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DETECTION OF CHICKEN ANAEMIA VIRUS AND AVIAN REOVIRUS BY POLYMERASE CHAIN REACTION AND FLUORESCENT ANTIBODY TEST IN VARIOUS TISSUES FROM EXPERIMENTALLY CO-INFECTED CHICKS

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Avian reovirus (ARV) is an economically important emerging avian pathogen associated with a variety of disease manifestations mainly viral arthritis, malabsorption syndrome and infectious proventriculitis, which are commonly observed in broilers. Chicken infectious anaemia (CIA) caused by chicken anaemia virus (CAV) has recently been reported in the country. These disease conditions many a times go unnoticed in field due to masking of pathognomic symptoms by secondary/mixed infections and nutritional disorders, which are commonly observed. Therefore, the present study was formulated with the objective to detect CAV and ARV specific antigen/nucleic acid by indirect immunofluorescent test (IIFT) / fluorescent antibody test (FAT) and polymerase chain reaction (PCR) / reverse transcription - polymerase chain reaction (RT-PCR), respectively. Detection of CAV and ARV infections by PCR/ RT-PCR and IIFT/FAT in various tissues of infected chicks proved to be rapid and reliable tests for confirmatory disease diagnosis and could well be used for the detection of these agents at an early stage.

The commercialization of poultry industry, which started as backyard rearing, has been achieved by adoption of advanced techniques and intensive poultry farming. This rapid growth has lead to a high pressure, especially on the broiler population in order to show better performance. As a result more stressed broiler population has become available for some opportunistic microorganisms to evolve into higher pathogenicity levels, and the already existing pathogens to enhance their virulence. Among these many viral agents cause economically significant clinical conditions in poultry. A glaring example of such an agent is Avian Reovirus (ARV).

ARV has been implicated in different manifestations viz. respiratory diseases (Hussain *et al*., 1981); enteric conditions including cloacal pasting and ulcerative enteritis in young chicks (stunted chick / malabsorption syndrome / helicopter disease) (Dutta and Pomeroy, 1967), pericarditis (Mustaffa-Babjee *et al*., 1973), hydropericardium (Spradbrow and Bains, 1974), infectious proventriculitis (Kouwenhoven *et al*., 1978), anaemia with swollen spleen and liver with petechiation of skeletal muscles (Vielitz and Landgraf, 1974) and viral arthritis (tenosynovitis / brittle bone disease / femoral head necrosis / infectious stunting and runting syndrome) (Kataria *et al*., 1986). Viral arthritis / tenosynovitis is seen mainly in heavy breeds of poultry, usually at about 5-7 weeks of age (Jones and Kibenge, 1984).

Chicken infectious anaemia (CIA) causing infectious anaemia in chickens is an emerging pathogen with worldwide distribution (Bulow and Schat, 1997). Chicken is the only natural host for CAV and it affects mainly young chickens causing poor weight gain, reduced haematocrit values, anaemia, aplasia of bone marrow and generalized lymphoid atrophy especially of the thymus leading to a concomitant immunosuppression (Bulow and Schat, 1997; Todd, 2000). The agent first isolated by Yuasa *et al*. (1979) from contaminated vaccines in Japan, has been recently designated in a newly created genus *Gyrovirus* (Pringle, 1999) within the family *Circoviridae*.

Under field conditions, CAV infections mostly go unnoticed but co-infection with other immunosuppressive agents like Marek's disease virus (Dhama *et al*., 2002), infectious bursal disease virus (Rosenberger and Cloud, 1989), Reticuloendotheliosis virus (Bulow *et al*., 1986), Newcastle disease virus (De Boer *et al*., 1994), FAV (IBH/HPS) (Rosenberger, 1992) or avian reovirus (McNeilly *et al*., 1995) will cause profound immunosuppression and synergistic effects of both agents. Reovirus infection also causes stress and is reported to act synergistically with other infections (McKenzie and Bains, 1976). In India the existence of CAV infection confirmed (Venugopalan *et al*., 1994; Kataria *et al*., 1999) makes it an important emerging pathogen along with ARV, posing a severe threat to Indian Poultry Industry.

