STUDIES ON AVIAN REOVIRUS INFECTION IN BROILER CHICKENS WITH SPECIAL REFERENCE TO STUNTING SYNDROME

By

ASHWINI GOEL

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in

VETERINARY PUBLIC HEALTH AND EPIDEMIOLOGY

COLLEGE OF VETERINARY SCIENCES
CH. CHARAN SINGH HARYANA AGRICULTURAL UNIVERSITY
HISAR

1993

DEDICATED

TO

MY PROFESSION

CERTIFICATE-I

This is to certify that the thesis entitled " Studies on avian reovirus infection in broiler chickens with special reference to stunting syndrome" submitted for the degree of Master of Veterinary Sciences, in the subject of Veterinary Public Health and Epidemiology to the CCS Haryana Agricultural University, Hisar, is a bonafide research work carried out by Dr.Ashwini Goel under my supervision and that no part of this thesis has been submitted for any other degree.

The assistance and help received during the course of investigation has been fully acknowledged.

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(Ashwini Goel)

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Introduction

1. INTRODUCTION

Poultry farming in India has developed from a mere backyard venture in the sixties to a flourishing and profitable industry in recent years. It is now one of the many livestock vocations to be chosen from as commercial poultry farming has shown a spectacular growth owing to its tremendous potential in terms of employment and financial gains. This is the reason that poultry production in India and elsewhere in the world is fast becoming an organized and specalized industry.

For the setting up of any industry, attention is paid to the factors which may hamper its productivity. The major factors that pose a great threat to the poultry industry include infectious and non-infectious diseases. Reovirus is one such infection which has been considered to cause significant economic losses abroad and is an emerging problem in India.

Avian reovirus, a prototype of family reoviridae, is a double stranded RNA virus. It is a non enveloped, icosahedral, double capsid virus with a diameter of about 65 nm and a genome of 10 segments. The virus was first isolated from the respiratory tract of chickens (Fahey and Crawley, 1954). Lateron, it was also found to be associated with a number of disease conditions like tenosynovitis, enteritis, respiratory disease and pericarditis. Kouwenhoven et al. (1978a) observed for the first time that reovirus was also associated with stunting syndrome in chickens. This syndrome, as the name suggests, restricts body growth and hampers weight gain in young broiler chickens. Besides retarded growth, elevated feed conversion ratio, poor feathering and leg weakness are the prominent factors responsible for economic losses.

Stunting syndrome has attained a world-wide distribution and has been reported from various developed countries like England, USA and Australia.

Several diagnostic tests including virus neutralization, complement fixation, immunofluorescence, enzyme-linked immunosorbent assay, agar gel precipitation, SDS-PAGE and western blotting have been used for the diagnosis of avian reovirus infection with promising results.

In India, although stunting syndrome has not been reported but stunted growth of broiler chicks has often been observed. However, sero-prevalence of reovirus infection in broiler chickens has been reported from India (Kataria et al., 1983, Verma and Verma, 1986). The demand for importing vaccine against avian reovirus infection has increased many folds in the recent years.

Keeping these points in view, the study on avian reovirus infection in chickens was undertaken with the following objectives :

1- To study the seroprevalence of avian reovirus infection in broiler chickens of Hisar and its vicinity.

2- To attempt isolation and identification of the reovirus from clinically sick birds exhibiting stunting syndrome.

3- To study the clinical signs and pathological lesions in chickens showing stunting syndrome.

Review of Literature

2. REVIEW OF LITERATURE

2.1 Epidemiology

2.1.1 Distribution

Reovirus infection as a cause of poor growth in chickens, was first reported in Netherland by Kouwenhoven et al. (1978a) and given the name "runting and leg weakness". Soon it acquired a world wide recognition and by 1980's, it was established as one of the important causes of stunting syndrome in chickens which was responsible for serious economic losses in England (Bracewell and Wyeth, 1981, Farmer and Taylor, 1985), Holland (Miltenburg et al., 1981), Italy (Ferrari et al., 1982), North America (Page et al., 1982), Australia (Pass et al., 1982, Barr et al., 1983, Reece et al., 1984), France (Schricke and Buttin, 1983), Malaysia (Chooi and Chulan, 1985), Sudan (Elmubarak et al., 1990) and Turkey (Carli et al., 1991).

2.1.2 Nomenclature

Depending on the clinical manifestations observed by various workers, this syndrome has been given different names in various parts of the world. Kouwenhoven et al. (1978a) called this syndrome as "runting and leg weakness" due to the lameness shown by affected chickens. Imperfectly developed primary wing feathers with a broken shaft gave the chickens a peculiar appearance and hence the name "helicopter chick" was given (Kouwenhoven et al., 1978a) . The inability of the birds to use dietary carotenoid in maize and therefore, failure to develop yellow colour of the skin has named this syndrome as "pale bird syndrome" (Van der Heide, 1982, Page et al., 1982). Disturbance in growth of chicks was observed by Kouwenhoven et al. (1983) and hence the name "runting and stunting" was given. Bracewell and Randall (1984) called it as "infectious stunting syndrome" which was considered most appropriate and is used in recent

literature.

Depending upon the post mortem lesions, this syndrome has also been named as "infectious proventriculitis" due to enlarged and distended proventriculus observed in stunted chicks (Kouwenhoven et al., 1978b). In some cases the disease showed osteoporosis of long bones and hence coined the name "femoral head necrosis" and "brittle bone disease" (Van der Heide et al., 1981). Page et al. (1982) observed undigested food material in the intestine of affected birds indicating improper absorption and digestion and called it as the "malabsorption syndrome".

2.1.3 Diagnostic techniques

2.1.3.1 Enzyme-linked immunosorbent assay (ELISA)

Slaght et al. (1978) used ELISA for detection of chicken anti reovirus antibodies against isolates S1133, UMI-203, Reo-25 and Winterfield. Schwarzmaier (1983) found ELISA to be very sensitive for antibody detection against reovirus infection in chickens. ELISA was recommended to be the most suitable for large scale diagnostic screening (Schwarzmaier et al., 1984). Indirect ELISA was applied on 78 broiler serum samples from birds with depressed growth in German Democratic Republic (GDR), of these, 65 were found positive for reovirus antibodies (Heider and Hlinak, 1985). Giambrone and Solano (1988) used ELISA and virus neutralization test for determining serological relatedness of 6 avian reovirus isolates (CO_8, S1133, 81-5, 2408, 1733 and UMI-203) and reported that except CO_8, all others belonged to the same serotype. Islam and Jones (1988) applied ELISA for measuring antibody titre of avian reovirus and recommended 0.01 M phosphate buffer saline (pH 7.5) as coating buffer which was considered the best and most suitable for getting optimum sensitivity as well as specificity of the test. Chen et al. (1991) found ELISA quite sensitive for detection of

antibodies against avian reovirus.

2.1.3.2 Agar gel precipitation test (AGPT)

Olson and Weiss (1972) demonstrated that avian reovirus share a common precipitating antigen using AGPT. Mustaffa-Babjee et al. (1973) showed the presence of precipitating antibodies in serum of chickens experimentally infected with reovirus using AGPT. With AGPT, the antigenicity of a new isolate of reovirus (S1133) was identified by Van der Heide et al. (1974). Sahu and Olson (1975) reported that serum from reovirus infected chicks showed a common precipitating line with agar gel precipitation test. Antisera raised against three strains of reovirus (S1133, Jones R1 and EK2286) were found to be cross reactive by AGPT while no precipitation was observed between antisera of Koster strain and any of these three reovirus strains (Wood et al., 1980). Robertson et al. (1984) screened 30 flocks of adult meat breeder chickens, 10 each from Victoria, New South Wales and Western Australia using AGPT and found 43.00, 39.00 and 44.00 per cent chickens positive for reovirus infection respectively. In an attempt to test sera for the presence of reovirus antibodies in 78 fowls in Northern Greece, Artopios et al. (1985) found 20.50 per cent positivity for reovirus using AGPT.

