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Full Length Research Paper

Rapid identification of chicken anemia virus in Nigerian backyard chickens by polymerase chain reaction combined with restriction endonuclease analysis

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Chicken infectious anemia (CIA) has not been routinely diagnosed in other avian species apart from commercial chickens. A sensitive polymerase chain reaction (PCR) combined with restriction endonuclease (RE) analysis was used for the detection and characterization of chicken anemia virus (CAV) in backyard chickens (*Gallus gallus domesticus*) in Nigeria. Using a pair of primers designed to amplify a 733 bp fragment in the VP1 (capsid protein) gene of CAV, the PCR assay detected CAV DNA in 9 of 12 serum samples from apparently healthy backyard chickens. RE digestion of the purified PCR products of commercial and backyard chickens with *Cfo*I yielded two separate restriction endonuclease patterns, suggesting that Nigerian backyard chicken CAVs differed from the commercial chicken isolates at the nucleotide sequence level. Results of RE analysis also suggested that the backyard chickens contained a mixed population of CAV strains. These findings confirm that backyard chickens are susceptible to CAV infection and could also harbor the virus.

Key words: Chicken anemia virus, polymerase chain reaction, backyard chickens, restriction endonuclease analysis.

INTRODUCTION

Chicken anemia virus (CAV) is a small, non-enveloped, icosahedral virus measuring 25 - 26.5 nm in diameter, with a negative sense, single-stranded circular DNA genome of about 2300 base pairs (bp) in length (Schat, 2003). The virus, which is the only member of the genus *Gyrovirus* of the *Circoviridae* (Pringle, 1999), is ubiquitous and worldwide in distribution. It causes chicken infectious anemia (CIA), a disease of 2 - 4 weeks old chickens characterized by aplastic anemia and generalized lymphoid atrophy with immunosuppression (Schat, 2003). As a result of the accompanying immunosuppression, field cases of CIA are frequently complicated by secondary bacterial infections (Engstrom and Luthman, 1984), Marek's disease vaccination breaks (Bulow et al., 1983) and enhanced severity of infectious bursal disease (IBD) (Rosenberger and Cloud, 1989). The economic losses due to CAV infections result from reduced performance and profitability in infected flocks caused by subclinical infections (McNulty et al., 1991). Older chickens are less susceptible to clinical disease but can still be infected.

Diagnosis of CAV infections can be made by detecting infectious virus, virus antigen, virus DNA or virus-specific antibodies. The virus has been isolated from commercial chickens worldwide (Schat, 2003), including from Nigeria (Oluwayelu et al., 2005). However, diagnosis of CAV infections by the use of conventional virus isolation methods is expensive and time-consuming (McNulty, 1991). The polymerase chain reaction (PCR) has been applied as a rapid diagnostic tool for the detection of virus genome in clinical specimens such as urine and sera (Arthur et al., 1989), faeces (Gouvea et al., 1990) and DNA extracted

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from fresh (Arthur et al., 1989; Rogers et al., 1990) and formalin-fixed, paraffin-embedded tissues (Rogers et al., 1990). Although molecular biologic techniques have been used to detect CAV DNA in tissues and sera from commercial chickens (Tham and Stanislawek, 1992; Todd et al., 1992; Soine et al., 1993), their role in the diagnosis of CAV infection in indigenous chickens remains largely unexplored.

All naturally occurring CAV isolates belong to the same serotype and are antigenically indistinguishable by serum neutralization tests (McNulty, 1991). However, it is possible to differentiate CAV isolates by using immunofluorescent staining patterns with monoclonal antibodies (McNulty et al., 1990), restriction endonuclease (RE) analysis (Todd et al., 1992), DNA sequence differences (Renshaw et al., 1996) and amino acid sequence comparisons (Islam et al., 2002). Although CIA has not been hitherto routinely diagnosed in backyard chickens, CAV-specific antibodies have been detected in Nigerian backyard chickens (*Gallus gallus domesticus*) (Emikpe et al., 2005; Oluwayelu, 2006) and in fancy chicken breeds (De Wit et al., 2004), which are similar to backyard chickens. Moreover, Ducatez et al. (2006) reported a comprehensive study of the CAV molecular epidemiology in Nigerian commercial chickens but not in backyard chickens.

In the present study, the PCR combined with RE analysis was used to rapidly detect and characterize CAV in Nigerian backyard chickens as a first step to elucidating their role in the epidemiology of CAV infections.