The immediate situation demands for a study on the interaction of CAV with other agents like ARV, which usually occurs under field conditions. In this context the present study has been conceived to study the experimental co-infection of CAV and ARV in broiler and application of suitable rapid diagnostic tests viz. PCR and FAT for confirmatory detection of the causative agents.

MATERIALS AND METHODS

Viruses: The ARV (VA-1) isolated from Chicken (*Gallus domesticus*) at 9th passage level in CEF and an Indian isolate of CAV at 7th passage level in MSB1 tissue culture used in the present study were obtained from Division of Avian Diseases, Indian Veterinary Research Institute (IVRI), Izatnagar.

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Embryonated chicken eggs and chickens: Unvaccinated day-old broiler chicks of both sex, and seven-day-old embryonated chicken eggs were obtained from Experimental Hatchery Unit of Central Avian Research Institute (CARI), Izatnagar. All biosecurity, sanitary and hygienic measures were followed to provide a pathogen free environment to chicks. The day-old broiler chicks used for this experimental study were monitored weekly for NDV, IBDV, MDV, reovirus, *Salmonella* Gallinarum, *E. coli* and avian mycoplasma infections following conventional standard procedures *viz*. agar gel precipitation test (AGPT), haemagglutination inhibition test (HI), plate agglutination and bacterial cultures. Chicks were also screened before experimental infection for the presence of maternal antibodies in their sera against avian reovirus by agar gel precipitation test (AGPT) carried out as described by Kawamura and Tsubahara (1966) and against CAV by indirect immunofluorescent test (IIFT) carried out as per the method described by McNulty *et al.* (1988).

Primers

For CAV: For PCR amplification, the primers amplifying the conserved region were used as described by Dhama *et al.*, 2002. These primers flanked a 454 base pair (bp) DNA sequence in size and were synthesized from Gibco BRL, USA (Table.1)

For ARV: Oligonucleotide primers from the S1 gene region of ARV used in the study were as reported by Koti (2000). The primer sequences were commercially synthesized (Gibco BRL, USA) and are as given (Table 2)

Propagation and titration of virus strain in CEF cultures: Chicken embryo fibroblast (CEF) cell cultures were prepared from 10-11 day-old chicken embryos as per the method of Merchant *et al*. (1960) with slight modifications. Once formed the monolayer was infected by inoculating 0.5 ml of 1:10 diluted virus and flasks were incubated at 37°C. Cytopathic effects (CPE) started after 48 h and after development of complete CPE, the flasks were transferred to deep freeze (-40°C). Rapid freeze-thawing was done at 37°C and -40°C for three times. For further passaging, the virus material was thawed, centrifuged and the supernatant after dilution in 1:10 in HBSS was inoculated on to fresh cultures. Titration of VA-1 isolates was carried out in CEF cell cultures in micro culture plates (96-well microtitre plates, Nunc, Denmark) and the 50% tissue culture infective dose (TCID50) was calculated using the method of Reed and Muench (1938).

Titration of CAV: A pretitrated ($10^{4.5}$ TCID50/0.1 ml) CAV isolate (at 7th passage level) maintained in MSB1 cell culture was used in this study.

Experimental infection: One hundred twenty (120) clinically healthy day-old broiler chicks hatched at CARI hatchery, Izatnagar, from the embryonated chicken eggs were used in the experimental studies. These were divided randomly into four groups A-D, having equal number of chicks. All the sixty day-old broiler chicks of Group A and C were inoculated intramuscularly (I/M) with 0.5 ml of $10^{4.5}$ TCID50/0.1ml of MSB1 cell culture passaged CIAV isolate (at 7th passage level). Chicks in-group D were inoculated I/M with 0.5 ml PBS. Ten days post-CAV infection, the chicks in groups B and C were infected with $10^{6.5}$ EID50/1ml of ARV, VA-1 isolate. The various groups were reared separately under strict isolated conditions and fed autoclaved feed and water, supplemented with vitamins and minerals, *ad libitum*. They were monitored for signs of disease and mortality, if any. Dead chicks were subjected to necropsy and tissues collected for polymerase chain reaction (PCR), fluorescent staining for CAV and ARV nucleic acid, antigen detection and histopathology at various intervals.