Kataria et al. (1983) used AGPT for antibody detection against avian reovirus in chicken, pigeon, pheasant, duck and quail in India. They observed reovirus antibodies in 41.00 per cent and 14.20 per cent of 1429 chicken sera and 972 quail sera respectively. None of the serum samples from pheasant, duck and pigeon was found positive. Verma and Verma (1983) screened 321 serum samples from chicken of different breeds, strain and age groups for reovirus infection in Manipur and found 149 (46.40%) to be positive for reovirus antibodies

using AGPT. Verma and Verma (1986) screened 537 samples using AGPT in Meghalaya and found 4.47 per cent seroprevalence of reovirus infection. Verma et al. (1987) carried out seroprevalence of avian reovirus infection in Arunachal Pradesh using AGPT and found 10.35 per cent positive of the 338 serum samples tested. Verma et al. (1989) recorded a seroprevalence of 13.60 per cent for reovirus infection in chickens in Tripura.

2.1.3.3 Electron microscopy

Mustaffa-Babjee et al. (1973) demonstrated the reovirus in cell culture by negative staining electron microscopy. The virion appeared as a spherical particle with two distinct layers of capsomeres with an average size of 75 nm. Page et al. (1982) attempted reovirus isolation from the intestinal tract of chicks with stunting syndrome and confirmed the isolate to be reovirus using electron microscopy. Based on electron microscopy, Hieronymus et al. (1983) identified reovirus from the intestine of broiler with malabsorption syndrome. Farmer and Taylor (1985) isolated a new viral antigen - FEW virus from the gut homogenate of a four day old chick with signs of infectious stunting syndrome using electron microscope which revealed a spherical particle 45-55 nm in diameter with a RNA genome. Kataria et al. (1985) used electron microscopy for characterization of a viral agent isolated from chicken suffering from arthritis and reported it to be a reovirus based upon its size (75 nm) and round to hexagonal shape. While examining the intestinal contents of 102 flocks with enteritis, Decaesstecker et al. (1988) found 54.00 per cent of them to be positive for reovirus by electron microscopy.

2.1.3.4 Comparison of diagnostic tests

Ide (1982) compared indirect fluorescent antibody test (IFAT) with AGPT and plaque reduction test and found AGPT the least sensitive and IFAT

the most sensitive for reovirus detection. Schwarzmaier (1983) found that ELISA was more sensitive than AGPT and as sensitive as immunofluorescence test. ELISA was later reported to be even more sensitive than immuno-fluorescence test (Schwarzmaier et al., 1984). Comparing virus neutralization (VN) and haemagglutination inhibition (HI) with ELISA, Thayer et al.(1987) found a good correlation of mean ELISA titre or enzyme immuno assay (EIA) system sample to positive (S/P) ratio with specific HI and VN titre. Kataria et al. (1989) compared the counter-immuno-electrophoresis (CIEP) test with AGPT to detect antibodies against reovirus infection in chickens and found the former to be more sensitive. Comparing AGPT and ELISA, Chen et al. (1991) found the latter test to be better as it gave 100 per cent positive rate of detection as compared to AGPT which gave only 65.00 per cent positive rate of detection. Hlinak et al. (1992) found ELISA to be more sensitive than serum neutralization test for detection of antibodies against avian reovirus.

2.2 Reovirus

2.2.1 Properties

Avian reovirus is a non-enveloped virus with a double capsid structure of icosahedral symmetry. According to Kawamura et al. (1965), the outer capsid shell of the virus particle consists of 92 capsomeres arranged in five fold symmetry. Krauss and Ueberschär (1966) calculated the capsomere number to be 122 or 132. Deshmukh et al. (1971) reported that three reovirus isolates from birds with cloacal pasting had a size of 56, 61, 71 nm respectively. The size was estimated ranging from 70 to 82 nm by Kawamura et al. (1965) and Van der Heide (1977).

The reovirus was reported to be a RNA virus with 18.70 per cent RNA and 81.30 per cent protein (Sekiguchi et al., 1968). The RNA of avian reovirus had both single stranded (ss) and double stranded (ds) RNA (Koide et al., 1968) with ss RNA representing approximately 30.00 per cent of the total RNA (Koide, 1970). The genome of avian reovirus was found to be a double stranded RNA (Glass et al., 1973) and consisted of 10 segments (Gouvea and Schnitzer, 1982).

Avian reovirus was reported to be resistant to ether and partially sensitive to chloroform (Glass et al., 1973, Carboni et al., 1975). Glass et al. (1973) found avian reovirus to be stable at a wide range of pH (3.0 to 9.0) for 4 h. But Carboni et al. (1975) reported partial sensitivity at pH 3.0 and 12.0 for 3 h causing reduction in infectivity of the virus. The reovirus was found stable at 50°C for 2 h (Kawamura et al., 1965, Rossi et al., 1969, Levisohn and Weisman, 1980), at 56°C for 1 h (Van der Heide and Kalbac, 1975) and at 56°C for 6 h (Hieronymus et al., 1983). However, earlier Dutta and Pomeroy (1967 b) reported partial inactivation of the virus at 56°C within 10 to 30 min. Spandidos and Graham (1976) found that the reovirus became completely inactive at 55°C in less than an hour. However, at lower temperature , avian reovirus could survive for three months at 4°C and for two months at room temperature (Dutta and Pomeroy, 1967 b).

2.2.2 Isolation

Reovirus has been isolated from a number of disease conditions of chickens which includes enteric disease (Dutta and Pomeroy, 1967 a, b) , respiratory disease (McFerran et al., 1971), heart and liver disease (Spradbrow and Bains, 1974), infectious bursal disease (Nick et al., 1975), tenosynovitis (Van der Heide, 1977) and the stunting syndrome (Kouwenhoven et al., 1978 a).

Olsen (1977) reported isolation of an avian reovirus from two week old chick with diarrhoea and stunting. Inoculation of crude intestinal homogenate of affected birds produced cloacal pasting as well as stunting in experimental day old chicks. Vertommen et al. (1980) isolated reovirus from the intestine of stunted birds and observed temporary growth retardation and diarrhoea in day old experimental chicks due to the virus isolate. Van der Heide et al. (1981) isolated an avian reovirus from intestine of birds with a history of stunting and diarrhoea. This isolate produced femoral head necrosis, osteoporosis and tenosynovitis in specific pathogen free chicks. Marchi and Zanella (1982) isolated three reovirus strains from stunted birds none of which could reproduce the disease. Page et al. (1982) isolated reovirus from the intestinal tract and the visceral organs of chicks with malabsorption syndroı and reported that these isolates could produce cytopathic effect (CPE) on chicken embryo kidney cells. Pass et al. (1982) isolated reovirus from thymus, proventriculus, intestinal contents, spleen and pancreas of 13 out of 33 samples and demonstrated the virus by electron microscopy. Kouwenhoven et al.(1983) isolated reovirus from intestinal homogenate of stunted birds but were unable to reproduce the disease experimentally. However, pre-inoculation of day old chicks with reovirus followed by intestinal homogenate at third day in chickens produced the disease. Robertson et al. (1984) isolated reovirus from the rectum, pancreas, spleen, thymus and proventriculus of chicken with stunting syndrome and tenosynovitis. Rectal contents of clinically normal three week old broilers were examined and found to be positive for the reovirus (Robertson et al., 1984). Gerganov et al. (1987) isolated reovirus from proventriculus and vaginal sheaths of four to five day old broiler chicks with helicopter disease from 20 out of 26 flocks of poultry. Saiffudin et al. (1989) isolated a reovirus from a broiler chicken flock with high mortality, which produced large syncytia in primary

chicken kidney cell culture. Carli et al. (1991) isolated reovirus from cloacal swab, liver, spleen and bursa of chicken showing stunting syndrome.

2.2.3 Cultivation

Avian reovirus has been cultivated successfully in embryonated chicken eggs (ECE) (Glass et al., 1973, Guneratne et al., 1982). Several workers have attempted isolation and propagation of virus from clinically sick birds by using this route. Three routes have been adopted for the inoculation of ECE.

