Nigerian Veterinary Journal (2003),Vol. 24 (2) 1-9

DETECTION OF INFECTIOUS BURSAL DISEASE VIRUS (IBDV) IN NATURALLY INFECTED CHICKENS IN NIGERIA BY THE REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (RT-PCR)

HARUNA*[1], E. S., ADENE[2], D. F. AND GERRIT[3], V. J.

[1] National Veterinary Research Institute, Vom, Nigeria.
[2] Department of Veterinary Medicine, University of Ibadan, Nigeria
[3] Molecular Biology and Applied Biotechnology Department, Onderstepoort Veterinary Institute, Onderstepoort, South Africa

***Correspondence**

SUMMARY

The Reverse Transcription - Polymerase Chain Reaction (RT-PCR) was used for the identification of Infectious bursal disease virus (IBDV). The technique was applied on bursa of Fabricius of infected chicken. Some of these bursae have been kept in the freezer for 16years under conditions of regular electric power fluctuations. A commercially produced IBD vaccine and two freeze dried IBD viruses grown on chicken embryo fibroblast cell were used as known source of IBD viruses. One pair of primers was produced from published sequences of serotype1 IBDV targeted at the VP2 region of the genomic RNA. These primers were designed to generate a product of 1325bp size. Out of 45 samples tested, IBD viral RNA could be detected in 43 samples by the RT-PCR. No reaction was obtained from the RNA extracted from the control bursae. The use of PCR for the diagnosis of diseases is fairly new in this country though the technology has been in use in most developed and some developing countries. It's application has been found to improve the efficiency and accuracy of disease diagnosis as the test is very specific. The Viral RNA gemone of IBDV could be detected even in bursal samples found to have degenerated due to storage problems.

KEYWORDS: Infectious bursal disease virus, diagnosis, reverse transcription Polymerase Chain Reaction, chicken

INTRODUCTION

Infectious bursal disease (IBD) first described in 1962 (Cosgrove, 1962) as a highly fatal and immuno-suppressive disease of chicken caused by the IBD virus (IBDV). The virus is an Avibirnavirus (Murphy *et. al.,* 1995) of the Family Birnaviridae (Dodos *et. al.*, 1979, Brown, 1986). It is a double stranded RNA virus with two segments, A and B which are 3300 base pairs bp and 2900 bp long respectively. Segment A has two open reading frame (ORF). The smaller ORF proceeds and partially overlaps the larger ORF (Kibenge *et al.*, 1991; 1996). It encodes a 17kda viral protein VP5 whose function may include that of virus induced pathogenicity (Mundt *et. al.*, 1997). The larger ORF is made of a 109 Kda precursor polyprotein which can be processed into two structural proteins, VP2 and VP3 and a 28-30kda putative viral protease VP4. VP2 is the major viral protein that elicit neutralising antibodies in infected birds and is also responsible for serotype specificity of the virus (Hudson *et. al.*, 1986; Becht *et. al.*, 1988 and Fahey *et. al.*, 1989). VP3 is responsible for structural integrity of the virion (Becht *et.al.,* 1988) and also serves

as a minor neutralising site (Jagadish and Azad, 1991; Mahardika and Becht, 1995). The B segment encodes the VP1 which is a putative viral polymerase involved in the replication of viral genome and synthesis of messenger RNA (Bruenn, 1991).

The virus target mainly the bursa of Fabricius of young chickens causing initial enlargement and edema and at later stages atrophy of the bursa. The infection results in reduced weight gain and feed efficiency, increased mortality and immuno-suppression (Lukert and Saif, 1997).

Since the first documented report of the disease in Nigeria (Ojo *et. al.*, 1973) it has been a source of major economic concern to the country's Poultry Industry. It has been reported in all parts of the country in commercial and locally kept backyard chicken (Adene *et. al.*, 1985; Salami *et. al.*, 1989). Of great concern is its occurrence in vaccinated and unvaccinated birds (Okoye 1984 b; c; Awolaja and Adene 1995; Abdu 1986).