Detection of virus in tissues of experimentally infected chicks

Detection of CAV antigen by indirect fluorescent antibody technique (IFAT): Tissues *viz*. liver, spleen, bursa and thymus collected from experimentally infected chicks were employed for detection of CAV antigen at different intervals. These tissues were tested for detection of virus specific antigen by IFAT (Dhama *et al*., 2002). Sections from uninfected chicks included as controls were stained and examined similarly for IF studies. The sections in triplicate were fixed on clean microscopic slides by keeping in chilled acetone for 10 min. The sections were washed thrice in PBS and 1:50 diluted anti-CAV chicken hyper immune serum was added. The slides were incubated for 1 h at 37ºC in a moist chamber. Then, these were washed thrice with

Table 1: Description of CAV specific primers used in the study

Forward	CA-1	22 mers	5'AGCCGACCCCGAACCGCAAGAA3'
Reverse	CA-2	24 mers	5'AGACCCGTCCGCAATCAACTCACC3'

Table 2: Description of ARV specific primers used in the study

Forward	ARVSF1	23 mer	5' TATCTAGCGGCGGGTGGTGGTTT 3'
Reverse	ARVSR2	23 mer	5' GCGTGTGGGAGAATAGTAGAGCA 3'

1Avian reovirus S1 gene Forward Primer
2Avian reovirus S1 gene Reverse Primer

PBST and 1:30 diluted anti-chicken FITC conjugate was added and incubated for 1 h at 37°C in a dark moist chamber. After washing thrice, the slides were mounted with 50% glycerol saline and examined under the UV microscope (Nikon, Japan) for characteristic granular bright green fluorescence, in the absence of reactions in uninfected controls, was considered positive for the presence of CAV antigen.

Detection of CAV DNA by PCR

Extraction of total DNA from CAV-infected tissues: For extraction of viral DNA from infected tissues method of Todd *et al.* (1992) was followed with slight modifications. Approximately 100 mg of pooled tissue comprising thymus, bone marrow, liver, spleen and bursa were homogenized thoroughly into a suspension in 1 ml of sterile TNE buffer. To 540 µl suspension, 30 µl of 10% SDS (0.5% final concentration) and 30 µl of 20 mg/ml proteinase-K (1 mg/ml final concentration) were added and the reaction mixture was incubated overnight at 370C for lysing the cells. For DNA extraction from the lysate, equal volume (600 µl) of phenol-chloroform-isoamyl alcohol (25:24:1) was added, vortex mixed gently and centrifuged at 10,000 rpm for 10 min at 4°C. After centrifugation, the supernatant (aqueous phase) was collected carefully and phenol-chloroform extraction procedure repeated once again. To the aquous phase collected, equal volume of chloroform-isoamyl alcohol (24:1) was added, vortex mixed gently and centrifuged at 10,000 rpm for 10 min at 40C. 360 µl of the above supernatant was collected carefully (aqueous phase) to which 40 µl of 3 M sodium acetate (1/10th volume, 0.3M final concentration, pH 5.2) was added along with 1ml (2.5 volumes) of chilled absolute ethanol. The reaction mixture was mixed thoroughly and kept overnight at -20°C. The precipitated DNA was centrifuged at 12,000 rpm for 10 min at 4°C and the supernatant discarded gently. The pellet obtained was dissolved gently in 1 ml of 70% ethanol, centrifuged at 12,000 rpm for 15 min at 4°C for washing of DNA and the supernatant discarded. DNA pellet was air dried completely, resuspended in 20 µl of Tris EDTA (TE) buffer and stored at -20°C for further use.