2.2.3.1 Yolk sac route

Dutta and Pomeroy (1967 a) used yolk sac route for multiplication of a reovirus isolate and observed that the virus caused death of embryo via this route. Yolk sac route has been used both for propagation of reovirus (Olson and Weiss, 1972) as well as for its isolation (Levisohn and Weisman, 1980). Glass et al. (1973) found yolk sac route the most sensitive amongst allantoic cavity route, chick embryo kidney route and yolk sac route for reovirus isolation. Olson (1975) reported the characteristic lesions of reovirus infection through yolk sac route inoculation which were purplish discolouration of embryo and its mortality within three to five days. According to Guneratne et al. (1982), death usually occurred within three to five days post infection via yolk sac route; those alive, were dwarf with necrotic yellow-greenish areas in the liver.

2.2.3.2 Allantoic cavity route

Fahey and Crawley (1954), Glass et al. (1973) and Bains et al. (1974) used allantoic cavity route for isolation of reovirus from chickens. However, Deshmukh and Pomeroy (1969) found allantoic cavity route unsuitable for isolation of reovirus. Guneratne et al. (1982) also used allantoic cavity route

of inoculation for isolation of reovirus but did not get significant results.

2.2.3.3 Chorioallantoic membrane (CAM) route

Chorioallantoic membrane is the most popular route amongst all embryonated chicken egg (ECE) inoculation routes and has been used both for isolation and propagation of reovirus by a number of workers (Dutta and Pomeroy, 1967 a , Spradbrow and Bains, 1974, Guneratne et al., 1982). Dutta and Pomeroy (1967a) reported that reovirus isolate (No. 1394) was easily adopted when inoculated via CAM route and the lesions could be reproduced very easily. CAM inoculation was considered the route of choice for reovirus isolation by Deshmukh and Pomery (1969). Mustaffa-Babjee and Spradbrow (1971) also used this route successfully for isolation of a strain of reovirus (RAM-1) from Australia. Spradbrow and Bains (1974) found that the presence of reovirus could be demonstrated more easily via the CAM route than the cell culture. Guneratne et al. (1982) used the CAM route for cultivation of reovirus and compared it with the yolk sac route. They recommended the latter for isolation and cultivation of reovirus as compared to other routes.

On inoculation of reovirus, the CAM became thick, oedematous with stunted growth of embryo, greenish discolouration of liver, spleenomegaly and heart lesions followed by death of the embryo in about three to five days post inoculation (Mustaffa-Babjee and Spradbrow, 1971, Bains et al., 1974, Olson, 1975).

2.3 Pathology

2.3.1 Necropsy

Grossly, the birds stunted due to reovirus infection are smaller in size with marked reduction in body weight as compared to the apparently healthy birds of corresponding age group (Kouwenhoven et al., 1978 a, Bracewell and Wyeth, 1981).

According to Bains et al. (1974), broilers affected with reovirus showed atrophied spleen, hydropericardium, distended gall bladder and little contents in the intestines. Bracewell and Wyeth (1981) found distended gall bladder, poorly digested food material in the intestines and atrophy of bursa in stunted birds. According to Randall et al. (1981), gross changes in pancreas varied from atrophy of one or more lobes to its complete replacement with a thin strip of fibrous tissue. Page et al. (1982) reported the involvement of proventriculus and found their size to be larger than those of healthy birds. Page et al. (1982) reported reduced gizzard size, catarrhal enteritis and hydropericardium. Pass et al. (1982) observed atrophy of the thymus and pancreas and distended caeca filled with gas and froath. Bracewell and Randall (1984) reported that the thymus of affected birds was reduced to a mere strip of tissue. The pancreas were pale, firm in texture, smaller in size with rounded borders particularly near the closed end of duodenal loop. Montgomery et al. (1985) noted a decrease in weight of bursa but the weight of spleen was found to be increased. The increase in spleen weight was also reported by Tang et al. (1987) who observed the size of spleen in the affected birds to be five to six times more than the normal size of spleen. Besides spleenomegaly, hepatic necrosis, hepatomegaly, pericarditis and myocarditis were reported in birds (Tang et al., 1987).

2.3.2 Histopathology

Mandelli et al. (1978) reported hepatocellular degeneration, cytoplasmic necrosis and disintegration of hepatocytes in birds infected with reovirus. According to Randall et al. (1981), the lesions in pancreas were confined to exocrine tissue and showed degeneration, atrophy and fibroplasia. Page et al. (1982) found that the pancreas in affected birds had diffused vacuolar degenerat

with extensive loss of acinar cells and fibrosis. In Australia, Pass et al. (1982) observed vacuolation of cytoplasm and necrosis of acinar cells of pancreas. The normal acini merged with acini containing vacuolated acinocytes as well as acinocytes with no zymogen granules. The intralobular and interlobular ducts were often dialated and irregular in outline. They also observed loss of corticomedullary differentiation in the thymic lobes of affected birds. Cortex of larger lobes in such birds had fewer lymphocytes than those in unaffected birds. Bracewell and Randall (1984) revealed blunted villi in the small instestine and observed that the crypts of Lieberkuhn were dialated with the lumen containing debris. Martland and Farmer (1986) observed that an acute inflammatory reaction in the wall of one or more duct of the pancreas occluded the lumen of duct causing obstruction to pancreatic drainage. According to Tang et al. (1987), hepatic stromal cell hyperplasia, intrahepatic inflammatory cell infiltration and bile stasis caused hepatomegaly and greenish discolouration of liver in affected birds. The bursa were found atrophied having small lymphoid follicles with thin cortex separated by increased amount of fibrous connective tissue. It was also reported that the lymphostromal cell hyperplasia caused spleenomegaly in affected birds (Tang et al., 1987). Martland (1989) reported cholangio-hepatitis with obstructive hepatitis and occlusion of bile ducts. He also observed necrosis and cystic dengeneration of the crypts of Lieberkuhn in the intestines.

Materials and methods

3. MATERIALS AND METHODS

3.1 Epidemiology

3.1.1 Survey

Eight broiler poultry farms around Hisar city located two each on Barwala road, Sirsa road, Dabra road and Gangwa road were chosen for the present study. For convenience sake, the farms on Barwala road were designated as 'A', those on Sirsa road as 'B' while those located on Dabra road and Gangwa road were designated as 'C' and 'D' respectively. Poultry farm situated near the city (Hisar) was designated as number-1 and away from the city as number-2 in each area (Fig. 1). These farms were visited personally and the history regarding total number of chickens on the farms, their source of purchase and vaccination schedule was collected from the farm owners. The age of chicks was recorded from the farm records and the average weight of the apparently healthy chickens was taken. The farms were surveyed for the presence of stunted chickens showing clinical manifestations like poor feathering, pasted vent and weakness. The average weight of stunted chickens was also recorded. Samples of feed being fed to the chicks under study were procured to estimate the aflatoxin content in the Toxicology Laboratory Department of Veterinary Public Health and Epidemiology, Ch. Charan Singh Haryana Agricultural University, Hisar.

A few stunted chickens characterized by poor weight gain and ruffled feathers and a few apparently healthy (control) chickens were procured from these farms and brought to the laboratory for further studies (Table-1). These chickens were sacrificed and blood was collected aseptically in sterile vials for seroprevalence studies. The gross pathological changes in the visceral organs were recorded and tissues from different organs for histopathology and virus isolation were collected in 10 per cent buffered formalin and sterile