MATERIALS AND METHODS

Clinical samples

Since there were no clinically ill or dead backyard chickens from which samples could be collected for this study, blood samples obtained by jugular venipuncture from 151 apparently healthy backyard chickens in several flocks in Ibadan, Oyo state, Nigeria were allowed to clot at room temperature. The sera, which were separated into Eppendorf tubes and heat-inactivated at 56°C for 30 min, were then stored at -80°C until tested. The chickens varied in age but all were greater than one year of age.

Serology

The presence of CAV-specific antibodies in the sera was detected using a modified blocking ELISA (MBE) which was developed and standardized (submitted manuscript). Briefly, 100 µl of a 1:200 dilution of coating antigen in PBS was added to each well of a Maxisorp ELISA plate and incubated overnight at 4°C. The plate was washed four times with ELISA wash solution (0.1% v/v Tween 20 in PBS pH 7.3). A 1:100 dilution of each test serum was carried out in duplicate wells by first doing a 1:10 dilution in PBS-Tween 20 (PBST) (0.05% v/v Tween 20 in PBS) in a microtitre plate. A further 1:10 dilution of each PBST-diluted serum was then made in ELISA diluent (3% v/v horse serum, 0.05% v/v Tween 20 in PBS) in the test (antigen-coated) plate to give a final dilution of 1:100 and the plate incubated at 37°C for 1 h. For development and evaluation purposes, each serum sample was tested in duplicate wells. Each plate contained two pairs of positive control (PC) and negative control (NC) serum samples as well as serum-free or "no serum" (NS) controls that contained 100 µl ELISA diluent only. Eleven microliters of a 1:2000 dilution of monoclonal antibody (Mab) 2A9 (15) in ELISA diluent was then added per well without washing off the serum to give a final monoclonal antibody dilution of 1:20000 and the plate further incubated for 1 h at 37°C. A 1:2000 dilution of horseradish peroxidase conjugated rabbit anti-mouse immunoglobulin (Nordic Immunological Laboratories, The Netherlands) was made by first doing a 1:200 dilution in PBST. A further 1:10 dilution of the PBST-diluted conjugate was then made in milk powder (3.3% w/v milk powder in PBST) to give a final conjugate dilution of 1:2000. The plate was then washed four times with ELISA wash solution, 100 µl of diluted conjugate added per well and the plate further incubated for 1 h at 37°C. After washing four times with ELISA wash, 100 µl TMB substrate was added per well and the plate left at room temperature for 10 min. The enzyme reaction was stopped with 25 µl of 0.5 M H_2SO_4 and absorbances were read at 450 nm using a Dynex Revelation 4.22 ELISA reader (Dynex Technologies, USA). A positive result was indicated for sera with Sample : Negative (S/N) values ≤ 0.80. Serum samples with S/N values > 0.80 were considered to be CAV antibody-free.

DNA extraction

Using 12 sera that gave strong positive results with the MBE, DNA extraction was carried out with the QIAamp DNA mini-extraction kit (QIAGEN Ltd., UK) according to the manufacturer's instructions, except that 100 µl of each serum was added to 100 µl of buffer ATL. Positive control DNA was extracted from Cuxhaven-1 (Cux-1) isolate-infected plasmid pCAA-3 (Meehan et al., 1992). The DNA extracts were kept at -20°C until analyzed by PCR.

PCR

PCR was carried out to amplify a 733 bp fragment in the VP1 (capsid protein) gene of CAV using the Taq PCR Master Mix kit (QIAGEN Ltd., UK) according to the published protocol (Oluwayelu et al., 2005). However, 35 cycles of amplification were used. The sequences of the oligonucleotide primers 5' - CTGTTCCGACACATTGAAACC - 3' and 5' - CCCCAGTACATGGTGCTGTT - 3' were designed based on the published DNA sequence of the Cux-1 CAV strain (Meehan et al., 1992).

Analysis of amplified products

25 µl aliquots of each PCR product was electrophoresed with 5 µl loading dye on 1% agarose gel stained with ethidium bromide, and the separated DNA bands were visualized by ultraviolet (UV) transillumination. A 100 bp ladder (Roche Diagnostics GmbH, Germany) and lambda DNA/Hind III fragments (Invitrogen, Carlsbad, CA) were used as size markers.

