Diagnosis and Molecular Characterization of Chicken Anaemia Virus

Nishant Singh Saini[1] and Anjan Dandapat[2]

Dairy Cattle Breeding Division,
National Dairy Research Institute, Karnal, Haryana - 132 001

Abstract

Chicken infectious anemia (CIA) is an emerging disease especially of young chickens and has proved considerable health problems and economic losses to the poultry industry worldwide. The disease is characterized by aplastic anemia, hemorrhages in the muscle and subcutaneous tissue, thymus atrophy and immunosuppression. CIA infection is relatively easy to identify based on the pathognomic signs and lesions exhibited by the affected flock.Tentatively it can usually be made based on flock history, clinical signs, haematological changes and gross pathological findings in affected birds. For confirmatory diagnosis isolation and identification of the CIAV is done. Reduced haematocrit (PCV) values are the sensitive indicator to identify clinically affected birds with CIAV following experimental exposure. Monitoring of CIAV infection by virus isolation, antigen and CIAV-specific antibody detection by enzyme linked immunosorbent assay (ELISA), virus neutralization test (VNT), immunofluorescent test (IFT) and immunoperoxidase test (IPT), along with application of molecular diagnostic tools such as polymerase chain reaction (PCR), nucleic acid hybridization and sequencing etc. can be used for confirmatory diagnosis of CIAV infection.

Keywords: Chicken, Diagnosis, Virus, Molecular characterisation, Economic, Industry.

Isolation

Embryonated eggs, cell culture (MDCC-MSBI cells), one-day-old specific pathogen free (SPF) chicks lacking maternally derived antibodies to CIAV are the suitable host systems for the isolation of CIAV from the suspected materials. Due to higher concentration of virus, liver is the best source for virus isolation. Maximum virus titre has been detected 7 days p.i. CIAV can also be isolated from rectal contents, thymus, buffy coat, splenic tissues, bone marrow, bursa, lung, heart and muscle (McNulty et al., 1989, McNulty, 1998).

Bioassay

The most specific method that is routinely followed for the primary isolation of CIAV is the inoculation of susceptible (free of anti-CIAV maternal antibody) one-day old / SPF chicks by intramuscular or intraperitoneal route with the suspension of suspected material. Preferably, 20% tissue homogenate is clarified, extracted with chloroform and heat treated for use as an inoculum. The inoculated chicks are examined for the presence of anaemia after 12-16 dpi which will be assessed by low hematocrit value (PCV < 25%), typical gross lesions and histopathological findings (Otaki et al., 1988, McNulty et al., 1989, McNulty, 1991, Brentano et al., 1991, Coombes and Crawford, 1996, Kataria et al., 1999, Dhama, 2002). Reproducibility of these lesions is used for identification of CIAV. Isolation of CIAV can be achieved in one or two passages, but the naive one-day-old chicks are not always available and the time lapse of 2 weeks per passage in- vivo before anaemia can be confirmed is the limitation (Yuasa et al., 1979, McNulty et al., 1989, McNulty, 1998).

Cell Culture

The most commonly used cell line is MDCC-MSB1 that is Marek's disease virus transformed chicken lymphocytes (T-cell) derived from a Marek's disease T-cell splenic lymphoma (Akiyama and Kato, 1974, Yuasa, 1983, Goryo et al., 1987). MDCC-MSB1 suspension cell cultures (2-3 x 105 cells/ml in RPMI-1640 medium) inoculated with appropriately prepared tissue homogenate containing CIAV will produce cytopathic effects (CPE) characterized by enlarged and misshapen cells with the nuclei containing small vacuoles and aggregation of chromatin, cell degeneration and lysis, along with alkalinity of the medium (due to pH remains increased) and the inability to subculture (Bulow et al., 1985, McNulty et

1. E-371, Subhash Nagar, Roorkee, Dist. Hardwar, Uttarakhand - 247 667 2. Ph.D. Scholar

al., 1989, McNulty, 1991, 1998). The titre of the virus is quantified by virus infectivity titration (TCID50/ml) assay which involves subculturing of the inocu!ated cells every 2-3 days until cells inoculated with the endpoint dilution of CIAV are destroyed (7-10 subculture) (Imai and Yuasa, 1990). The growth of virus in cell culture is routinely confirmed by indirect immunoflourescent technique (IIFT) described by McNulty et al. (1988). This virus can grow well in- vitro in tumorous cells, as kinase enzyme activity is rich in these cell lines, which is required for phosphorylation of the apoptotic protein of virus replication. Some CIAV strains could not adapt to MDCC-MSB1 cell line. Hence, MDCC-CU 147 (Monoclonal lesion derived T-cell line) was found to be an alternative cell line after successful isolation of 10 fold low doses of virus that is used for MDCC-MSB 1 cell lines. Other cell lines suitable of propagation and assay include MDCC-JP2 (T cell, MDV transformed), LSCC-l104/ X5 B1 (B-cell, induced by ALV) and LSCC-HD11 (AMV transformed) lymphoblastoid cell line (Calnek et al., 2000). The standard cell cultures are not susceptible for the CIAV infection. Only few of the specific lymphoblastoid cell lines are employed for virus isolation.

