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Original Research Article

Detection of Marek's Disease Virus Serotypes 1, 2 and 3 in Poultry Flocks using PCR Technique in South-Western-Nigeria

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ABSTRACT

Marek's disease virus (MDV) causes immunosuppression and tumors in poultry. Lymphoma induction in Marek's disease (MD) has been associated with serotype-1. The study employed the use of two-step-PCR with primers directed to gB-gene of MDV to determine and differentiate MDV-serotypes associated with MD-outbreaks in poultry-flocks using tissue samples with neoplastic lesions collected from carcasses during postmortem examination. A total of 58 different flocks categorized into pullets, broiler, turkey and duck suspected of MD were sampled using PCR-techniques. Forty three out of the 58 flocks sampled were positive for MDV employing first and nested PCR analysis using serotype-specific primer pairs in the nested PCR, 38 (65.5%), 22 (37.93%) and 20 (34.48%) were positive for MDV-serotypes 1, 2 and 3 respectively. The distribution of three-serotypes of MDV was quite different among investigated poultry-flocks. Fourteen (73.68%), 7 (36.84%) and 6 (31.57%) farms were positive for MDV-serotypes-1, 2 and 3 respectively in pullet flocks with age range between 8-20 weeks. In layer flocks, 22 (66.66%), 13 (39.39%) and 11 (33.33%) were positive for MDV-serotypes 1, 2 and 3. One broiler flock was positive for MDV-serotype-2 and 3, only one turkey flock positive for MDV-serotypes 1, 2 and 3, one duck farm was positive for MDV- serotype 1. Multiple infections involving two or three of the serotypes were observed in 8-20 weeks old pullets with 4 (21.1%) of the flocks showing MDV-1 and MDV-2, 3 (15.79%) with MDV-1 and 3 and 3 (15.79%) with all the three-serotypes. This study revealed increased virulence of MDV-serotypes-2 and 3 that have been classified as non-oncogenic in field MD-outbreaks.

.**Key words:** Chicken, Marek's disease virus, PCR, serotypes.

INTRODUCTION

Marek's disease (MD) is a common lymphoproliferative disease of chickens and sometimes turkeys. It is an economically important disease in poultry production costing the poultry industry each year millions in veterinary expenses. It is characterized by mononuclear cellular infiltration in peripheral nerves and various other organs and tissues including iris and skin (Witter & Schant, 2003).The aetiology of the disease is Marek's disease virus (MDV) which is a cell associated oncogenic herpesvirus with lymphoproliferative properties. This virus is highly contagious since it is transmitted readily by direct or indirect contact between chickens, apparently by the airborne route (Biggs, 1985). The transmission is through the inhalation of virus present in feather follicle epithelium and dander (Calnek *et al*., 1970). Many strains of MDV have been

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identified. Oncogenic strains are classified as serotype 1 (MDV1) while the naturally non on-cogenic chicken strains (Cho & Kenzy, 1972) and herpesvirus of turkeys (HVT) (Witter *et al*.,1970) belong to serotypes 2 (MDV-2) and 3, respectively. Marek's disease was first described as polyneuritis (Marek, 1907). Pathologic changes in MD consist mainly of nerve lesions and visceral lymphomas (Payne,1985) which may occur in one or more of a variety organs and tissues. Over the years, virulence of MDV strains has increased and investigation has shown that the evolution in virulence of MDV continues (Witter, 1996). The principal methods to identify the presence of infection are isolation and identification of the virus and demonstration of viral DNA or antigens in tissues (Witter & Schat, 2003). A number of molecular techniques have been used to identify viral genome in tissues (Sharma, 1998). These include Polymerase chain reaction, dot-blot hybridization and in-situ hybridization. Polymerase chain reaction (PCR) has an additional diagnostic tool offering advantages of serotype specificity (Davidson *et al*., 1995) and the ability to differentiate between vaccinal and wild strain of MDV serotype-1 (Handberg *et al.,* 2001). Marek's disease virus can be easily detected by PCR in lymphoid tumors of infected chicken (Davidson & Borenstein, 1999), demonstrating that viral DNA is present in tumors. Viral MDV can also be demonstrated in different organs of infected chickens (Davidson & Borenstein, 1999). Only Serotype 1 MDV has been claimed to be pathogenic to chicken while serotypes 2 and 3 have not been associated with MD. This study employed PCR technique to investigate the presence of different serotypes of MDV in lymphoma tissues from MD cases from some chicken, turkey and one duck flock in some poultry flocks in South Western Nigeria.