Restriction endonuclease (RE) analysis

The amplicons were purified with the Wizard® PCR Preps DNA purification kit (Promega, Madison, WI). Purified DNAs of the Cux-1 CAV isolate, a Nigerian commercial chicken isolate (NGR-5) sequenced in a previous study (unpublished data) and two backyard chicken sera (LC49 and LC52) that gave strong bands following agarose gel electrophoresis of PCR products in this study were

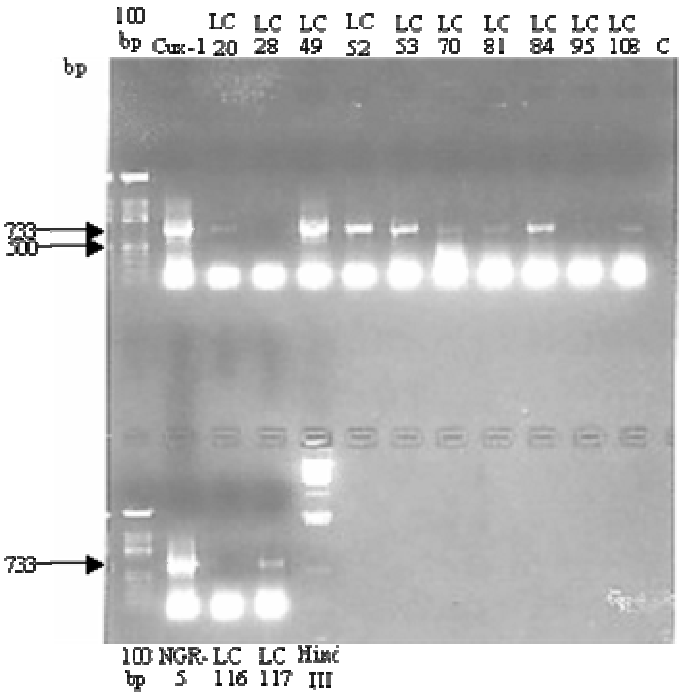

Figure 1. PCR amplification of CAV DNA from 9 of the 12 Nigerian backyard chickens tested. Lanes 1 and 14: 100 bp DNA fragments (size marker); Lane 2: positive control obtained by amplification of DNA extracted from Cux-1 isolate-infected plasmid pCAA-3; Lanes 3 - 12, 16, 17: products obtained by amplification of DNA extracted from backyard chicken sera (LC20, LC28, LC49, LC52, LC53, LC70, LC81, LC84, LC95, LC108, LC116, LC117); Lane 13: negative control (sterile distilled water); Lane 15: product obtained by amplification of NGR-5 DNA; Lane 18: phage λ DNA digested with Hind III (size marker).

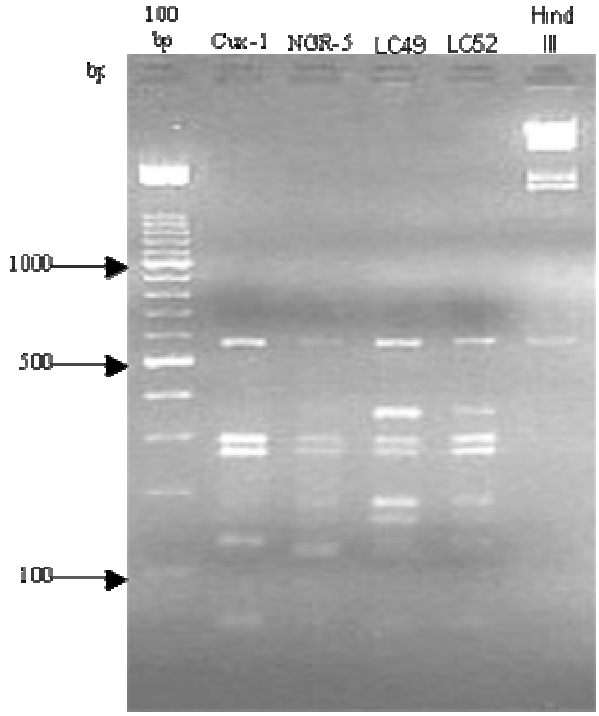

Figure 2. Agarose gel electrophoretic patterns generated following *Cfo*I digestion of purified DNAs of Cux-1 CAV (Lane 2), NGR-5 (Lane 3), LC49 (Lane 4) and LC52 (Lane 5); 100 bp DNA fragments and phage λ DNA digested with Hind III (Lanes 1 and 6 respectively).

selected and digested with *Cfo*I endonuclease (Sigma, St. Louis, MO) in 20 µl reaction volumes. The restriction digests on 2% agarose gel were stained with ethidium bromide and visualized by UV transillumination.

RESULTS

Serology

100 (66.2%) of the 151 backyard chicken sera screened by the MBE were positive for CAV antibodies.

Detection of CAV DNA in sera by PCR

A single DNA fragment of 733 bp was produced following agarose gel electrophoresis of 9 (75.0%) of the 12 backyard chicken samples amplified. The size of the PCR product was the same as that produced using DNA specific to the Cux-1 CAV isolate (Figure 1).