Identification

The virus can be identified by using direct diagnostic techniques in which CIAV antigen or the virus itself can be demonstrated in the tissues of affected chicks, or by using indirect diagnostic technique which involves detection of serum antibodies to CIAV using standard immunological tests and/or employing the diagnostic techniques for the detection of CIAV-DNA.

Detection of Virus / Antigen

Clinical signs and the direct demonstration of virus/antigen in clinical samples submitted are reasonable criteria for diagnosis of this disease. The usefulness of immunoassays for CIAV antigen detection in infected tissues/ MDCC-MSB 1 cells are being well employed. Tissue impression smears and cryostat sections, fixed with acetone are used for either direct / indirect immunofluorescence staining employing polyclonal chicken or rabbit hyperimmune serum or monoclonal antibodies to CIAV (McNulty et al., 1990, McNulty et al., 1991, Dhama et al, 2002). Infected MSB1 cells should be collected just prior to cell lysis, i.e., 36-42 hr after inoculation, are smeared onto glass slides, acetone fixed and reacted with reference (anti-CIAV) serum and then with FITC labelled rabbit anti-chicken IgG (McNulty et al., 1988, Zhou et al., 1997) and then observed under the fluorescent microscope. Fluorescent staining of small, irregularly shaped granules in the nucleus of enlarged cells is observed in positive cases of specific immunofluorescent antigens.

Serological Assay

Serology can be employed for epidemiological study of CIAV in the flock. The antibody tests are more reliable for screening of the flocks for CIAV antibody but these does not indicate whether the chickens are currently infected or earlier infected. Serological testing for CIAV infection is important in two areas of poultry production for seromonitoring of SPF/breeder flocks for CIAV specific antibody to avoid the risk of CIAV contamination of avian vaccines and clinical disease in progeny flocks, respectively. Antibodies to CIAV in chicken sera or egg yolk can be assessed by virus neutralization (VN), enzyme linked immuno-sorbent assay (ELISA), immunofluorescence (IIFA) and immunoperoxidase (IP) tests (Bulow et al., 1985, McNulty et al., 1988, Brewer et al., 1994, McNulty, 1998).

Indirect Immunofluorescence Test (IFAT)

Surveillance of antibody against CIAV is usually done with Indirect IFA technique (Bulow et al., 1985, Yuasa et al., 1985, McNulty et al., 1988, 1989) as it detects antibody better than antigen. IIFT has been effectively employed for screening of hybridoma supernatant fluid (McNulty et al., 1990). CIAV infected MSB1 cells are used as positive antigen for detecting antibody to CIAV. IFA titers of 1:40 and higher are generally considered positive. Although, it has been conventionally used in most serological surveys for breeder flocks(McNulty., 1991) the test however , lacks sensitivity in detecting low levels of antibody to CIAV as compared to SNT, and is prone to non-specific or false positive results (Bulow, 1988, Otaki et al., 1988). Employing mAbs against VP3 (CIAV specific protein) infected cells can also be efficiently stained in an IIFT (Todd et al., 1990, Noteborn et al., 1994).

Detection of CIAV at DNA level

Conventional diagnostic methods, such as diagnosis of CIAV infections based on histopathological lesions alone cannot be done, electron microscopy and routine virus isolation techniques, which are often have certain shortcomings such as highly expensive, time consuming and unable to detect CIAV strains which, failed to replicate in MSB1 or other lymphoblastoid cell lines. Serological tests have certain limitations and also do not indicate the time of infection. So, highly sensitive and specific, time and labour saving techniques of molecular biology such as Polymerase chain reaction (PCR), hybridization assay, Restriction Enzyme (RE) analysis, sequencing needs attention for the detection and confirmatory diagnosis of CIAV-DNA and will be of

great value for the molecular epizootiological studies and diagnostic laboratories in avian medicine.