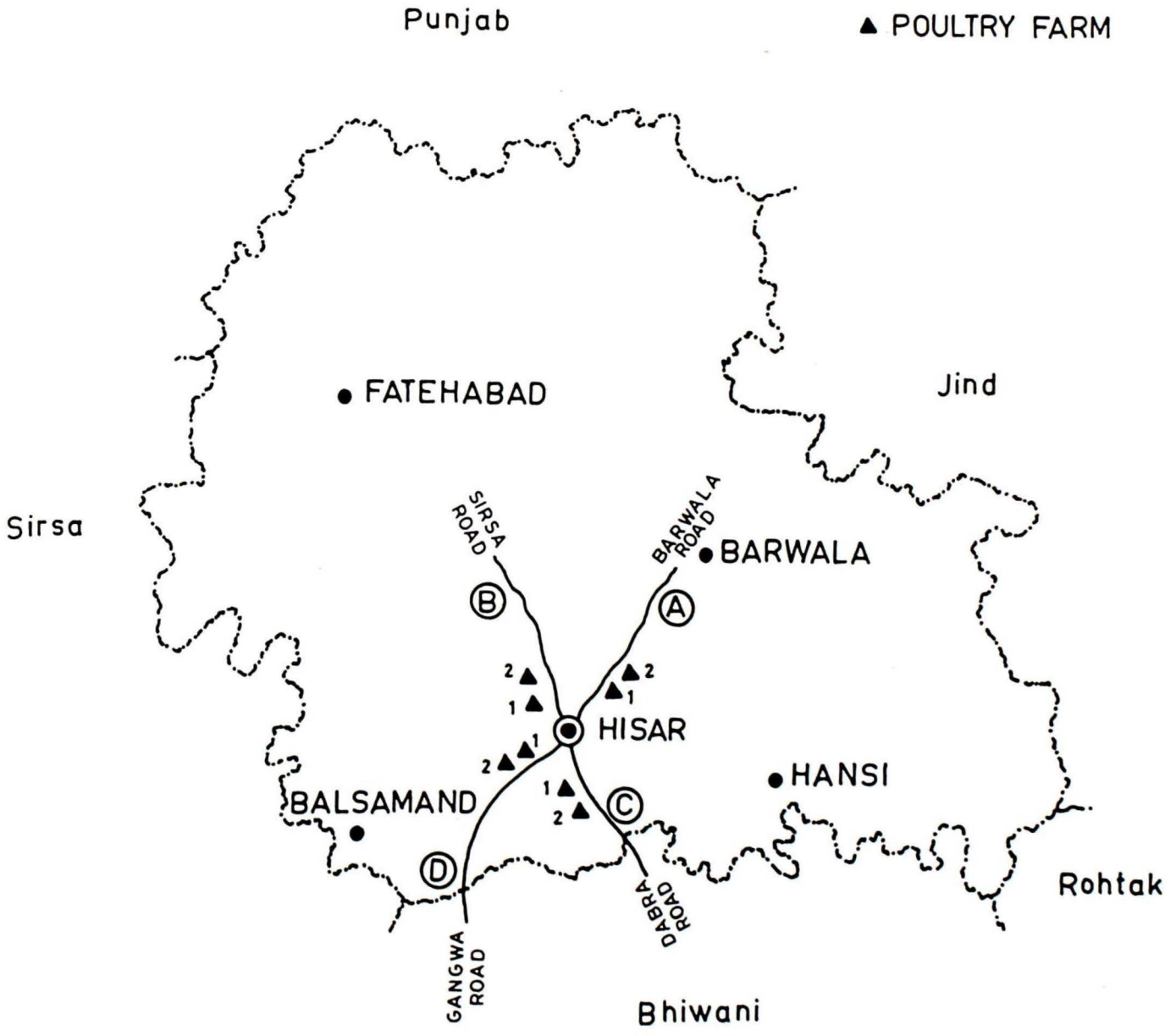

FIG. 1. AREA UNDER STUDY

Table 1 : Number of chickens procured for virus isolation

Area	Stunted			Apparently healthy		
	Farm-1	Farm-2	Total	Farm-1	Farm-2	Total
A	3	3	6	2	2	4
B	22	18	40	4	4	8
C	3	2	5	2	2	4
D	3	2	5	2	2	4
Total	31	25	56	10	10	20

petridishes respectively.

3.1.2 Seroprevalence

3.1.2.1 Collection of serum samples

The blood samples of broilers at the farms under study were collected at the time of slaughter in 15 ml screw capped sterile vials. These samples were brought to the laboratory where serum was separated. Serum was also separated from blood collected from stunted and apparently healthy (control) chickens procured from various farms at the time of survey. All the serum samples were stored in sterilized 5 ml vials at -20°C till further use.

3.1.2.2 Avian reovirus antigen and hyperimmune serum

The avian reovirus antigen and hyperimmune serum were procured from the Division of Avian Diseases, Indian Veterinary Research Institute (IVRI), Izatnagar. The hyperimmune serum was also procured from the Poultry Health Institute, Doorn (Holland).

3.1.2.3 Serological tests

Two tests viz., enzyme-linked immunosorbent assay and counter-immuno-electrophoresis were employed to find out the presence of antibodies against avian reovirus in the serum samples.

3.1.2.3.1 Enzyme-linked immunosorbent assay (ELISA)

The procedure as described by Ellens and DeLeeuw (1977) and Slaght et al. (1978) was adopted with slight modifications. The test was standardized by the checker board method. (Kurstak, 1985). Serial dilutions of the known antigen (1:10, 1:20, 1:40, 1:80, 1:160 and 1:320) were made in 0.01 M PBS (pH 7.5). Positive and negative serum were serially diluted (1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200, 1:6400 and 1:12800) in washing solution. The highest dilution of antigen giving ELISA value of $\geqslant 2$ of the

known positive and negative serum was chosen as the optimum dilution of the antigen and the test sera. It was found that a dilution of 1:40 for antigen and 1:200 for the serum was most suitable.

Following the procedure described by Ellen and DeLeeuw (1977) and Slaght et al. (1978), the polysterene immuno-plates were coated with 100 µl/well of the reovirus antigen diluted in the ratio of 1:40 in 0.01 M phosphate buffer saline (PBS, pH 7.5). The plates were kept at 4°C overnight for coating of the antigen. The plates were washed thrice with the washing solution containing 0.05 per cent tween-20 in PBS (pH 7.2) and tapping of the plate was done against a soft towel after each wash.

To block the non-specific reaction, 100 µl of five per cent milk powder (Amul Spray) in washing solution was poured in each well and incubated at 37°C for 2 h. Three washings were given again as described above after incubation. The serum samples collected at the time of slaughter were diluted 1:200 in washing solution containing two per cent bovine serum albumin (BSA) and 100 µl of a sample was pipetted in a well. The last four wells were pipetted with a similar amount of known negative serum in two wells and known positive serum in two wells. After 1 h of incubation at 37°C, the plates were washed and tapped three times as described earlier. 100 µl of antichicken IgG peroxidase conjugate (Sigma Chemical Co., USA) diluted as 1:1500 in washing solution was pipetted per well and incubated at 37°C for 1 h. After three washings and tapping of the plate, 100 µl of enzyme substrate, orthophenylenediamine dihydrochloride (OPD) containing 0.01 percent hydrogen peroxide was pipetted in each well. The plates were incubated for 15 min at room temperature. The results were read with an ELISA reader at 492 nm. Any sample giving a reading more than double from the reading of known negative serum was considered to be

positive (Kurstak, 1985).

3.1.2.3.2 Counter-immuno-electrophoresis (CIEP) test

The procedure described by Grauballe et al. (1981) and Kataria et al. (1989) was followed with slight modifications. One per cent agarose (Centron) was prepared in 0.2 M veronal buffer (pH 8.6). About 15 ml molten agarose was poured on a clean slide (75 mm x 75 mm) and allowed to solidify at room temperature for 5 min followed by solidification in refrigerator at 4°C for 1 h. Wells of 5 mm diameter were cut on each slide in two rows at a distance of 5 mm from each other. The bottom of these wells were sealed with a drop of 0.1 per cent agarose. The wells on one side of the slide were charged with reovirus antigen obtained from IVRI. The wells on the other side were charged with the test serum samples alongwith one positive and one negative control. The slides were placed on the platform of electrophoretic tank containing veronal buffer (pH 8.6) in such a way that the wells containing antigen were towards cathode and those of serum samples were towards anode. The slides were connected to the electrode vessel containing veronal buffer with the help of filter paper wicks. The wicks were soaked in the buffer to ensure a satisfactory bridge with the agarose layer. Electrophoresis was carried out at 150 volts for 1 h at room temperature. After 1 h, power supply was put off and the slides were observed for the development of a precipitating band between the wells holding the slide against a dark background. For better interpretation, the slides were stained with amido black. The slides were washed in 0.85 per cent sodium chloride, transferred to distilled water and kept in it for 2 h. The slides were dried and stained with amido black for 5 n At 45:10:45 solution of methanol, glacial acetic acid and distilled water was used to destain the slide. They were dried and was examined in a view box.