MATERIALS AND METHODS

COLLECTION OF SAMPLES

A total number of 87 samples were collected from neoplastic lesions of liver, spleen and kidney, from 58 different farms. Samples were collected from carcasses during postmortem examination in the poultry clinic of the Department of Veterinary Medicine, post mortem unit of the Department of Veterinary Pathology, University of Ibadan and also during visit to various commercial poultry farms. The samples were collected into 1.5ml eppendorf tubes containing viral transport medium and placed in ice packs for transportation to the laboratory. Those samples that were not used immediately were stored at - 20°C.

ETHICAL APPROV AL

Samples were collected from carcasses during postmortem examination in the poultry clinic of the Department of Veterinary Medicine, post mortem unit of the Department of Veterinary Pathology, University of Ibadan and also during visit to various commercial poultry farms. Birds were not raised for the research hence no ethical approval.

NUCLEIC ACID EXTRACTION AND PCR

The extraction and isolation of DNA was done using Qiagen® (QIAGEN GmbH, QIAGEN Strasse 1* 40724, Germany). About 3g of each lymphoma tissue sample was manually homogenized in a medium of viral transport medium and used for the extraction following kit manufacturers recommended protocol. Marek's disease vaccine Biovac (VIR 107, HVT-FC 126 strain, Batch 1-071056) were used as positive controls and subjected to similar treatment. The extraction was followed by PCR amplification using primers gBF and gBR specific to the highly conserved region of the gB MDV gene for all serotypes (13). Primer pairs gBMDV-1F and gBMDV-1R, gBMDV -2F with gBMDV-2R and gBMDV-3F with gBMDV-3R (Aminev *et al*., 1998) that are specific for the hyper variable region of gBMDV of serotype 1, 2 and 3 MDV respectively were used. PCR mixtures in a reaction mixture of 25µl per sample consist of 18.2µl of RNAse free water, 2.5µl PCR buffer, 1.5µl of 50mM of $Mgcl_2$, 0.5µl of 10mM of DNTP, 0.1µl of each of 25µM of forward and reverse primers and 0.1µl of 5U/µl of Taq polymerase (Invitrogen life technologies® USA) and 2µl of extracted DNA sample. PCR thermal programme were done as follows: Initial denaturation at 94°C for 5 minutes, 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 1 minute after which final extension of 72°C for 7 minutes. Differentiation of the three MDV serotypes were done as nested PCR using appropriate primer pairs. The PCR conditions were the same. The PCR product were separated at 120mV in a 2.0% agarose gel, stained by ethidium bromide and visualized using a UV light source (Sigma Aldrich®). One Kb ladder (Invitrogen life technologies, USA) was used as DNA maker. A 491 base pair (bp) band represented MDV while 421bp, 386bp and 380bp represented MDV serotypes 1, 2, 3 respectively (Aminev *et al*., 1998).

RESULTS

Forty three (43) out of the 58 flocks sampled were positive for MDV by PCR analysis using MDV specific primers. Fifteen out of the 21 pullet flocks, 25 out of 33 layers, 1 broiler flock, 1 turkey flock and 1 out of two duck flocks sampled were positive for MDV (Table 1).