1. Polymerase Chain Reaction (PCR): It has been reported that the molecular technique of PCR assay, a sequence specific target gene amplification, highly specific and extremely sensitive for the direct detection of CIAV DNA in infected specimens. PCR assay has been applied for the detection of CIAV DNA from various samples such as cell-free virus, infected MDCC-MSBI cells, unfixed liver/lymphoid organ homogenates (fresh/frozen organs), formalin fixed liver homogenate or formalin fixed paraffin embedded (FFPE) tissues (thymus etc.) and blood smears from experimentally or field infected chicks and in serum samples from disease free chickens (Noteborn et al., 1992, Tham and Stanislawek, 1992).

CIAV DNA genome of various CIAV strains has been cloned and sequenced enabling construction of oligonucleotide primers to specifically amplify the particular gene of interest (Noteborn et al., 1991). Todd et al. (1992) employed primers of 20-25 bp in size flanking 675 bp DNA fragments encompassing part of the putative gene for the capsid protein. The ability of these primers to amplify DNAs of 14 different isolates (to generate a PCR product of expected 675 bp) from different countries indicated towards the possession of similar primary structures in their capsid proteins indicating also the sequence of this region apparently to be highly conserved among the various strains. Tham and Stanislawek (1992) reported that the PCR assay could detect a single infected cell or 10-1.5 TCID50 of cell free virus or 1fg (100 genome copies) of CIAV replicative form of DNA, which indicates the higher sensitivity of PCR assay over hybridization assay. Tham and Stanislawek (1992) utilized a set of complementary oligonucleotide primers (20 bp long, nt/bp positions of 485-504, 1067-1048), amplifying the coding gene sequence of CIAV-DNA genome, yielding amplified product of 583bp which was confirmed by a unique Hind III RE cleavage pattern at position 791. Primers were selected on the basis of published DNA sequence of Cuxhaven-l strain of CIAV (Noteborn et al., 1991). Thus, PCR can detect a small number of CIAV particles directly from clinical specimens. Nucleotide sequences appearing in the database had 100% identity to the primer pairs used indicating to be highly conserved and also the region amplified was highly conserved among the strain. Using two pairs of primers designed to cover the whole genome, the CIAV genome of 2300 bp was amplified into two fragments of 1500 bp and 800 bp (Soine et al., 1993). Nested PCR (primers-nt position 2303, 1528) enhanced the sensitivity of PCR. Compared with culture in MDCC-MSB1 cells and chick inoculation assay of CIAV in avian biological products by PCR was more sensitive (Rodenberg et al., 1994). Goodwin et al. (1996) established the suitability of PCR for the detection of CIAV in formalin fixed paraffin embedded (FFPE) thymus section. Use of PCR assay with FFPE tissues is of high diagnostic value, since it allows detection of both microscopic lesions and viral DNA. It also allows retrospective studies of the disease caused by or associated with CIAV.

Kataria et al. (1999) detected CIAV-DNA in tissues of chicks showing clinical sings of infectious anaemia by PCR. By employing PCR, transmission studies and virus isolations Kataria et al. (1999) confirmed the occurrence of CIAV in India. Subsequently, Dhama (2002) utilized PCR for diagnosing CIAV infection in clinical samples/ experimental studies and its detection during in vitro isolations and passages in MSB1 cells. Cardona et al. (2000), Senthilkumar et al. (2003) and Brentano et al. (2005) employing nested PCR reported wide distribution and longer persistance of CIAV in the reproductive tissues of infected birds. Yamaguchi et al. (2000) established competitive PCR, the quantitative method for CIAV detection which is rapid, highly reproducible, sensitive and reliable method for quantification of CIAV by possessing several advantages over conventional infectivity titration methods. Markowski (2002) developed a strain-specific real-time PCR for quantitation of PCR. Caterina et al. (2004) developed a multiplex polymerase chain reaction (mPCR) for the simultaneous detection and differentiation of avian reovirus (ARV), avian adenovirus group I (AAV-I), infectious bursal disease virus (IBDV), and chicken anemia virus (CIAV). The mPCR DNA products were visualized by gel electrophoresis and consisted of fragments of 365 bp for IBDV, 421 bp for AAV-I, 532 bp for ARV, and 676 bp for CIAV. The mPCR assay developed in this study was found to be sensitive and specific method for detection of virus in droppings. It is expected that PCR assay will be a highly efficient replacement of current laboratory, Test for CIAV diagnosis and particularly for the study of molecular epizootiology of CIA.