Table 1 : Number of chickens procured for virus isolation

Area	Stunted			Apparently healthy		
	Farm-1	Farm-2	Total	Farm-1	Farm-2	Total
A	3	3	6	2	2	4
B	22	18	40	4	4	8
C	3	2	5	2	2	4
D	3	2	5	2	2	4
Total	31	25	56	10	10	20

petridishes respectively.

3.1.2 Seroprevalence

3.1.2.1 Collection of serum samples

The blood samples of broilers at the farms under study were collected at the time of slaughter in 15 ml screw capped sterile vials. These samples were brought to the laboratory where serum was separated. Serum was also separated from blood collected from stunted and apparently healthy (control) chickens procured from various farms at the time of survey. All the serum samples were stored in sterilized 5 ml vials at -20°C till further use.

3.1.2.2 Avian reovirus antigen and hyperimmune serum

The avian reovirus antigen and hyperimmune serum were procured from the Division of Avian Diseases, Indian Veterinary Research Institute (IVRI), Izatnagar. The hyperimmune serum was also procured from the Poultry Health Institute, Doorn (Holland).

3.1.2.3 Serological tests

Two tests viz., enzyme-linked immunosorbent assay and counter-immuno electrophoresis were employed to find out the presence of antibodies against avian reovirus in the serum samples.

3.1.2.3.1 Enzyme-linked immunosorbent assay (ELISA)

The procedure as described by Ellens and DeLeeuw (1977) and Slaght et al. (1978) was adopted with slight modifications. The test was standardized by the checker board method. (Kurstak, 1985). Serial dilutions of the known antigen (1:10, 1:20, 1:40, 1:80, 1:160 and 1:320) were made in 0.01 M PBS (pH 7.5). Positive and negative serum were serially dilute (1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200, 1:6400 and 1:12800) in washing solution. The highest dilution of antigen giving ELISA value of ≥ 2 of the

known positive and negative serum was chosen as the optimum dilution of the antigen and the test sera. It was found that a dilution of 1:40 for antigen and 1:200 for the serum was most suitable.

Following the procedure described by Ellen and DeLeeuw (1977) and Slaght et al. (1978), the polysterene immuno-plates were coated with 100 µl/well of the reovirus antigen diluted in the ratio of 1:40 in 0.01 M phosphate buffer saline (PBS, pH 7.5). The plates were kept at 4°C overnight for coating of the antigen. The plates were washed thrice with the washing solution containing 0.05 per cent tween-20 in PBS (pH 7.2) and tapping of the plate was done against a soft towel after each wash.

To block the non-specific reaction, 100 µl of five per cent milk powder (Amul Spray) in washing solution was poured in each well and incubated at 37°C for 2 h. Three washings were given again as described above after incubation. The serum samples collected at the time of slaughter were diluted 1:200 in washing solution containing two per cent bovine serum albumin (BSA) and 100 µl of a sample was pipetted in a well. The last four wells were pipetted with a similar amount of known negative serum in two wells and known positive serum in two wells. After 1 h of incubation at 37°C, the plates were washed and tapped three times as described earlier. 100 µl of antichicken IgG peroxidase conjugate (Sigma Chemical Co., USA) diluted as 1:1500 in washing solution was pipetted per well and incubated at 37°C for 1 h. After three washings and tapping of the plate, 100 µl of enzyme substrate, orthophenylenediamine dihydrochloride (OPD) containing 0.01 percent hydrogen peroxide was pipetted in each well. The plates were incubated for 15 min at room temperature. The results were read with an ELISA reader at 492 nm. Any sample giving a reading more than double from the reading of known negative serum was considered to be

positive (Kurstak, 1985).

3.1.2.3.2 Counter-immuno-electrophoresis (CIEP) test

The procedure described by Grauballe et al. (1981) and Kataria et al. (1989) was followed with slight modifications. One per cent agarose (Centron) was prepared in 0.2 M veronal buffer (pH 8.6). About 15 ml molten agarose was poured on a clean slide (75 mm x 75 mm) and allowed to solidify at room temperature for 5 min followed by solidification in refrigerator at 4°C for 1 h. Wells of 5 mm diameter were cut on each slide in two rows at a distance of 5 mm from each other. The bottom of these wells were sealed with a drop of 0.1 per cent agarose. The wells on one side of the slide were charged with reovirus antigen obtained from IVRI. The wells on the other side were charged with the test serum samples alongwith one positive and one negative control. The slides were placed on the platform of electrophoretic tank containing veronal buffer (pH 8.6) in such a way that the wells containing antigen were towards cathode and those of serum samples were towards anode. The slides were connected to the electrode vessel containing veronal buffer with the help of filter paper wicks. The wicks were soaked in the buffer to ensure a satisfactory bridge with the agarose layer. Electrophoresis was carried out at 150 volts for 1 h at room temperature. After 1 h, power supply was put off and the slides were observed for the development of a precipitating band between the wells holding the slide against a dark background. For better interpretation, the slides were stained with amido black. The slides were washed in 0.85 per cent sodium chloride, transferred to distilled water and kept in it for 2 h. The slides were dried and stained with amido black for 5 min. At 45:10:45 solution of methanol, glacial acetic acid and distilled water was used to destain the slide. They were dried and was examined in a view box.

3.2 Isolation

3.2.1 Preparation of inoculum

Isolation of reovirus was attempted by the method of Dutta and Pomeroy (1967a) from the small intestines and pancreas of 16 stunted chickens which were found seropositive for reovirus infection and 10 apparently healthy procured chickens which were found seronegative. The 16 stunted chickens included one each from farm-1 and farm-2 of area A; one from farm-1 of area C; eight from farm-1 of area B and five from farm-2 of area B. None of the samples from farm-2 of area C and both the farms of area D were attempted for virus isolation being negative for reovirus antibodies with ELISA. The chickens were sacrificed and the small intestines and pancreas were collected in sterile petridishes. About 10 cm of each of the three parts of the small intestines (i.e. duodenum, jejunum and ileum) of each chicken were cut open and the gross intestinal contents were removed. Their mucosal surfaces were scrapped and collected in a graduated centrifuge tube under sterile conditions. A 10 per cent suspension of the scrapped cells was prepared with the Hank's balanced salt solution (HBSS).

The pancreas of each chicken was collected separately and triturated in sterile paste and mortar and 10 per cent suspension was prepared in HBSS. Both the suspensions were separately frozen and thawed twice at -70°C and 37°C respectively to dissociate the virus from the cells. This homogenate was then centrifuged at 6000 rpm for 30 min at 4°C in refrigerated centrifuge to remove the coarse material. The supernatant was collected and filtered through a 0.22 um millipore filter to obtain a bacteria free filtrate. The filtrate was then treated with 100 IU of pencillin and 2.5 mg streptomycin per ml. In addition, mycostatin was also added at the rate of 100 IU/ml. The filtrate was

incubated at room temperature for 1 h and tested for sterility on tryptose soya agar plates before inoculating in the embryonated eggs. Any filtrate sample showing bacterial growth was refiltered through 0.22 um millipore filter and tested for sterility again till it showed no further growth. The filtrate was stored at freezing temperature till further use.

3.2.2 Inoculation of embryonated chickens eggs (ECE)

Ten day old incubated embryonated chicken eggs obtained from the Department of Animal Breeding of the University and Government Hatchery, Hisar were used for inoculation of the filtrate. The eggs were cleaned and disinfected with ethanol. About 0.2 ml inoculum was deposited on dropped chorioallantoic membrane (CAM) with a tuberculin syringe using a 26 gauge half inch needle (Dutta and Pomeroy, 1967a). The holes on the egg shell were sealed with melted paraffin wax. Three eggs were inoculated with each sample. The inoculum prepared from apparently healthy chickens was also inoculated on CAM of eggs in a similar way. The control eggs were inoculated with 0.2 ml of sterile HBSS instead of the filtrate. The eggs were rotated once and incubated at 37°C for 72 h with the artificial air sac facing upward. After 24 h of incubation, the eggs were candled and the dead ones discarded considering their death as non-specific.