Detection of CAV DNA in infected tissues by PCR: All DNA amplifications were performed in an automatic thermal cycler (PTC 200 MJ Research, USA) in 25 µl reaction mixture volumes using primer pair CA-1 and CA-2 in 0.2 ml thin walled PCR tubes as per Dhama *et al*. (2002). The amplified PCR products were confirmed for their expected size (454 bp) by agarose gel electrophoresis on 1.5% (w/v) agarose gel in 0.5X tris borate EDTA (TBE) buffer as per the method of Sambrook *et al*. (1989).

Detection of ARV antigen by fluorescent antibody technique (FAT): Distribution of ARV antigen in tissues of experimentally infected chicks was carried out by FAT as described by Menendez *et al.* (1975) with some modifications. Tissues *viz.* liver, spleen, bursa and thymus collected from experimentally infected chicks were employed for standardization of FAT for detection of ARV antigen in tissues of experimentally infected chicks.

Tissues were cryosectioned at -20°C and fixed in cold acetone at room temperature for 10 min. Slides were air dried and washed three times in PBS (pH - 7.4), each time for 10 min. FITC conjugated anti-ARV globulin (0.2 ml), available at the Division of Avian Diseases, was poured over the sections and incubated for 30 min at 37°C in a dark moist chamber in order to prevent drying during staining. After incubation, the sections were thoroughly washed in PBS and mounted in 50% buffered glycerin (pH - 7.4). The slides were then examined for the presence of apple green fluorescence under fluorescent microscope (Nikon Eclipse E200, Japan). Sections from uninfected broiler chicks included as controls were stained and examined similarly for FAT studies.

Detection of ARV RNA by RT-PCR

Extraction of total RNA from reovirus-infected tissues : Extraction of RNA from reovirus-infected tissues was done as per Koti (2000). Approximately 100 mg of spleen, liver, bursal and joint tissues were triturated and a 20% suspension was prepared in sterile PBS. From this, 400 ml suspension was taken and extracted with equal volume of chloroform: isoamyl alcohol (24:1) at 12,000 rpm for 5 min. The aqueous phase was collected and treated with 0.5% SDS and 1 mg/ml proteinase K (final concentration) at 37°C for 2 h. Total RNA from lysed cells was extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1) and once with chloroform:isoamyl alcohol (24:1). Viral RNA was precipitated by 0.3 M sodium acetate (final concentration), pH 5.2 and 2.5 volumes of absolute alcohol. The RNA pellet was obtained by centrifugation at 13,000 rpm for 20 min and washed with 70% ethanol. Dried RNA pellet was dissolved in 10 ml to 20 ml of nuclease-free water and stored at -20°C for further use.

Table 3: Experimental co-infection

Group	No. of chicks	Treatment	Route	Age
A	30	CIAV	I/M	Day old
B	30	ARV	Foot pad	10 days age
C	30	CIAV	I/M	Day old
		ARV	Foot pad	10 days post CIAV infection
D	30	PBS	I/M	Day old

Detection of ARV RNA in infected tissues: Viral RNA extracted from tissues *viz*. liver, spleen, bursa, thymus and joints, of experimentally infected chicks were tested by the PCR technique as described by Koti (2000) to detect the presence of ARV RNA by checking specific amplification of the S1 gene using oligonucleotide primers ARV SR and SF. The amplified PCR products were confirmed by agarose gel electrophoresis on 1.5% gel in 0.5X TBE buffer.

