to identify pathogenic MDV isolates in clinical samples from flocks with MD outbreaks that had been vaccinated.

### Materials and Methods

#### Collection of tissue samples and processing

Tissue samples from live chickens with MD symptoms and dead birds were collected during autopsy. Samples consisting of spleen, liver, skin, feather follicles, and peripheral nerves were collected in 50% glycerol saline and, whole blood with heparin on ice from live suspected birds were collected from different farms in Tamil Nadu, Andhra Pradesh, Karnataka and Kerala with suspected MD outbreaks. The tissue samples were processed by trituration and sonication on ice (Bremson-Model 250. Sonifier, 1/8 inches, Tapered tip) for 5 minutes at 30 x 30 seconds bursts with 30-sec intervals between each sonication and centrifuged at 3200 g for 10 minutes using a refrigerated centrifuge (Eppendorf). The supernatants were collected and used for AGID, LAT, Dot-ELISA and, for PCR the trituated tissues as well as the supernatants were used for DNA extraction.

## Hyperimmune serum

### Preparation of antiserum and immunoglobulins

Anti-HVT – hyperimmune serum was prepared as per standard methods [14] using 6-week old white leghorn cockerels using the HVT-vaccine virus, procured from the Institute of Veterinary Preventive Medicine, Ranipet, India.

The globulins were sequentially precipitated using 45% saturated ammonium sulphate solution (pH 7.2, adjusted with 1M Sulphuric acid) and the final precipitate was dissolved in minimum quantity of PBS (pH, 7.2) and extensively dialysed against PBS at 4°C for 48 hours.

### Sensitization of latex beads

Sensitization of latex beads was performed as per the protocol described earlier [15] with minor modifications. Hundred  $\mu$ L of 10% suspension of 0.6  $\mu$ m latex beads (Sigma-Aldrich, USA) was mixed with 900  $\mu$ L of carbonate-bicarbonate buffer (pH 9.6) to make 1% suspension. This suspension was adjusted to 2  $\mu$ g/mL and the dialysed immunoglobulin suspension was added to these beads and kept at 4°C overnight. The coated beads were centrifuged and the non-specific sites on the beads were blocked with BSA (Sigma-Aldrich, USA) in PBS (pH 7.2) at 37°C for 2 hrs. The beads were washed in carbonate-bicarbonate buffer and finally made up to a 0.6% suspension for conducting tests.

### Latex agglutination test (LAT)

One drop of clarified supernatants of the MDV suspected sample was placed on a glass slide and mixed with one drop of sensitised beads. HVT vaccine virus as a positive control and tissue extract from the normal un-vaccinated chicken as a negative control was also included in the test for comparison. The positivity of the sample was indicated by the agglutination of the beads.

### Dot-enzyme linked immuno sorbent assay (Dot-ELISA)

This test was conducted as per the method described earlier [16] with certain modifications. One  $\mu$ L of test samples and the HVT vaccine virus as a positive control and tissue extract from the normal un-vaccinated chicken as negative control were dotted onto a 5 cm x 5 cm nitrocellulose (NC) membrane. The NC membrane was air-dried and kept in a vacuum oven at 80°C for 45 minutes for fixing the antigen onto the membrane. The unsaturated sites were blocked with 1% gelatin in distilled water and incubated at 37°C for 1 hr. The NC membrane was washed in PBS plus Tween 20 (PBST) three times by changing the buffer every 10 min. The NC membrane was then incubated in 1:1000 anti-HVT chicken hyperimmune serum containing antibody to MDV, at 37°C for 45 minutes. The NC membrane was washed in PBST three times and incubated in 1:1000 diluted anti-chicken IgG-HRP conjugate (Sigma, USA) and incubated at 37°C for 30 minutes. The NC membrane was washed in PBST thrice and treated with freshly prepared substrate solution. (0.05% of 3-3 diaminobenzidine tetrachloride in PBST). A positive reaction was indicated by the appearance of brown dots. Rinsing the membrane in tap water stopped the reaction.