OCCURRENCE OF MDV SEROTYPES

Thirty eight (65.5%), 22 (37.93%) and 20 (34.48%) of the flocks were positive for MDV serotype 1, 2 and 3 respectively. In the pullet flocks with age less than 20 weeks, 14(66.7%) were positive for MDV 1 while 7 (33.3%) farms were positive for each of MDV serotype 2. In the layer flocks, 22 (66.66%), 13 (39.39%) and 11 (33.33%) were positive for MDV serotype 1, 2 and 3 respectively. The only

broiler flock investigated had mixed infection with MDV serotypes 2 and 3 also the only turkey flock was positive for MDV serotypes 1 2 and 3 (mixed infection) . One of the two duck farms was positive for MDV serotype 1 (Table II).

The distribution of different serotypes of the virus was quite different among investigated flocks. The total flocks positive for the MDV among the pullet farms with age less than 20 weeks was 1 (50%) and the farm was positive for single infection with MDV serotype 3. In the flock with age ranges between 8-20 weeks, the total number of farms positive for MDV were 14 (73.68%) out of the 19 flocks investigated with 4 (21.1%) positive for single infection with MDV-1.The layer flocks had 25 (75.75%) farms positive for MDV out of 33flocks investigated with 6 (18.18%) positive for single infection with MDV 1, 1 (3%) positive for single infection with MDV 2 and 1 (3%) positive for single infection with MDV 3. One (1) out of the two duck flocks investigated was positive for single infection with MDV serotype 1. (Table III).

Multiple infections involving two or three of the serotypes were also observed and in the case of the pullet flocks with age ranges between 8-20 weeks, 4 (21.1%) different flocks were infected by both MDV-1 and MDV-2, 3 (15.79%) flocks with MDV1 and 3 and 3 (15.79%) with all the three serotypes. The layers had 7 (21.2%) different flocks infected with MDV1 and 2, 5 (15.15%) with MDV 1 and 3, 1 (3%) with MDV2 and 3 and 4 (12.12%) flocks infected with all the serotypes. The 8 week old broiler flock investigated had multiple infections with both serotype 2 and 3 while the turkey flock was infected with the combination of the three serotypes (Table IV).

DISCUSSION

In the present study, a diagnostic PCR technique that allows detection of MDV and the different serotypes in clinical specimens from infected commercial chickens is described. The methods to detect MDV are continuously developing and several PCR applications have been reported (Becker *et al*., 1992; Davidson & Borenstein, 1999). The study employed the use of a primer that is specific to the highly conserved region of the gB MDV gene and also primers specific to the hypervariable region of gB MDV gene of the serotype 1, 2 and 3 MDV, to determine the distribution of MDV throughout different chicken population. The amplification of the gBMDV gene is necessary in diagnosis because of its importance for cell attachment and/or penetration (Ikuta *et al*., 1984) and the advantage of this is the possibility to distinguish the MDV serotypes present in infected chickens.

The first round PCR assay performed confirmed 27 (46.55%) of the tested tissue samples as positive for MDV (Table 2). Some samples that were negative at this stage became positive with the use of the internal primers that were specific for the hypervariable region of the gBMDV gene. This support the work of (Bossak *et al.,* 2001) which described the specificity of the internal primers used. It was found that among the clinical specimens 38 (65.5%) were infected with MDV-1, 22 (37.93%) with MDV2 and 20 (34.48%) with MDV3 (Table 3). The study shows that the distribution of three serotypes of virus DNA was quite different among investigated chicken flocks and the level of infection could be influenced by many factors. These factors may include virus strain which the birds are exposed to because the virulence of MDV strains varies widely and appears to have increased over time (Witter, 1997), virus dose and route of exposure, host genetics, age at exposure and environmental factors and stress.

Table 1: The distribution of MDV among different population of chickens.

Type of Flocks	Number of MD suspected flocks	Number Positive for MDV (%)
Pullets (< 20 weeks)	21	15 (71.4)
Layers(>20 weeks)	33	25 (75.75)
Broiler	1	1 (100)
Turkey	1	1(100)
Duck	2	1 (50)
Total	58	43(74.14)

Table II: The occurrence of the MDV serotypes among the flocks.