Various test have been used for the diagnosis of the clinical and subclinical immuno-suppressive forms of the disease. These include the agar gel precipitation test, virus isolation, virus neutralisation, electron microscope, immunofluoresence test, Enzyme linked immunosorbent assay (ELISA) and monoclonal antibody assay (Lukert and Saif, 1997; Meulemans *et al.*, 1977; Lee, 1992; Snyder *et.al.*, 1988). While these test are good for routine and confirmatory diagnosis they may not detect low levels antigens in tissues and are time consuming, labour intensive and may be non specific. More sensitive techniques such as the Radio labelled (Jackwood *et. al.*, 1989) and non radio labelled cDNA probes (Jackwood *et. al.*, 1990; Lee, 1992) have been tried. These are however expensive and harzadous. With the development of the polymerase chain reaction (PCR) where by a single copy of DNA is amplified many folds at a specific region, the sensitivity of diagnosis has been greatly increased and time required reduced. Reverse transcription PCR (RT-PCR) was also developed to translate the RNA genome into DNA before the PCR. This RT-PCR has been used in the diagnosis of IBDV infection (Lee *et. al.*, 1992, Wu *et. al.*, 1992; Jackwood, 1994; Jackwood *et. al.*, 1996).

We report the use of RT-PCR for the detection of IBDV from bursae of naturally infected chickens from various parts of the country.

MATERIALS AND METHODS

Clinical Samples

A total of 45 samples were used for this work. Of these 42 samples were bursal tissue collected from suspected and also confirmed cases of IBD outbreaks in various parts of Nigeria between September, 1984 and May 2001(Table 1). Two samples were freeze dried IBD viruses grown chicken embryo fibroblast (CEF) and one was a vaccine virus (Fibro Gombovac, NVRI Vom). All samples were stored in the deep freezer (-20°C). Some of the bursal tissues collected had undergone many cycles of freezing and thawing due to electric power fluctuation. Bursae of six week old non-infected birds were harvested three days before running the test and used as non-infected controls.

RNA extraction

Each bursal tissue was thawed weighed and chopped into tiny pieces with sterile scalpel blade. A 10% suspension was made using PBS. This suspension was frozen at -70°C, thawed 3 times and clarified by centrifugation at 13000 rpm for 2 mins. The freeze dried viral samples and the vaccine virus were reconstituted in 2mls. of nuclease free water. Viral RNA isolation was done from 140ul of supernatant using the QIA amp Viral RNA Mini Kit (QIAGEN - Germany) according to manufacturers instructions. The RNA eluted from the column membrane was stored at -70oC until used.

Primers

A pair of oligonucleotide primers were designed from the published sequence of segment A genome of IBDV strain PBG-98 (Gen Bank Assession No. D00868). The sequence of the Primers and nucleotide position on the genome are given in Table 2. This primer pair was designed to give a 1325 base pair size DNA sequence and were synthesize and supplied by MWG Biotech AG, Roche Products (Pty) Ltd, Randbung, South Africa. A 100 pmol/ul stock solution was prepared and stored in aliquot at -20 C. For the PCR 10 pmol/ul solution was made and used.

Reverse Transcriptase-PCR and Analysis

This was carried out using methods as described by Bremer and Viljoen (1998) and Wu *et. al.*, (1992). 6 ul of total RNA was added to a mixture of 1 ul each of the two primers (P1 and P6) and 12ul of nuclease free water (PromegaR). The samples were heated at 96°C for 3 mins and snap cool on ice for 1 min. A 30ul mixture of 5 ul of 5X MMLVRT Buffer (Promega[R]), 0.2ul MMLVRT enzyme (Promega[R]), 2 ul of 10mM dNTP mix and 0.1ul RNAsin in 22.7 ul of Nuclease free water was then added. Samples were incubated at 42oC for 40 mins. PCR Amplification was done by adding 10ul of cDNA to a 40ul mixture containing 1x Dynazyme Buffer (Mg^{2+} free), (10mM Tris-HCL pH8.8, 50mM KCl, 1% Triton X-100), 2mM $MgCl_2$, 200mM each dNTP, 4U of thermostable DNA polymerase and 10pmol of each primer all in 23.5 ul of water. The reaction was carried out in a Perkin Elmer 9700 PCR machine as follows; 94o C for 2 mins followed by 40 cycles of 94oC for 30 sec., 53o C for 30 sec. and 72o C for 2 mins.10ul of the RT-PCR amplicon were analysed by gel electrophoresis using 1.5% Agarose gel containing ethidium bromide. A 50 to 1000 bp marker (PCR Marker Promega[R]) was used to compare the amplicon size. Amplicon size movement was observed using a UV illumination and documented by the Lumi-Imager system (Roche [R]).