## Polymerase chain reaction (PCR)

### Extraction of DNA from affected tissues and cell culture

DNA was extracted from blood and other tissue samples. DNA was extracted as per the methods described earlier [17] with slight modifications.

Three mL of the homogenised tissue suspensions or heparinized blood or cell cultures was mixed with 9 mL of lysis buffer containing 0.17M ammonium chloride and incubated for 15 min. on ice. White cells were pelleted by centrifugation at 2000 g for 10 min at 4°C. The pellet was suspended in 10 mL of sodium chloride – EDTA (Sigma-Aldrich, USA) buffer with Proteinase K (Sigma-Aldrich, USA) added at 100  $\mu$ g/mL and 3mL of 20 per cent sodium dodecyl sulphate (SDS, Sigma-Aldrich, USA) solution and incubated for 4 - 6 hours at 37°C. The solution was then extracted with phenol-chloroform mixture (5: 24) 2 to 3 times. The upper aqueous phase was removed and 3 M sodium acetate, pH 5.5 was added to 1/10th of its original volume. The solution was mixed, and one volume of isopropanol was added to it. The DNA strands were gently spooled out in a glass rod by gently mixing the solution. The DNA was dissolved in 200  $\mu$ L of Tris-EDTA (TE) buffer and was used for PCR reactions.

**PCR amplification of the target DNA**

Total DNA preparations from naturally infected tissue samples were used as templates for PCR amplification.

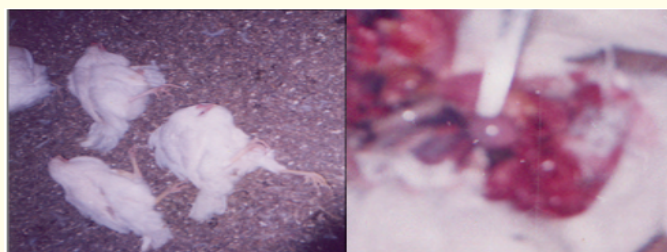
Primers were selected from the nucleotide sequence that is unique for MDV-1 antigen A [7]. The forward primer for MDV-1 antigen A gene was 5' GAG GTA CCT CAT GGA CGT TCC ACA 3' (24 mer), reverse primer for MDV-1 antigen A gene was 5' ACA TTC TTT TCG TTG GCG TGG TAT 3' (24 mer). Primers that were described by Becker, *et al.* 1982 were selected to detect 132 bp nucleotide repeat sequence located within the Bam H1-H fragment [18] by amplifying the sequences flanking the tandem repeat sequence. The forward primer consisted of 5' TAC TTC CTA TAT AGA TTG AGA CGT 3' (24 mer) and the reverse primer consisted of 5' GAG ATC CTC GTA AGG TGT AAT ATA 3' (24 mer). The direct primer is located 65 bp 5' to the tandem repeat and the reverse primer is 105 bp downstream. The expected amplified DNA product size with a primer of 132 bp tandem repeat was 434 bp [19]. Primers were procured from Genie, Bangalore, India.

One to two µL of total DNA (as template) was mixed with 10 µL of reaction buffer with the 1.5 µL of magnesium chloride, 2 µg of dNTP mixture (Thermo-Fisher Scientific), 4 µL of both primers (10 picomole of each primer) and 0.5 µL of Taq Polymerase (Thermo-Fisher Scientific) enzyme were mixed and reaction mix was made up to 50 µL with distilled water. The DNA was amplified in a thermal cycler (Perkin Elmer, Italy), by denaturing the DNA at 94°C for 5 minutes initially and amplified at 94°C for 1 minute, 55°C for 1 minute, 73°C for 1 minute with a final elongation temperature of 72°C for 5 minutes. The reaction was carried out for 35 cycles and the PCR products were run on a 2% agarose gel.