Type of flocks (Age in weeks)	No of flocks tested	Serotype 1 positive (%)	Serotype 2 positive (%)	Serotype 3 positive (%)
Pullet (< 20)	21	14 (66.7)	7(33.3)	7(33.3)
Layers (> 20)	33	22 (66.66)	13 (39.39)	11 (33.33)
Broiler (7)	1	0	1 (100)	1 (100)
Turkey (Adult)	1	1 (100)	1 (100)	1 (100)
Duck (Adult)	2	1 (50)	0	0
Total	58	38 (65.5)	22 (37.93)	20 (34.48)

Table III: Summary of single and multiple infection with different serotypes of MDV

Type of flocks	Number of flocks tested	Serotype 1 only	Serotype 2 only	Serotype 3 only	Number and Percentage infected with MDV1 and 2 (%)	Number and Percentage infected with MDV 1 and 3 (%)	Number and Percentage infected with MDV2 and 3 (%)	No and Percentage infected with all serotypes (%)
Pullet (< 20 weeks)	21				4	3	0	3
Layers (>20 weeks)	33				7 (21.2)	5 (15.15)	1 (3)	4 (12.12)
Broiler	1				0	0	1(100)	0
Turkey	1				0	0	0	1 (100%)
Duck	2				0	0	0	0
Total	58				11 (18.97)	8 (13.79)	2 (3.45)	8 (13.79)

Table IV: Summary of Multiple infections involving the different serotypes of MDV. SRT; serotype

Type of flocks	Number of flocks tested	Serotype 1 only	Serotype 2 only	Serotype 3 only	Number and Percentage infected with MDV1 and 2 (%)	Number and Percentage infected with MDV 1 and 3 (%)	Number and Percentage infected with MDV2 and 3 (%)	No and Percentage infected with all serotypes (%)
Pullet (< 20 weeks)	21				4	3	0	3
Layers (>20 weeks)	33				7 (21.2)	5 (15.15)	1 (3)	4 (12.12)
Broiler	1				0	0	1 (100)	0
Turkey	1				0	0	0	1 100%
Duck	2				0	0	0	0
Total	58				11 (18.97)	8 (13.79)	2 (3.45)	8 (13.79)

The result show a higher proportion of specimen from the layer flocks 22(66.66%) to be positive for MDV serotype 1. MDV serotype 1 was also confirmed in the turkey and duck specimen investigated. This support the report by Davidson & Borenstein, (1999) that MDV can be easily detected by PCR in lymphoid tumors of infected chickens demonstrating that viral DNA is present in tumors. Serotype 2 MDV infection was also detected in the clinical samples by this study with specimens from the layer flocks having a large amount 13 (39.39%) positive for MDV 2. The specimen from the only investigated broiler flock was positive for MDV2 and also specimen from the turkey flock. This did not support the work of (Schat & Kalnek, 1978)) who reported that serotype 2 strain did not produce neoplastic lesions in either normal or immunosuppressed chickens and also with the work of Cho, (1976) who reported the absence of lymphoma induction with serotype 2 strains of MDV. The presence of MDV serotype 3 which is Herpesvirus of turkey in 1 of the pullet flocks with age less than 8 weeks,6 (31.57%) in pullets with age ranges between 8-20 weeks and 11 (33.33%) in layer flock is not in accord with what was reported by Kurt & Jorgansen, (2000) that HVT, using PCR approach was not detected in commercial chickens though it has been used widely as vaccine and not in accord with Kalnek & Witter, (1997) who reported that the serotype 3 are not clear and need further investigation. The infection caused by only MDV serotype 1 in this result support the