TABLE II: Primer sequence and nucleotide positions

Name	Sequence	Nucleotide Position	Direction
IBDV P1	5'-tga tgc caa caa ccg gac-3'	1-18	Fw
IBDV P6	5'-aca gct atc ctc ctt atg gcc-3'	1325-1304	Rew.

TABLE 1: Bursal samples collected by year, geographical region and the Species/Type of bird

S/No	Sample No	Year collected	Geographical Area	Specie/Type of Bird
1	PL/JE/99/NV	1999	Central(C)	Pullets
2	OG/1192/IB	1992	South west (SW)	"
3	BDN/6/BEN	-	Midwest (MW)	-
4	PL/OK/299	1999	C	"
5	BO/6/MAD	1997	Northeast(NE)	-
6	KD/8037/ZA	1988	North-central (NC)	-
7	BO/3/MAD	1997	NE	-
8	PL/LB/698/NV	1998	C	Pullets
9	KD/8002/ZA	1998	NC	-
10	PL/BB/599	1999	C	Bantam Crosses
11	KD/8454/ZA	1999	NC	-
12	PL/MK/500	2000	C	Pullets
13	KD/2606/ZA	-	NC	-
14	KD/8170/ZA	-	NC	-
15	KD/7973/ZA	-	NC	-
16	PL/EPU/696	1996	C	-
17	PL/EPU/88	1988	C	-
18	IB/799/OGB	1999	SW	-
19	KB/101/VN	2001	Northwest (NW)	Pullets
20	PL/984/AJ	1984	C	-
21	PL/984/SEED	1984	C	-
22	HA/501/KAF	2001	NC	Pullets
23	KD/8464/ZA	-	NC	-
24	KD/640/ZA	-	NC	-
25	PL/NVRI/1099/VN	1999	C	Pullets
26	KD/3375/ZA	-	NC	-
27	KD/7281/ZA	-	NC	-
28	KD/8374/ZA	-	NC	-
29	KD/8476/ZA	-	NC	-
30	KD/8480/ZA	-	NC	-
31	KD/8427/ZA	-	NC	-
32	NVRI/VAC/101	2001	C	Vaccine
33	IB/501	2001	SW	-
34	PL/NVRI/194	1994	C	-
35	IB/1101	2001	SW	Broilers
36	PL/NVRI/789	1989	C	Pullets
37	PL/NVRI/594	1994	C	"
38	PL/NVRI/301	2001	C	"
39	PL/NVRI/599	1999	C	"
40	PL/NVRI/788	1988	C	"
41	BA/595/1	1995	NE	"
42	BA/595/2	1995	NE	"
43	PL/NVRI/588	1988	C	"
44	PL/NVRI/393	1993	C	"
45	BO/MA/97	1997	NE	"

RESULTS

The primer sequence analysis alignment comparison with published sequences indicated that these same sequences could be found in IBDV strains STC (Ass No. D00499), GLS (Ass No. M97346) UK strain 661 (X927600 and Israeli strain (L42284). This set of primers were able to amplify a section of the 43 samples out of 45 IBDV RNA extracted. An amplicon fragment of 1325 bp in length was observed for the IBDV RNA extracted (Fig 1). No band was observed with the non-infected control bursae.