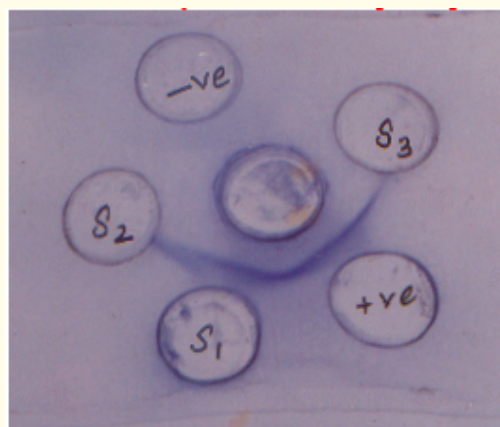
**Results**

**AGID, LAT and Dot-ELISA**

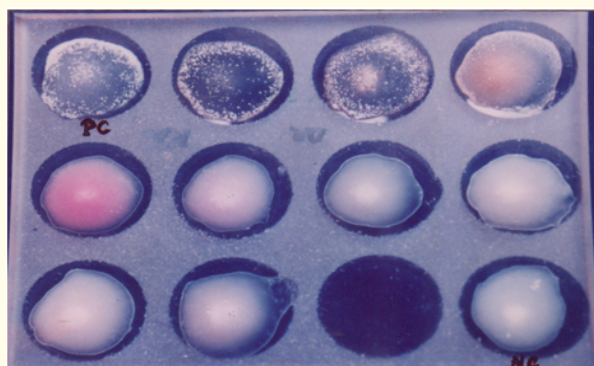
For all three immunoglobulin-based diagnostic techniques, hyperimmune serum raised using HVT vaccine virus in chickens was used. Figure 1 shows the farms with chickens exhibiting classical range paralytic birds and the dead birds showing enlarged spleen on post-mortem or autopsy. Figure 2, 3 and 4 show the AGID, LAT and DOT-ELISA test results, respectively.



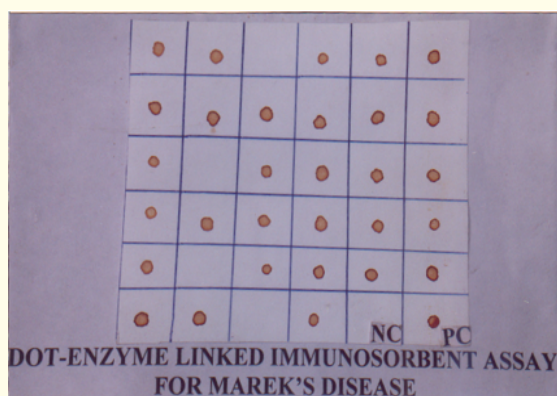
**Figure 1:** Vaccinated farms with outbreak of Marek's Disease; birds showing typical range paralysis. Post Mortem lesions of dead bird showing enlargement of spleen.



**Figure 2:** Agar Gel Immuno Diffusion Test - Center well- Hyper Immune serum. (+) e: Positive control; (-) ve: Negative control; S1, S2, S3: Samples.



**Figure 3:** Latex Agglutination Test of field samples with MD outbreak.  
PC: Positive control; NC: Negative control and samples.



**Figure 4:** Dot - Enzyme-Linked Immuno Sorbent Assay. Nitrocellulose membrane fixed with MDV suspected samples.  
PC: Positive control; NC: Negative control and samples.