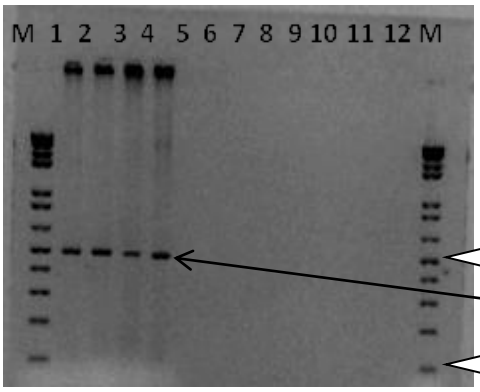

Figure I: PCR result for MDV detection
Lane M= DNA Marker; Bands in lanes 1, 2, 3 and 4 are positive for MDV (491bp), Lane 4= Positive control; Lane 5 =Negative control
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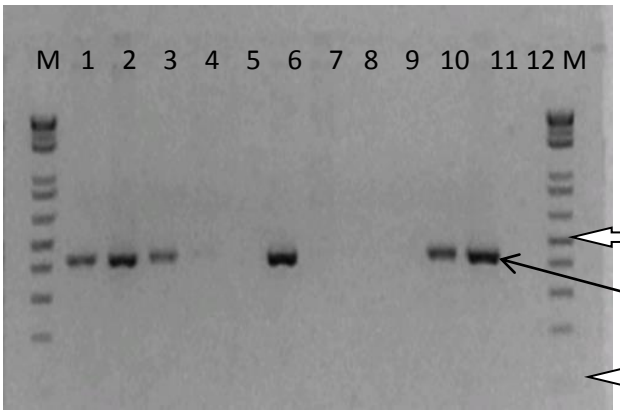

Figure II: PCR result for MDV serotype 1 detection
Lane M= DNA Marker; Bands in lanes 1, 2, 3, 6, 10 and 11, are positive for MDV serotype 1 (421bp); Lane 9= Positive control; Lane 12 =Negative control.

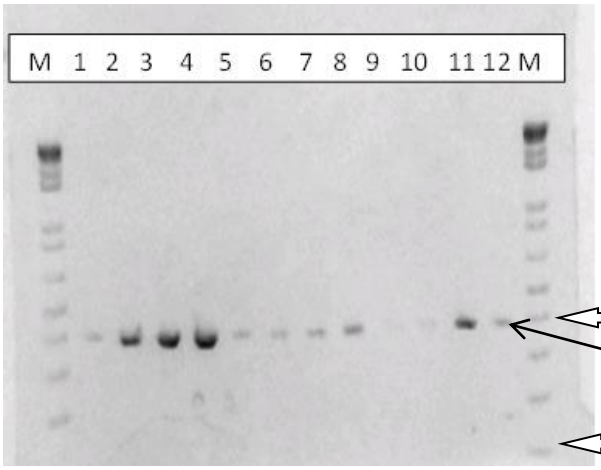

Fig: 3 PCR result for MDV serotype 2 detection
Lane M= DNA Marker; Lanes1,2,3,4,5,6,7,8,11,12 are positive for MDV serotype 2 (386bp). Lane 10= negative control; Lane 12= positive control

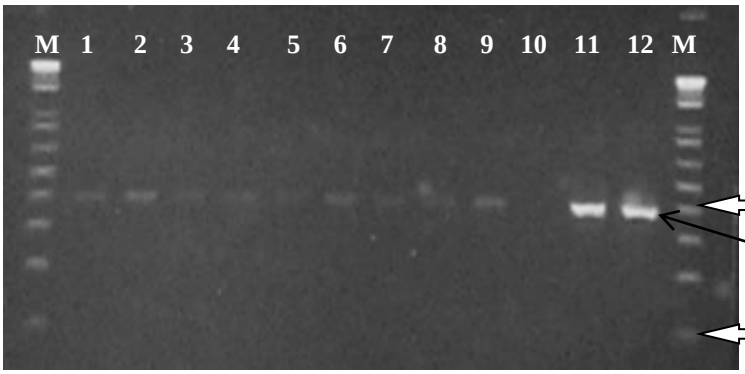

Figure IV. PCR result for MDV serotype 3
Lanes 1-9 are positive for MDV serotype 3 (380bp), Lane 10= Negative control, Lanes 11 and 12= Positive control which was positive for serotype 3