3.2.3 Harvesting of eggs

After 72 h of incubation, the eggs were swabbed with 70 per cent ethanol and the egg shell along with shell membrane was removed under sterile conditions with a pair of sterilized scissors and forceps. The CAM was separated, washed thrice in cold sterile HBSS and examined for the presence of cytopathic effects like thickening, congestion, pock lesions and liver lesions against a dark background. Three blind passages were given before declaring

any sample as negative.

3.2.4 Titration of the virus

The method described by Reed and Muench(1938) was adopted. Ten day old embryonated chicken eggs were used for titration of the virus. The intestine and pancreatic suspension of the chickens showing CPE on CAM were pooled after 6th passage. Serial 10 fold dilutions of infected CAM suspension were prepared in HBSS and 0.2 ml of each dilution was inoculated by the method described earlier. Four eggs per dilution were inoculated and the same number of eggs were inoculated with sterile HBSS which acted as control. After 72 h, the CAM were harvested, washed in HBSS and examined for the presence of thickening and pock lesions.

3.2.5 Identification of virus

3.2.5.1 Electron microscopy

After the 8th passage, CAM showing CPE were taken and small blocks of 2 mm x 2 mm size were cut and fixed in 2.5 per cent glutaraldehyde for 24 h at 4°C. They were transported to All India Institute of Medical Sciences (AIIMS), New Delhi for electron microscopy. The tissue was processed by the method of Doane and Anderson (1987). The blocks were post fixed in two per cent osmium tetraoxide for 2 h. They were then washed in 0.05 M phosphate buffer for 12 h, each washing being changed after a period of 2 h. Dehydration was done in graded alcohol followed by impragnation with epoxy resin using the technique described by Luft (1961). The blocks were transferred to gelatin capsules containing the resin mixture and polymerised in an ovan at 60°C for 24 to 48 h. Ultrathin sections were cut on an ultramicrotome taken on copper grid and stained with saturated solution of uranyl acetate in 50 per cent ethanol and 0.2 per cent lead citrate. The grids were then examined

on electron microscope (Philips -300) for the presence of viral particles and ultra structural changes in the cells.

3.2.5.2 Dot immunobinding assay

This test was applied for confirmation of the isolated virus using standard known avian reovirus serum procured from IVRI, Izatnagar and from Poultry Health Institute, Doorn (Holland). The nitrocellulose strips were charged with a drop of the isolated virus having EID_{50} 10^6 and dried at room temperature (Chauhan and Singh, 1992). In the positive control, known antigen was used instead of the isolate. The negative control strips were charged with homogenate of CAM of control eggs. The non-specific sites were blocked by dipping the strips in five per cent milk powder (Amul Spray) in washing solution and incubated at 37°C for 2 h. Three washing of 5 min each were given in washing solution containing PBS (pH 7.2) and tween-20 and the strips were dipped separately in known positive serum for 1 h. Again three washings were given with vigorous shaking in washing solution and the strips were transferred to anti chicken IgG peroxidase congugate (Sigma Chemical Co., USA) diluted 1:1500 in washing solution containing two per cent BSA. After 1 h of incubation at 37°C, the strips were given three washings with vigorous shaking in washing solution. The strips were then dipped in the substrate, 3,3'-diaminobenzidine tetrahydrochloride (DAB) for 2 min. The reaction was considered positive when a brown dot appeared at the site where antigen was placed.

3.3 Pathology

The stunted and apparently healthy procured chickens were sacrificed and examined for the gross pathological changes in the intestines, pancreas, liver and gall bladder.

The visceral organs for histopathological studies included pancreas, intestines, bursa and proventriculus. These organs were collected from stunted as well as apparently healthy procured chickens in 10 per cent buffered formalin which was replaced after 24 h. The blocks of tissues were cut and were washed in running water overnight, dehydrated in ascending series of ethanol, cleared in xylene, embedded in paraffin and sections of 5 to 6 µ were cut using microtome. The tissue sections were dried and stained with Hematoxylin and Eosin stain using the standard procedure (Lillie, 1965). The slides were examined under the light microscope for interpreting the reaction of affected tissues.

Results

Results

4. RESULTS

4.1 Epidemiology

4.1.1 Characteristics of chickens population

The preliminary survey revealed the suitability of eight poultry farms situated in four different directions around Hisar city. Two farms in each direction marked as area A, B, C and D had chicken population of 2500, 2300, 1500 and 1800 respectively. The history revealed that chicks at the six farms in area A, C and D were procured from a private hatchery at Hisar and at both the farms in area B from a private hatchery at Jhajjar. It was further revealed that only F_1 vaccination against Ranikhet disease had been done and no other vaccination including reovirus infection was performed.

As per records of the poultry farms, the average age of chickens was found to be 27 days, 25 days, 21 days and 32 days in area A, B, C and D respectively. The average weight of apparently healthy chickens was 650 gm, 600 gm, 500 gm and 720 gm in area A, B, C and D respectively (Table-2).

4.1.2 Observations on stunted chicks

Number of stunted chicks at each farm, their average weight and clinical manifestations are presented in Table-3. It is clear from the table that out of 2500 chickens in area A, 8 (0.32%) had stunted growth while at farms in area B, 300 (13.04%) out of 2300 chickens were stunted. Out of 1500 chickens at farms in area C and 1800 on farms in area D, 10 (0.66%) and 9 (0.50%) chickens respectively were found to be stunted. Clinical manifestations at both the farms in area B were different from those observed at the farms in area A, C and D. Stunted chickens at farms in area B showed poor feathering, weakness and pasted vent (Figs. 2, 3 and 4) as compared to the chickens at farms in area A,C and D where either poor feathering or

Table 2 : Age and weight of apparently healthy chicken population under study

Area	Population of chickens			Age (days)			Weight (gm)		
	Farm-1	Farm-2	Total	Farm-1	Farm-2	Average age	Farm-1	Farm-2	Average weight
A	1200	1300	2500	26	28	27	630	670	650
B	1250	1050	2300	25	25	25	590	610	600
C	800	700	1500	22	20	21	530	470	500
D	1000	800	1800	32	32	32	710	730	720

Table 3 : Observations on stunted chickens

Area	Number of stunted / Population of chickens			Average weight (gm)			Clinical observations
	Farm-1	Farm-2	Total	Farm-1	Farm-2	Total	
A	3/1200	5/1300	8/2500 (0.32)	320	280	300	Poor feathering
B	190/1250	110/1050	300/2300 (13.04)	160	140	150	Poor feathering , weakness and pasted vent
C	4/800	6/700	10/1500 (0.66)	190	170	180	Weakness
D	5/1000	4/800	9/1800 (0.50)	310	290	300	Weakness
Total	202/4250	125/3850	327/8100 (4.04)	--	--	--	

Figures in parentheses are in percent

Fig. 2 Photograph of a stunted chicken (25 days, close up) shwoing pasted vent and ruffled feathers.

Fig. 3 Photograph of chickens (25 days) showing stunted growth and ruffled feathers.

Fig. 4 Photograph of chickens (25 days) showing apparently healthy (1) and stunted (2) birds with irregular feathers.


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symptoms of weakness were observed. The average weight of stunted chickens was 300 gm in area A, 150 gm in area B, 180 gm in area C and 300 gm in area D (Table-3) against the average weight of 650 gm, 600 gm, 500 gm and 720 gm of apparently healthy chickens in these areas respectively (Fig. 5). The average weight of stunted chickens beloning to the poultry farms in area B was too low as compared to the stunted chickens at other farms. The loss of weight in stunted chickens in comparison to the average weight of apparently healthy chickens of the same hatch was 75.00 per cent in area B. The loss of weight in chickens in area A, C and D was 53.84 per cent, 64.00 per cent and 58.33 per cent respectively (Table-4).