2. Restriction Enzyme (RE) Mapping: RE analysis remains one of the molecular methods that can effectively differentiate the DNA/amplified DNA fragments of different virus isolates. Noteborn et al. (1992) compared the DNA genome of various CIAV isolates to that of cloned CIAV DNA more closely by R.E. digestion (EcoR I, Acc I, Bgl II, Hind III, Sst I, Bam HI, Xba I) and showed that R.E. patterns of the analysed DNAs were very similar to each other and also to that of cloned CIAV DNA. Inspite of the overall high degree of similarity, some of the interesting minor

differences among the CIAV isolates were noticed. Some CIAV field isolates lack an Acc I site (position 356) or Hind III (Position 791, located in ORF) and only a minority contain an EcoR I site. RE analysis of the PCR amplified DNAs (675 bp PCR product) (Todd et al., 1992) with the enzymes Hae III, Hinf I and Hpa II, cleaving at 5, 3 and 3 sites, respectively (generating fragment sizes 30-300 bp) indicated that the CIAV isolates (14) from several countries can be assigned to seven groups (1- 7), the isolates from different countries usually exhibiting the greatest number of restriction site differences. Hae III proved to be the most useful enzyme, producing 6 different patterns. RE analysis of the Japanese isolates by Imai et al. (1998) could not detect strains lacking Hind III site (position 791) as detected by Noteborn et al. (1992) in the two Dutch strains. Restriction fragment length polymorphism (RFLP) patterns of Chinese isolates of CIAV indicated similarity between (Chen et al., 1999). Santeen et al. (2001) demonstrated the possibility to use RE analysis of the PCR products, instead of sequencing, to characterize and distinguish different CIAV sequences with potentially important amino acid differences in VP1 and reported the usefulness of the RE enzymes Bam HI, Hha I and Sst II for epidemiological studies in addition to those suggested by Todd et al. (1992). Variation among the six Indian CIAV isolates has been detected on the basis of RE analysis of PCR amplified products (Dhama, 2002). RE patterns indicated that CIAV isolates from closely related outbreaks are identical while isolates from unrelated outbreaks within the same country could sometimes be differentiated. Senthilkumar (2004) has detected variation among five Indian CIAV isolates based on RE analysis of PCR amplified VP1 region and found RE enzymes viz. Hha I, Dde I, Sac I and Hae III were useful for the differentiation of the CIAV isolates.

3. SDS-PAGE: There are very few reports regarding SDA-PAGE in CIAV. Todd et al. (1991) did southern blotting of CIAV isolate and revealed that the CAA-specific 1.3- and 1.0-kbp fragments generated by treatment of pCAA-1 with Hind III were fractionated by electrophoresis in 1% agarose, and the DNA was Southern blotted (14) onto nylon membranes (Hybond-N; Amersham). Blots were hybridized with 32P-labeled DNA probes prepared by the oligo priming method from either the 1.3- or the 1.0-kbp fragment present in slices of low-melting-point agarose after electrophoretic separation.

PCR fragments were separated in 1.5% agarose gels, exposed to ultraviolet light for 2 min, denatured and transferred to a nylon membrane using standard Southern blotting techniques (Sambrook et al., 1989). The DNA was cross-linked to the membrane in a Spectolinker XL-1500 UV cross-linker (Spectronics). The Genius probe labelling and detection kit (Boehringer Mannheim) was used for hybridization and detection. The probe used was a nick-translation labelled clone of the CIAV genome. Briefly, pCIA-AB (Soiné et al., 1994) was digested with EcoRI and fractionated on a 0.75% agarose gel ; the smaller band (2.3 kb) was cut out from the gel and the DNA was purified using the Concert gel purification kit.

4. Nucleotide Sequencing: Farkas et al. (1996) reported nucleotide differences introducing amino acid changes within the three hyper variable regions, with most of the changes in VP1 among the strains, which might influence the antigenic behavior of different VP's. Renshaw et al. (1996) highlighted a possible functional role for the natural changes in the hypervariable (HV) region contributing to differences in efficiency of viral replication or rate of spread in vitro. Cardona et al. (2000) studied chickens from five flocks representing three different strains and examined for the presence of CIAV using nested PCR. The SH-l strain of CIAV was isolated from these tissues and partially sequenced. Only minor sequence differences were found compared to CIA-l and Cux-l. Analysis of the nucleotide sequence of 1766 bp from position 386 to 2151 revealed 12-52 nucleotides variation in Indian CIAV isolates (Senthilkumar, 2004).

PCR based detection of CIAV from blood samples might be an efficient method for diagnostic and epidemiological purposes. The PCR assay will offer a highly efficient replacement of current laboratory tests for CIAV diagnosis and research and for the study of molecular epizootiology of CIA. RFLP and gene sequencing have often been used to study variation among the field viruses as well as their relationship with other reported viruses and the vaccine virus. Such studies are prerequisite for molecular characterization of the pathogen, so as to understand the molecular epidemiology of the disease, as well as to identify suitable vaccine candidates. The application of gene sequencing of CIAV is useful for detection of variation of virus circulating in field.

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