report that serotype 1 MDV is the oncogenic serotype for the group of MDV and also that it is the strain that induce disease in chickens (Biggs, 2001). Single infection and lymphoma formation with MDV-2 and MDV-3 suggest the evolution of virulent MDV2 and MDV 3 in the field and this may not support the report of Kalnek *et al.*, (1981) that viruses of serotype 2 and serotype 3 are nononcogenic. The dual infection with both viruses types MDV1 and MDV2 also MDV1 and MDV3 was observed in this study.These dual infection may be as a result of the presence of more than one serotype of the virus present in the affected farm thereby causing mixed infection with more than one serotype. Marek's disease virus-induced tumors are widespread in chickens and have been extensively studied, but in turkeys they have received much less attention because they occur so infrequent. The confirmation of MDV in turkey in this study using the polymerase chain reaction is in support of the work of Davidson *et al.* (2002) who also amplified MDV in 70% of tumor samples from turkeys in Israel. The study also confirmed MDV ducks which has been reported to be refractory to MDV infection by Baxendale, (1996). The presence of MDV- induced tumors in turkeys and ducks in this study may be as a result of evolution of recent MDV strains to an increase virulence in chickens, resulting in a shift in infectivity from natural host of MDV to the turkey and ducks or may be as a result of raising chickens, turkeys and ducks together which contribute to interspecies transmission of MDV. The types of vaccines used by hatchery operators for some flocks studied include HVT, Rispens and a combination of HVT/Rispens. The major strategy of the vaccine is to prevent early infection, slow the acquisition of virulence of field strain and to provide superior immune responses (Witter, 1997). The presence of MDV serotype 1 in flocks that had history of vaccination with Rispens (CV1988) and HVT/ Rispens suggests that the vaccine used had failed to protect the birds or the birds were exposed to a high virulent strain in the field. It may also be that the vaccines had reverted to a more virulent one which now causes disease. The birds might be exposed early to the field virus before the establishment of immunity. Other factor that contributes to this may include administration of inappropriate dose of vaccine which failed to provide adequate immunity. It is also possible that the vaccines used had lost its potency before administration. It is not possible to be the vaccine virus because Davidson *et al*. (2002) only reported the presence of the vaccine virus (CV1988) in chicks up to 4 weeks of age and that CV1988 DNA is present in vaccinated birds in a low quantity and it is difficult to detect directly probably because vaccine viruses are latent in-vivo. The flocks that were positive for MDV2 and MDV3 despite vaccination with either Rispens/or HVT suggests that the vaccine has failed to protect the birds and this may be as a result of the use of vaccine that has lost its potency before given to the birds due to poor storage temperature, reconstitution technique, choice of diluents and holding time and temperature after reconstitution (Halvorson & Mitchell, 1979).The presence of serotype 3 (HVT) in chickens is strange because turkeys harbor HVT as a ubiquitous virus as initially shown by Witter & Solomon, (1971). The presence of HVT in tumor specimen suggests that there could be a vaccination break or emergence of more virulent type in the field. Marek's disease is most prevalent in flocks between the ages 10 and 35 weeks old (Qie, 2000), although under field conditions, MD outbreaks sometimes occur in unvaccinated layer chickens as young as 3-4 weeks (Witter & Schat, 2003). Most of the serious cases begin after 8-9 weeks, but also sometimes commence well after the onset of egg production (Kreager, 1997). This study detected MDV in flocks less than 8 weeks despite vaccination and also in ages greater than 8 weeks.

CONCLUSION

We can conclude from this study that other serotypes of MDV could be responsible for MD outbreaks. This observation could explain reason for unpredictable MD outbreaks despite vaccination in commercial poultry and to some extent explain increasing virulence of the field MDV serotype 2 and 3 already classified as non-oncogenic.

CONFLICT OF INTEREST

There is no conflicjt of interest regarding the research.

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