4.1.3 Feed analysis

In order to rule out the possibility of aflatoxin in feed, samples from the feed were collected from each farm on the day of visit and analysed for aflatoxin. The analysis report for aflatoxin is presented in Table-5. The aflatoxin content in feed at farm-1 and farm-2 in area A was 40 and 60 ppb respectively while it was 28 ppb and 24 ppb at farms in area B, 30 ppb and 36 ppb at farms in area C and 30 ppb each at both the farms in area D. The aflatoxin content was found to be comparatively high in the feed being fed to the chickens at farms in area A as compared to the farms in area B, C and D.

4.1.4 Seroprevalence

A total of 1029 serum samples were tested for reovirus infection. Out of these, 973 sera were collected from the apparently healthy chickens at the time of slaughter. It also included 20 apparently healthy (control) chickens. Fifty-six sera were collected from stunted chickens. The sera were screened by using counter-immuno-electrophoresis (CIEP) test and enzyme-linked immunosorbent assay (ELISA) for the presence of antibodies against reovirus. The known positive

Table 4 : Average weight of apparently healthy and stunted chickens in different areas

Area	Average weight of apparently healthy chickens (gm)	Average weight of stunted chickens (gm)	Percent loss in body weight
A	650	300	53.84
B	600	150	75.00
C	500	180	64.00
D	720	300	58.33

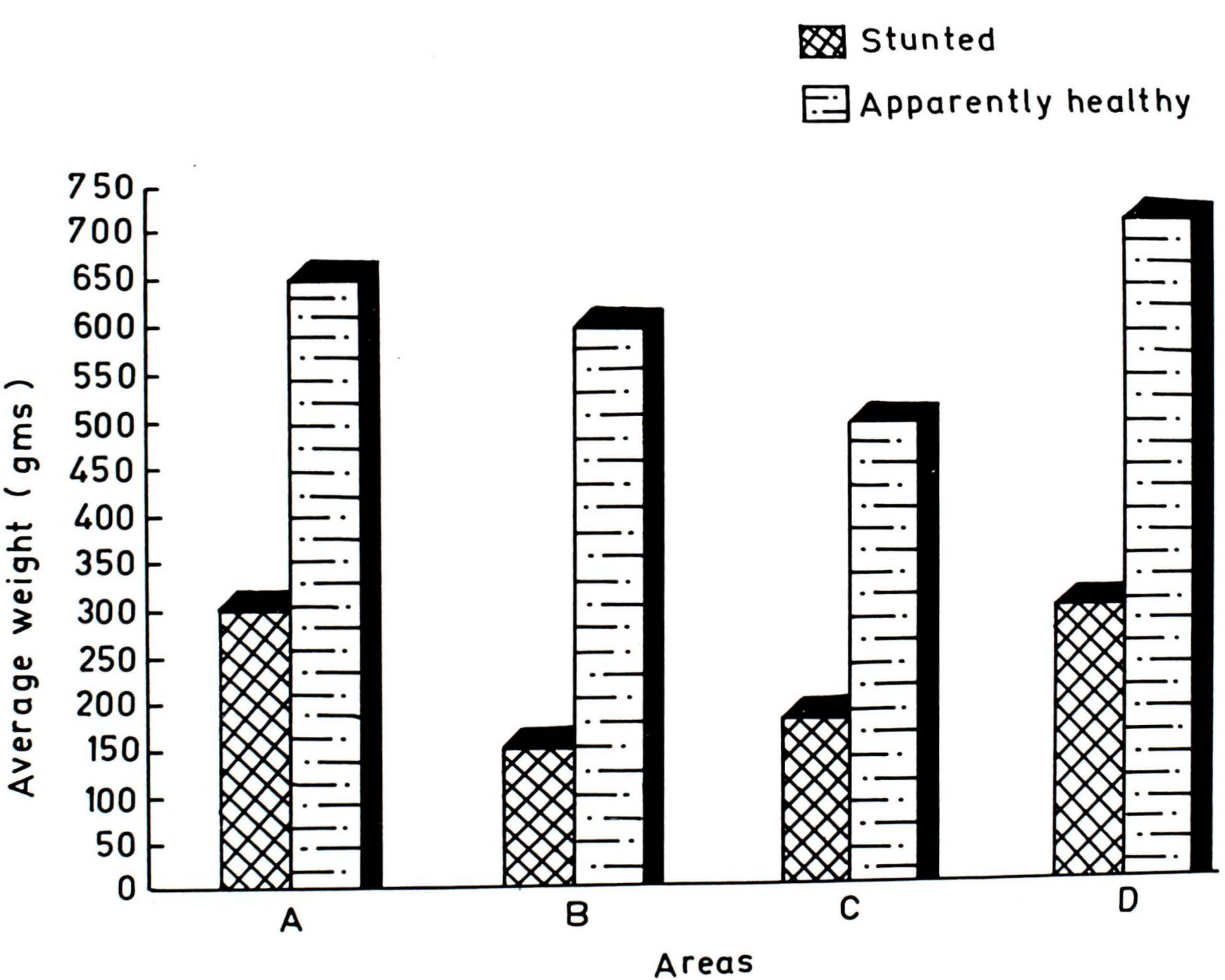


FIG. 5. AVERAGE WEIGHT OF STUNTED AND APPARENTLY HEALTHY CHICKENS OF SAME HATCH AT DIFFERENT FARMS

Table 5 : Status of aflatoxin in feed samples collected from poultry farms in different areas

Area	Number of feed samples		Aflatoxin contents (ppb)*	
	Farm-1	Farm-2	Farm-1	Farm-2
A	1	1	40	60
B	2	1	28**	24
C	1	2	30	36**
D	1	1	30	30

* Normal permissible limit of aflatoxin is 30 ppb.

** Values represent mean of two feed samples.

serum against reovirus was used as positive control in all the tests.

Of the 973 serum samples tested, 39 (4.00%) were found positive for reovirus infection with CIEP (Fig. 6). Out of these, 4 (1.60%) belonged to area A; two each from poultry farm-1 and farm-2. Remaining 35 (11.29%) positive samples were from the poultry farms in area B. Of these, 21(12.35%) were from farm-1 and 14 (10.00%) from farm-2. All the serum samples collected from poultry farms in area C and area D were found negative with CIEP (Table-6 ; Fig. 7).

The same serum samples when tested with ELISA, 55 (5.65%) samples were found to be positive for reovirus infection. Out of these, 9 (3.61%) belonged to area A. Of these, 5 (3.85%) were from poultry farm-1 and 4 (3.36%) from farm-2. Out of the remaining 46 (14.83%) positive samples, 28 (16.47%) were from poultry farm-1 and 18 (12.86%) from farm-2 of area B. All the serum samples collected from poultry farms in area C and D were found negative with ELISA (Table-7, Fig. 7).

A total of 56 sera from stunted chickens were tested with CIEP and 25 (44.64%) were found positive. Of these, 1 (33.33%) was from poultry farm-1 of area A and the remaining 24 were from the farms in area B. Out of the positive samples from area B, 14 (63.64%) were from poultry farm-1 and 10 (55.56%) from farm-2. The serum samples collected from area C and D were found negative with CIEP (Fig. 8). All the serum samples of apparently healthy chickens (control) were also found negative (Table-8).

The same serum samples when tested with ELISA, 35 (62.50%) were found positive for reovirus infection. Of these, 1 (33.33%) sample was from farm-1 and 1 (33.33%) from farm-2 of area A, while 32 (80.00%) were from area B. Out of the positive samples from area B, 18 (81.82%) were from

Fig. 6 Photograph of counter-immuno-electrophoresis (CIEP) showing a precipitating line between the wells of avian reovirus antigen and antibodies.