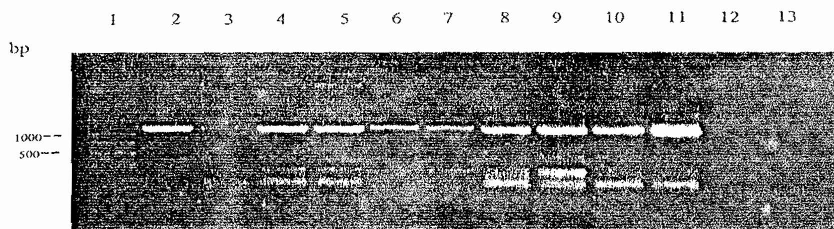

Fig. 1: Detection of Infectious bursal disease virus (IBDV) by RT-PCR on 1.5% Agarose gel Electrophoresis of PCR amplified cDNA fragment (1325bp). Lane 1, Mol. marker [50-1000bp]; Lane 2, OG/1192/IB; Lane 3, BDN/6/BEN; Lane 4, PL/LB/698/NV; Lane 5, KD/8002/ZA; Lane 6, KB/101/VN; Lane 7, PL/984/AJ; Lane 8, KD/8454/Z; Lane 9, BA/595/1; Lane 10, BO/MA/97; Lane 11, NVRI/VAC/10; Lane 12, IBDV Negative Bursa; Lane 13, Water control

DISCUSSION

Infectious bursal disease virus infection in chicken in Nigeria has been diagnose mainly by such serological methods like Agar gel precipitation test (AGPT). Counter immunoelectrophoresis (CIEOP) ands virus neutralisation test (NT) (Okoye, 1984; Adene *et. al.*, 1985, Durojaye *et. al.*, 1985) and by Gross and histopathology (Okoye, 1985; Okoye and Uzoukwu 1982). Some of these techniques are however less sensitive and time consuming. Thus, they may not detect low level infection in tissues.

With the introduction of PCR in the late 80s and its modification and adoption in the detection of IBDV, the sensitivity and time intervals, for diagnosis has been greatly improved. Many workers have reported of the increased sensitivity and reduction in time required for IBDV diagnosis using the RT-PCR technique (Kataria *et. al.*,1998; Jackwood *et. al.*, 1996; Stram *et. al.*, 1994, Lee *et. al.*, 1992). By this technique the tedious process of isolation and identification is eliminated as clinical samples can directly be used. It also has the added advantage of detected organisms in samples that have not been properly stored.

In this study the RT-PCR technique was used for the detection of IBDV from bursal tissue of recent as well as previous outbreaks. These samples were collected between 1984 and 2001. Earlier samples stored in the deep freezer -20oC had under gone many cycles of freezing and thawing due to power fluctuation.

During this work Reverse Transcription was carried out separately from the PCR using the Reverse Trascriptase enzyme in the presence of primers. A one step RT-PCR in which both the RT and PCR steps are done in one tube has been described with equally good and reproducible results (Lee *et. al.*, 1992). The primers used were chosen to amplify a region 1325 bp in length. This consist of the region varying from VP5 VP2 and part of VP4. Thus the whole genome encoding the VP2 region was amplified. The VP2 region as has been reported (Hudson *et. al.,* 1986, Becht *et. al.,* 1988) is a very important part of the viral genome responsible for serotype specificity of the virus and eliciting of Neutralising antibodies. It was observed that RNA isolation, RT and PCR could be accomplished within 5 hours and the procedure is repeated with same results.

The RT PCR described in this paper therefore shows that the technique can be used for routine laboratory diagnosis and retrospective studies of tissues. Though some of the bursal tissue samples had under gone many cycles or freezing and thawing the RNA genome could still be detected in these samples. The amplification of this large amplicon length which spans the length of the VP5 to parts of VP4 can allow the use of the product for Restriction Enzyme analysis and direct sequencing of the whole VP2 region of the IBDV genome.

ACKNOWLEDGEMENTS

The authors acknowledge with thanks the support, encourage and provision of funds for the work by the Director NVRI, Vom. We are grateful to staff of OVI PCR Laboratory, J.O. Mitchell, Claudia and Christine. We are also thankful to Dr. P. Abdu of Ahmadu Bello University, Zaria for providing some of the bursal samples used.

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