	Tamil Nadu			Andhra Pradesh			Karnataka			Kerala			Total
	Blood	Spleen	Organ	Blood	Spleen	Organ	Blood	Spleen	Organ	Blood	Spleen	Organ	
<b>LAT</b>													
Total	44	54	46	14	30	8	31	34	17	13	16	0	
Pos	38	29	30	10	21	6	25	28	15	10	13	0	225
<b>Dot-ELISA</b>													
Total	44	54	46	14	30	8	31	34	17	13	16	0	
Pos	38	29	31	10	21	6	25	29	15	11	13	0	228
<b>AGID</b>													
Total	44	54	46	14	30	8	31	34	17	13	16	0	
Pos	28	27	22	6	19	3	19	18	12	9	9	0	172
<b>PCR</b>													
Total	44	54	46	14	30	8	31	34	17	13	16	0	
Pos	39	32	30	11	23	6	26	29	15	11	12	0	234

**Table 1:** This table provides the details of samples collected from each state in Southern India and test results from each diagnostic technique.

Table 2 provides the details of the samples and the test results of all three immunoglobulin-based detection methods and PCR results.

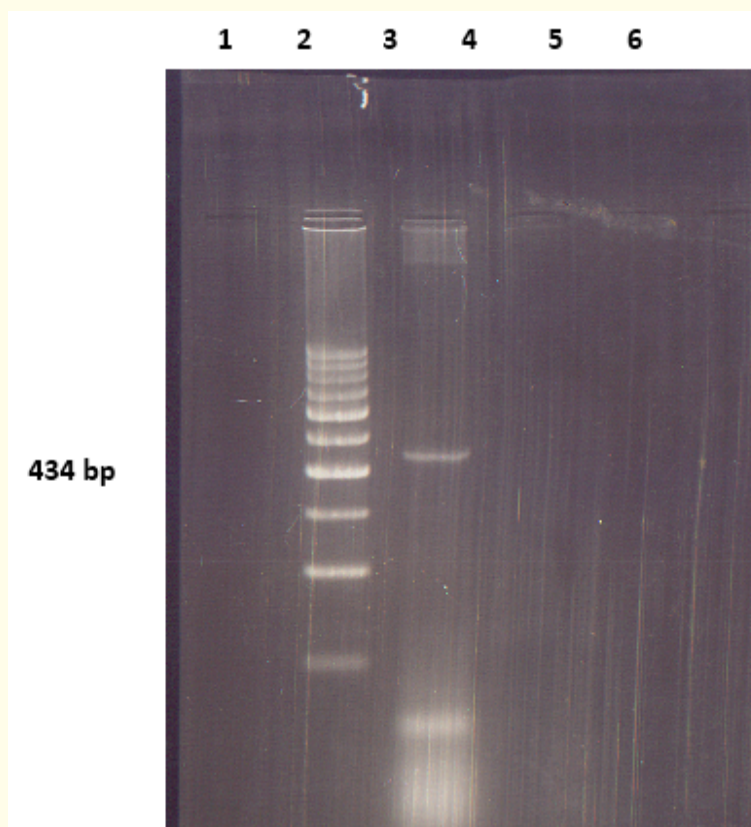
The results from AGID, LAT and Dot-ELISA showed that LAT and Dot-ELISA were more sensitive in detecting MDV compared to AGID (Table 2). PCR was relatively more sensitive than the immuno-based methods.

State	LAT	95% CI		Dot-ELISA	95% CI		AGID	95% CI		PCR	95% CI	
TN	86.4	72.6	94.8	86.4	72.6	94.8	63.6	47.8	77.6	88.6	75.4	96.2
TN	53.7	39.6	67.4	53.7	39.6	67.4	50.0	36.1	63.9	59.3	45.0	72.4
TN	65.2	49.8	78.6	67.4	52.0	80.5	47.8	32.9	63.1	65.2	49.8	78.6
AP	71.4	41.9	91.6	71.4	41.9	91.6	42.9	17.7	71.1	78.6	49.2	95.3
AP	70.0	50.6	85.3	70.0	50.6	85.3	63.3	43.9	80.1	76.7	57.7	90.1
AP	75.0	34.9	96.8	75.0	34.9	96.8	50.0	15.7	84.3	75.0	34.9	96.8
KK	80.6	62.5	92.5	80.6	62.5	92.5	61.3	42.2	78.2	83.9	66.3	94.5
KK	82.4	65.5	93.2	85.3	68.9	95.0	52.9	35.1	70.2	85.3	68.9	95.0
KK	88.2	63.6	98.5	88.2	63.6	98.5	70.6	44.0	89.7	88.2	63.6	98.5
KL	76.9	46.2	95.0	84.6	54.6	98.1	69.2	38.6	90.9	84.6	54.6	98.1
KL	81.3	54.4	96.0	81.3	54.4	96.0	56.3	29.9	80.2	75.0	47.6	92.7
KL	0.0	0.0	100.0	0.0	0.0	100.0	0.0	0.0	100.0	0.0	0.0	100.0