RESULTS

Propagation and titration of experimental viruses: The VA-1 isolate of avian reovirus (ARV) used in the present study was propagated and serially passaged 2-3 times in primary chicken embryo fibroblast (CEF) cell culture. The cytopathic effects (CPE) were characterized by cell degeneration, formation of multinucleated cells (syncytia), appearance of intracytoplasmic eosinophilic inclusions that later became basophilic with appearance of small plaques due to sloughing of the infected cells by 72 h PI. By 96 h PI, most of the cells fell off the surface, however, long cytoplasmic extensions were intact and large spaces appeared in cell sheet. The CAV isolate having a titre of 104.5 TCID50/0.1 ml of MSB1 cell culture (at 7th passage level) was obtained. The virus infectivity titres of the infected cell culture fluids, at 9th passage level was found to be 6.25 TCID50/ml.

Screening of experimental chicks: Pre-infection sera collected from day-old chicks before the experimental CAV infection were negative for the presence of antibodies to common viral pathogens *viz*. NDV, MDV, IBDV, CAV, ARV and also for *Salmonella* Gallinarum and *Mycoplasma gallisepticum.* Bacterial cultures for isolation of *E. coli* and *Salmonella* Gallinarum monitored during the course of the study were found negative. No evidence of other diseases was detected in chicks during the experimental studies.

Pathogenicity of ARV in chicks co-infected with CAV: Chicks experimentally infected with CAV showed acute course of disease with 100% morbidity. Infected chicks exhibited signs of anorexia and weakness at 10 days post CAV infection and developed signs of anaemia and loss in body weight gain. At 2-5 days DPI with ARV, clinical signs became more pronounced as evident by stunting, depression, paleness and ruffled feathers. Chicks infected with ARV alone (group B) and co-infected chicks (group C) exhibited growth retardation, swelling of foot pad and hock, abnormal gait and inability to stand (stand with legs wide apart), which was more intense in chicks of co-infected group (group-C) than the group B infected with ARV alone. Subsequently the chicks became dull and depressed and at 14 DPI showing extensive swelling of foot pad and hock (Fig. 1), which was comparatively mild in ARV alone group (B). Due to abnormal swelling, the normal morphology of the feet was deformed in the co-infected chicks. Gross and histopathological lesions in co-infected chicks sacrificed at various intervals were characterized by atrophied thymus, spleen and bursa as compared to control chicks. These lesions persisted even after 21 DPI in co-infected chicks. In chicks infected only with ARV (group- B), most of the changes were like those of chicks of co-infected group but less in severity. Swelling at the hock and foot pad region started declining at 14 DPI but feet appeared deformed due to uneven swelling.

Detection of viruses in tissues of experimentally infected chicks

Detection of CIAV antigen by FAT: Cryosections of liver and thymus tissues, collected from CAV infected chicks sacrificed at 7 and 10 DPI, were treated with reference serum and rabbit anti-chicken IgG FITC conjugate for the presence of specific apple green fluorescence. The optimized working dilution of reference sera at 1:100 and conjugate at 1:30 in PBS (pH 7.4) gave the specific fluorescence. IIFT stained liver and thymus sections showed CAV specific intra-nuclear fluorescence (Fig. 2). Tissues collected from uninfected control birds (group-D) were negative for specific immunofluorescence.

Detection of CIAV DNA by PCR: Simultaneously the samples of liver and thymus collected from chicks sacrificed at 7 and 10 DPI were screened by PCR for the presence of viral DNA. Total genomic DNA was extracted from these tissues. The 454 bp CAV specific product was amplified by PCR with DNA extracted from tissues of sacrificed chicks (Fig. 3). DNA extracted from tissues of uninfected controls was negative for the amplification of CAV DNA by PCR.

Detection of ARV antigen by FAT: Cryosections of various tissues *viz*. liver, thymus, bursa, spleen and joints collected from chicks experimentally infected with ARV at 7 DPI were stained with anti-ARV FITC conjugate for the presence of specific fluorescence. The optimized working dilution of conjugate (1:50) gave distinct viral specific intracytoplasmic apple green fluorescence in all the above-mentioned tissues (Fig. 4). Tissues collected from uninfected control birds (group D) were negative for specific fluorescence on FAT.