Restriction endonuclease analysis

Purified DNAs of the Cux-1 CAV and NGR-5 were cleaved by *Cfo*I into four fragments of estimated sizes of 288, 266, 128 and 51 bp. The RE profile for the CAVs from backyard chickens (LC49 and LC52) contained four fragments of sizes as described for the commercial chickens as well as additional fragments of about 340, 180 and 160 bp (Figure 2).

DISCUSSION

Apart from the reports of serologic evidence of CAV infection in backyard chickens (Emikpe et al., 2005; Oluwayelu, 2006; De Wit et al., 2004), the demonstration of CAV from naturally occurring, clinically or subclinically infected backyard chickens and characterization of the virus obtained from them has not been reported before. A knowledge of the CAV strains that infect backyard chickens may give an insight into understanding the CIA epidemiology, especially considering the role backyard chickens play in the epidemiology of some other viral diseases of poultry (Adene et al., 1985; Adu et al., 1985;

Gutierrez-Ruiz et al., 2000; Tan et al., 2004). In this study, the PCR combined with RE analysis has been used for rapid identification and characterization of CAV in Nigerian backyard chickens.

The PCR detection of CAV gene sequences in backyard chicken sera in this study is consistent with previous reports of detection of DNA of infectious agents in serum samples (Arthur et al., 1989; Tham and Stanislawek, 1992). The PCR assay used for this study has been shown (Oluwayelu et al., 2005) to have a sensitivity limit of 0.1 *fg* of target CAV DNA. Therefore, the detection of CAV DNA in backyard chicken sera by PCR confirms the susceptibility of these chickens to CAV infections since DNA presence is evidence that they were actually infected with the virus. Restriction endonuclease analysis of the 733 bp PCR products using *Cfo*I yielded two different R.E. patterns (Figure 2). The Cux-1 CAV and NGR-5 had the same pattern which was different from that obtained for the two backyard chicken CAVs. This suggests that Nigerian backyard chicken CAVs differ from the commercial chicken isolate at the nucleotide sequence level. Whereas the latter had a RE profile resembling that of the Cux-1 CAV strain, the backyard chickens had a unique profile combining that of the Cux-1 isolate with three additional fragments (Figure 2). This finding suggests that the backyard chickens contain mixtures of CAV strains that have different RE profiles. Cloning and sequencing of the PCR-amplified fragments will be required to determine the extent of genetic diversity that exists within the CAVs that infect backyard chickens and to determine the relationship between these CAVs and those that infect commercial chickens in Nigeria. Furthermore, the effect of this RE pattern difference on the biological characteristics of Nigerian backyard chicken CAV strains such as pathogenicity in one-day-old specific-pathogen-free chicks and growth characteristics in MDCC-MSB1 cell culture needs to be investigated.

Since neither vaccination nor other preventive measures are in practice in backyard chickens in Nigeria, the detection of CAV DNA and antibodies in the sera of apparently healthy, free-roaming Nigerian backyard chickens in this study indicates natural exposure to the virus and implicates them as a potential source of the infection to commercial chickens. Moreover, the high detection rate of CAV DNA in the sera of backyard chickens was unexpected since it is presumed that the presence of virus-specific antibody will remove virus from serum. Given the age profile of the birds tested and high seroprevalence, it is highly unlikely that all of these backyard chickens were recently infected. It is possible that the backyard chickens were persistently infected and that CAV is continually present in their sera. Cardona et al. (2000) reported that neutralizing antibodies will not necessarily eliminate virus from the bird and that a latent infection may become established in the reproductive tissues. Also, Yuasa et al. (1983) noted that CAV infectivity appeared to persist in chicken tissues even in the presence of circulating antibodies. Unlike these previous studies, CAV DNA was found in the sera of healthy naturally-infected backyard chickens in the present study, suggesting a persistent viremia.

Clinical disease due to CAV is uncommon but subclinical disease of commercial broilers is more common and results in reduced performance, poor growth and economic losses (McNulty et al., 1991). The backyard chickens sampled for this study had no apparent clinical illness or mortalities and were older than one year of age. The detection of CAV-specific antibodies in their sera is therefore an evidence of subclinical infection with the virus. Soine et al. (1993) had reported that the PCR can be used for detecting subclinical CAV infection in chickens. On the basis of the results obtained in this study, it can be concluded that lower productivity and poor performance generally associated with backyard chickens in Nigeria, among other factors, are also related to CAV infections. In addition, backyard chickens provide a rich milieu for the generation of novel genotypes of CAV that may alter the epidemiologic picture of this virus in future.

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