**Table 2:** This table provides the percentage positive by LAT, Dot-ELISA, AGID and PCR technique in detecting MDV is different sample types from South India and lower and upper limits at 95% confidence interval. TN: Tamil Nadu; AP: Andhra Pradesh; KK: Karnataka; KL: Kerala.

**PCR analysis of the 434 bp nucleotide sequence of the tandem repeat located within the Bam H 1-H fragment (PCR-I)**

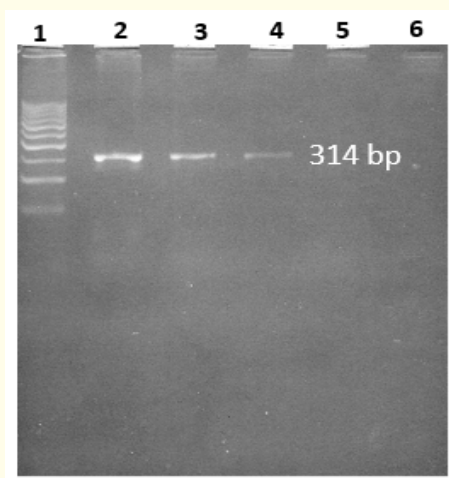
In this study, DNA from samples of organs and DNA from the culture of HVT vaccine virus were tested by PCR to amplify 434 bp nucleotide sequence of Bam H1-H fragment. The expected amplicon size of 434 bp fragment was visualized on agarose gel with the 100bp marker run alongside the samples (Figure 5 - Lane 2, 3, 4). The primers did not detect HVT (MDV-3) DNA (Figure 5 - Lane 5) from the cell culture DNA. The results indicated that the dimmers of the 132 bp tandem repeat were characteristic of oncogenic MDV [12].



**Figure 5:** The figure represents the PCR reaction of MDV samples amplified using the primers to amplify 132 bp tandem repeats in the BAM H1-H region. Lane 1- HVT (MDV-3); Lane 2- 100 bp marker; Lane 3- vv MDV- field sample spleen; Lane 4- Sample- blood; Lane 5- Sample- tumour; Lane 6- Sample- liver.

**PCR analysis of the 314 bp nucleotide sequence of glycoprotein A gene that is specific for MDV-1 (PCR-II)**

Further to the tandem repeats, amplification of MDV-1 antigen A gene sequence with no homology to the nucleotide sequence of antigen A gene of MDV 3 (vaccine virus) enabled the further confirmation of very virulent MDV. When MDV-1 antigen A gene-specific primers were used to amplify DNA extracts from tissue samples, an amplicon of 314 bp was detected, but the same primer when used to amplify vaccine viral DNA they did not amplify (Figure 6).



**Figure 6:** PCR analysis of the 314 bp nucleotide sequence of glycoprotein A gene. Lane 1- 100 bp marker; Lane 2- vv MDV- field sample-blood; Lane 3- vv MDV- field sample - spleen; Lane 4- vv MDV- field sample tumour; Lane 5- HVT (MDV-3); Lane 6- Sample-liver.