Detection of ARV RNA by RT-PCR: Samples of liver, thymus, bursa, spleen and joints collected from dead/-sacrificed chicks were screened by RT-PCR for the presence of viral RNA. Total genomic RNA was extracted from tissues following the SDS-proteinase K, phenol-chloroform extraction and ethanol precipitation method. The OD260/280 ratio of the extracted RNA ranged from 1.7 to 1.9 for different tissues indicating that it was free of protein contamination. The 422 bp ARV specific product was amplified by RT-PCR from RNA extracted from tissues of sacrificed chicks (Fig. 5, 6). RNA extracted from tissues of uninfected controls was negative for the amplification by RT-PCR.

DISCUSSION

Avian reovirus infection in young chicks cause major economic problems for commercial poultry producers throughout the world and has been associated with a variety of diseases including viral arthritis/infectious tenosynovitis, stunting and

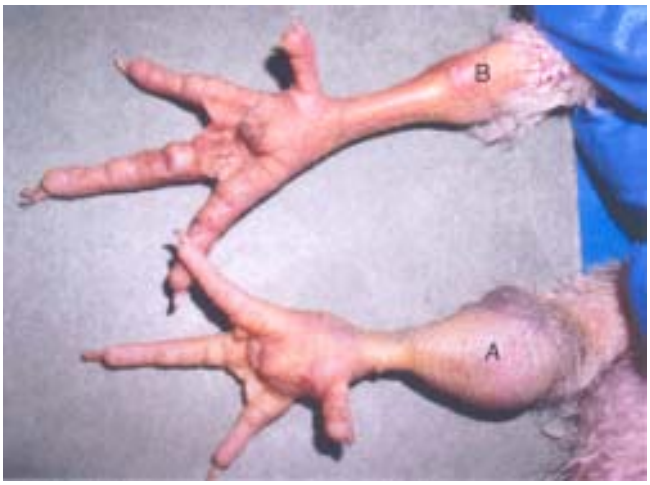

Fig. 1. A- Extensive swelling of hock joint and foot pad of co-infected chick at 14 DPI
B- Control

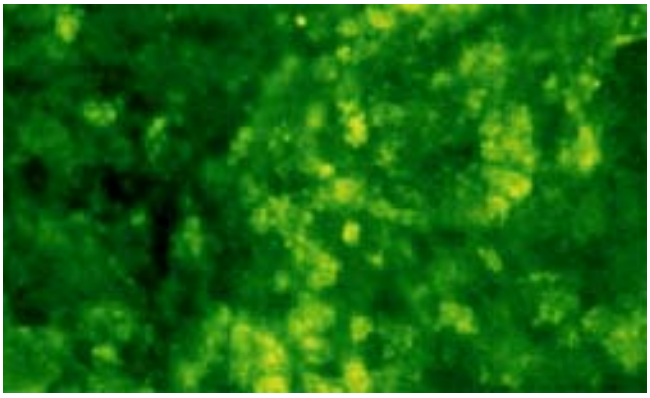

Fig. 2. Chick infected with CAV showing intranuclear immuno-fluorescence in hepatocytes (10 DPI)

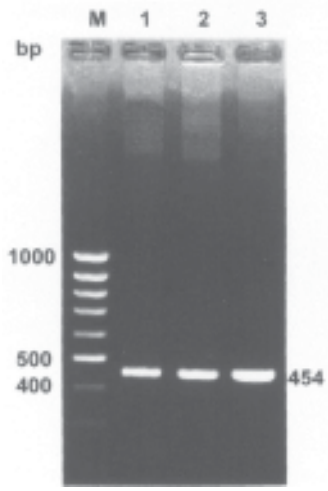

Fig. 3. PCR detection of 454 bp CAV antigen in tissue samples
Lane M: PCR marker; Lane 1: Liver; Lane 2: Spleen; Lane 3: Thymus