**Discussion**

Marek's Disease is a lymphoproliferative and neuropathic disease of domestic chickens and less commonly in turkeys and quails [20,21]. Immuno-incompetence is a major risk factor that plays a major role in the pathogenesis of MD and a lack of immunocompetence or immunocompromise leads to cytolytic infection and chickens suffer from immune suppression [22,23]. Immunity has been reported to develop better when chickens are vaccinated at one week of age- than one day-old vaccination [24].

Immunization at 23 days has shown better protection and it has been shown that immunity against MD is age-dependent [25,26]. It has been observed that development of immunity needed ample time interval between the vaccination and exposure to the disease which offered better protection than chickens being exposed within a short interval time of vaccination [27]. Furthermore, re-vaccination has been regarded as a best option for effective protection against MD [28]. While considering that re-vaccination is not a practice in countries like India for MDV, it is always a risk of chickens contracting the virus and succumbing to the disease.

The primary aim of this study was to detect the virulent and very virulent MDV antigens in HVT vaccinated flocks in Indian poultry farms, where this is the standard Indian poultry industry practice of vaccinating the flocks HVT vaccine virus at one day of age. We used AGID the gold standard, LAT, Dot-ELISA and PCR to detect MDV in vaccinated flocks and to differentiate the pathogenic and non-pathogenic MDV using PCR.

AGID detection of MDV was found to be limited when compared to LAT and Dot-ELISA where all three were immunoglobulin-based techniques (Table 1). We found that the antigen-antibody precipitation was not detected even in the samples that were collected from apparently diseased birds with classical MD symptoms, while those samples were tested positive for MDV by LAT and Dot-ELISA. Studies that have employed AGID and ELISA for other viruses have found ELISA being more sensitive than AGID in detecting antibodies [29,30] and our findings agree with these reports.

However, in our study, we wanted to reconfirm or validate the test results for the samples that were tested positive by all three immunoglobulin-based techniques to rule out false positives. At the same time, we acknowledge that we used HVT vaccine virus to raise hyperimmune serum and it may detect the asymptomatic HVT virus which may be persisting in the flock. Likewise, the mere absence of the antibodies could not be taken as negative, therefore we employed two different PCRs to identify the very virulent oncogenic MDV. All tumor-bearing samples were positive by PCR and few samples that were negative by the immunoglobulin-based techniques were tested positive by PCR.

The PCR designed for the 132 bp tandem repeat of BAM H1-H fragment sequence [18] amplified a product size of 434 bp from the field isolates but the primer did not amplify the vaccine virus HVT. This confirmed the absence of non-pathogenic virus among the flocks and the test results we obtained using the immunoglobulin-based techniques were reliable. However, we recommend that immuno-globulins raised against very virulent MDV specific antigens will serve better in the detection in field outbreaks.

Further, we employed a second PCR to differentiate between pathogenic and non- pathogenic MDV viruses and the vaccine virus of serotype 3 using specific nucleotide primers from the glycoprotein A genes of MDV-1 and the primer did not detect HVT but it allowed the amplification of 314 bp from the field isolates. This confirmed the prevalence of very virulent MDV among the flocks tested.

The results of the study revealed that the PCRs were very sensitive in detecting vvMDV antigens and, that LAT and Dot-ELISA were equally sensitive. Nevertheless, using vvMDV specific antibodies will improve the confidence in the test results under field conditions particularly during outbreaks such as this study. We also observed that blood DNA and the spleen DNA were easy to detect the MDV antigens over other tissues and/or tumour-bearing tissues as reported by other workers [7,19]. This might be ascribed to the cellular oncogenes that are involved in the tumour formation in conjunction with deregulation of genes controlling the normal cycle [19].

## Conclusion

In summary, PCR appears to be a sensitive method for MDV diagnosis when compared to the other immunoglobulin-based diagnostic techniques. Furthermore, we recommend that use of vvMDV specific antibodies will be useful in detecting vvMDV for field outbreak investigations.

## Competing Interests

All authors declare no competing interests.

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