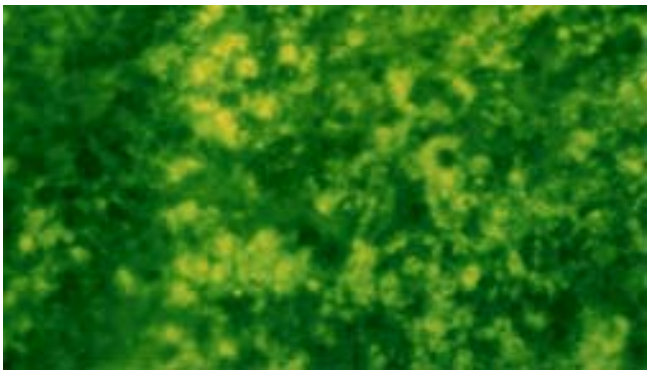

Fig. 4. Chick infected with ARV showing intracytoplasmic immunofluorescence in joints (7 DPI)

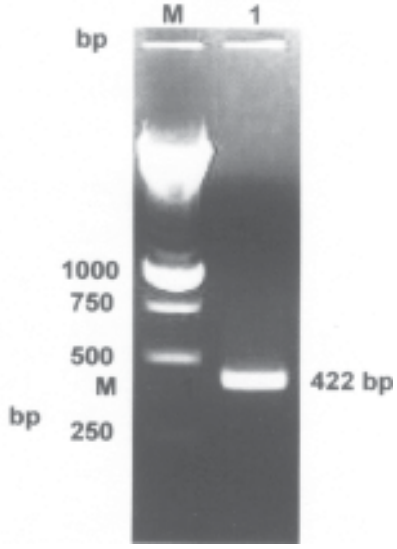

Fig. 5. Amplified PCR product of VA-1 isolate
Lane M: PCR marker; Lane 1: 422 bp PCR product of VA-1 isolate

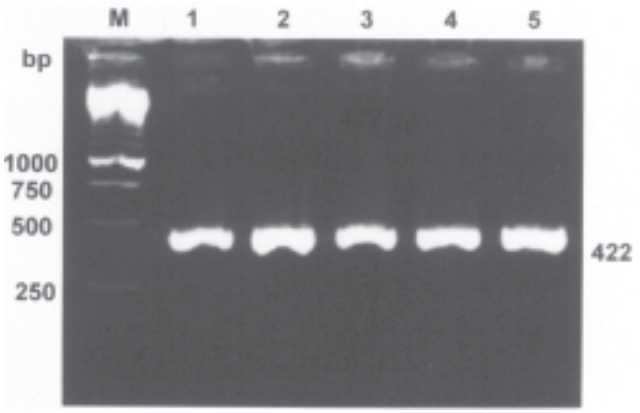

Fig. 6. PCR detection of 422 bp ARV antigen in tissue samples
Lane M: PCR marker; Lane 1: Liver; Lane 2: Spleen; Lane 3: Bursa; Lane 4: Thymus; Lane 5: Joint

malabsorption syndrome (Rosenberger and Olson, 1997). Fahey and Crawley (1954) isolated ARV for the first time from the respiratory tract of chickens with chronic respiratory disease. Reovirus infections are prevalent worldwide in chickens, turkeys and other avian species. Viral arthritis/tenosynovitis is found primarily in meat-type chickens but has been diagnosed in egg-type chicks also. Viral tenosynovitis is a worldwide disease associated with reovirus infection seen primarily in meat type chickens (Kibenge and Wilcox, 1983; Robertson and Wilcox, 1986). Chicks infected by these viruses have clinical signs and lesions of lameness, which include reluctance to walk, hock swelling and tendon rupture or fibrosis (Kibenge and Wilcox, 1983). Affected chicks usually have difficulty in reaching food and water and finally become emaciated (Kibenge and Wilcox, 1983) leading to death.

The present study was formulated with the objective to detect CAV and ARV specific antigen/nucleic acid by IIFT/ FAT and PCR/RT-PCR, respectively, in various tissues from experimentally co-infected chicks, to provide confirmatory diagnosis. The ARV isolate VA-1 was propagated in CEF cell culture and produced CPE characterized by syncytia and formation of micro-plaques as reported by Koti (2000). Also VA-1 isolate multiplied to a good titre when subjected to titration in tissue culture.

CAV causing infectious anemia in chicken is an emerging pathogen with worldwide distribution (Bulow and Schat, 1997), and chicken being the only natural host. This virus also effects young chicken mainly causing generalized lymphoid atrophy, bone marrow aplasia, anaemia, reduced haematocrit values, poor weight gain and increased mortality (Rosenberger and Cloud, 1989). Under field conditions, CAV infection goes unnoticed mostly, but co-infection with other immunosuppressive agents aggravates the condition. It has been reported (McNeilly *et al*., 1995) that CAV and ARV interacts in a synergistic manner following dual inoculation in susceptible day-old chicks. Similar reports were given by Engstrom *et al.* (1988) who reported the synergism between CAV and ARV from a field case of 'Blue Wing Disease' of chicken in Sweden.

In India the presence of CAV has been confirmed in the recent past (Kataria *et al*., 1999). Therefore a study was planned to experimentally co-infect the young broiler chicks with CAV and ARV, examine the characteristic symptoms and lesions and the conditions seen in co-infection (pathogenicity) and the application of rapid confirmatory diagnostics viz. PCR and FAT for confirmation of these pathogens. In the present study day-old broiler chicks were inoculated with an Indian isolate of CAV and subsequently challenged with ARV at 10-day age. The objective was to know how prior exposure to CAV affects the pathogenicity and immunogenecity of ARV infection in chicks and also to study whether prior immunosuppression would increase the severity and duration of ARV infection, as chickens have been reported most susceptible to ARV in the immediate post-hatching period and become increasingly resistant to infection with age (Rosenberger and Olson, 1997).

The present study has thrown light on the clinical picture of CAV and ARV infection in broilers, which is similar to their existence in field conditions, where they complicate the disease condition. It can be inferred that concomitant immunosuppression with agents like CAV can lead to extensive damage with subsequent ARV infection. The mechanism leading ultimately to exaggerated lesions of ARV infection in chicks appear to be immunosuppression and synergism acting at tandem.

Since both CAV and ARV affects primarily broilers, the emerging situation warranted a study to assess the changes produced in CAV infected chicks as a result of subsequent ARV infection and to unveil the interaction between CAV and ARV simulating field conditions as only few studies have been conducted to study the interaction of these two agents under natural conditions.

The detection of CAV antigen and DNA by IIFT and PCR from liver and thymus as early as 7 DPI of CAV infection as also reported by Dhama *et al.* (2002), explains the importance of these diagnostic tests in the early and accurate diagnosis of the disease. The detection of ARV antigen and RNA in various tissues *viz.* liver, spleen, bursa, joint in ARV infected/co-infected chicks at 7 days post ARV inoculation, by FAT and RT-PCR, respectively prove their suitability in early diagnosis of the condition and confirmatory detection of etiological agent. Koti (2000) used RT-PCR to detect ARV in infected liver, spleen, bursa and joint at 1, 2, 3 and 4 weeks post-infection. Kataria (1985) demonstrated the ARV antigen in cells of synovial membrane of experimentally infected chicks at 24 h of infection with VA-1. Though disease can be tentatively diagnosed by symptoms but co-infection conditions can be confused due to overlapping of symptoms/ lesions and also secondary infections.

In conclusion, the study highlighted the usefulness of PCR and IIFT for detection of CAV and ARV in the clinical samples in the wake of mixed infection with both these agents. Both PCR and IIFT were found to be rapid and reliable tests for confirmatory diagnosis of CAV and ARV. Overall results of the study revealed that PCR/RT-PCR and FAT are highly effective for detection of ARV isolates.

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