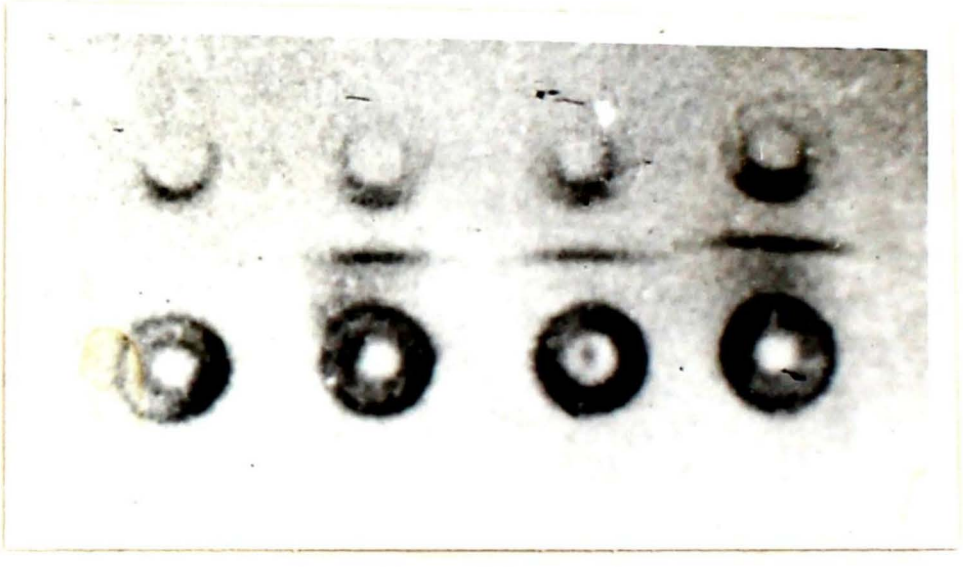


Table 6 : Seroprevalence of reovirus infection in apparently healthy chickens with counter-immuno-electrophoresis (CIEP)

Area	Number of sera tested			Number of sera positive		
	Farm-1	Farm-2	Total	Farm-1	Farm-2	Total
A	130	119	249	2(1.54)	2(1.68)	4(1.60)
B	170	140	310	21(12.35)	14(10.00)	35(11.29)
C	90	90	180	0(0.00)	0(0.00)	0(0.00)
D	132	102	234	0(0.00)	0(0.00)	0(0.00)
Total	522	451	973*	23(4.41)	16(3.54)	39(4.00)

* Figures include 20 sera of control chickens.

Figures in parentheses are in percent.

Table 7 : Seroprevalence of reovirus infection in apparently healthy chickens with enzyme-linked immunosorbent assay (ELISA)

Area	Number of sera tested			Number of sera positive		
	Farm-1	Farm-2	Total	Farm-1	Farm-2	Total
A	130	119	249	5(3.85)	4(3.36)	9(3.61)
B	170	140	310	28(16.47)	18(12.86)	46(14.83)
C	90	90	180	0(0.00)	0(0.00)	0(0.00)
D	132	102	234	0(0.00)	0(0.00)	0(0.00)
Total	522	451	973*	33(6.32)	22(4.87)	55(5.65)

* Figures include 20 sera of control chickens.

Figures in parentheses are in percent.

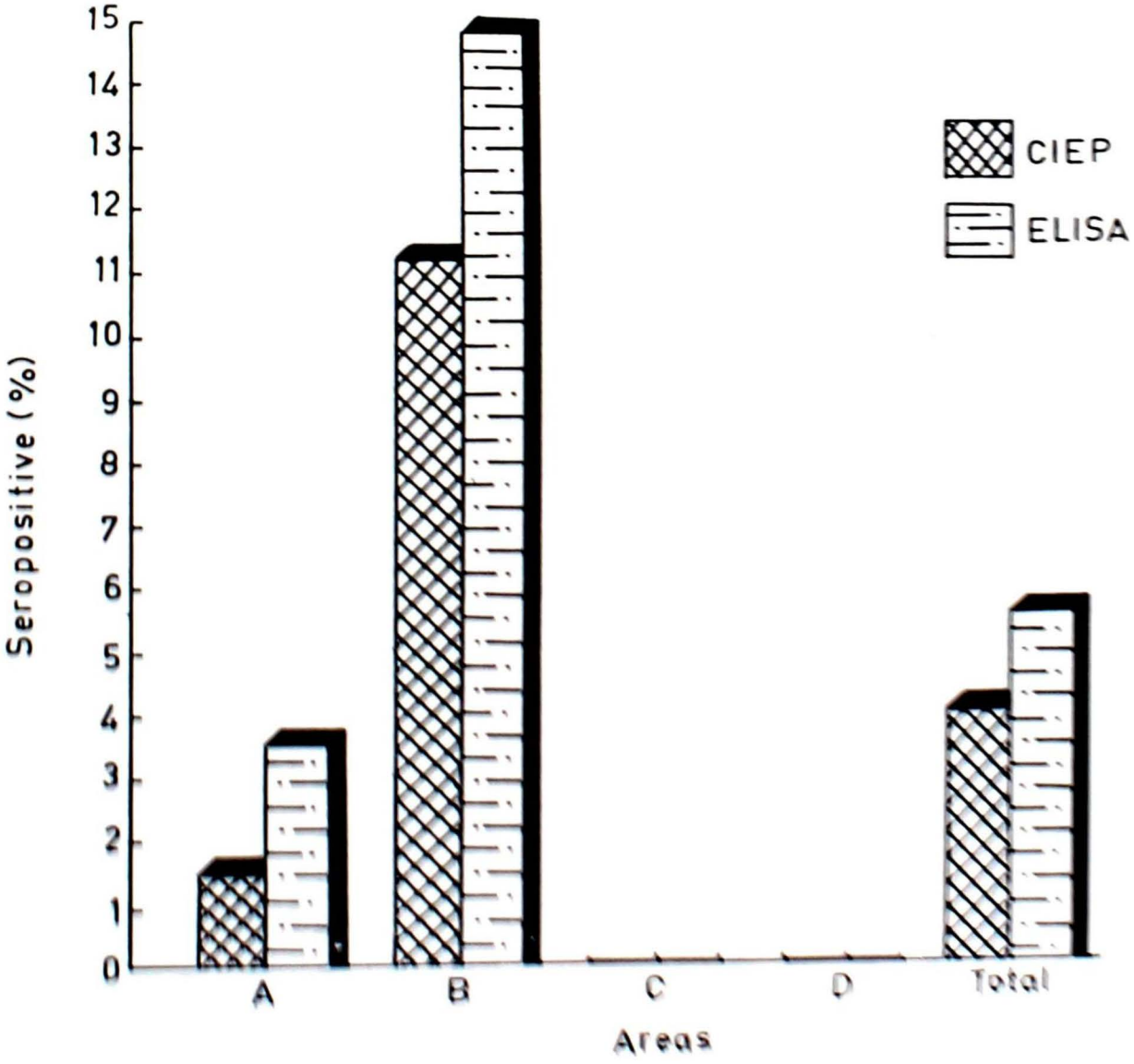


FIG. 7. SEROPREVALENCE OF REOVIRUS INFECTION IN APPARENTLY HEALTHY CHICKENS WITH CIEP AND ELISA

farm-1 and 14 (77.77%) were from farm-2. One (33.33%) serum sample from farm-1 of area C was also found positive while all the samples from farm-2 in area C and both the farms in area D were found negative (Fig. 8). Two serum samples out of 20 apparently healthy chickens (control) were found positive with ELISA. Both of these samples were from farm-1 of area B (Table-9).

Table-10 represents the total number of serum samples found positive by CIEP and ELISA. Out of a total of 1029 serum samples (973 samples of apparently healthy chickens and 56 of stunted chickens) tested, 64 (6.22%) were found to be possitive with CIEP and 90 (8.75%) with ELISA. On comparison, ELISA appeared to be more sensitive as it not only detected more samples positive but all samples which showed positive reaction with CIEP were also found positive with ELISA.

4.2 Isolation of reovirus

Isolation of the avian reovirus was attempted from the small intestines and pancreas of 16 stunted chickens fround seropositive with ELISA and 10 apparently healthy procured chickens found seronegative with ELISA.

The bacteria free filtrate prepared from 16 intestines and 16 pancreas of stunted chickens was inoculated on chorioallantoic membrane (CAM) of 10 day old embryonated chicken eggs. Of these only two intestinal and one pancreatic filtrate showed cytopathic effect (CPE) on CAM. All the remaining samples did not produce any CPE even after three blind passages and were considered to be negative and discarded. Three samples showing CPE were from two stunted chickens belonging to area B. None of the 10 intestinal and 10 pancreatic inoculum from apparently healthy chickens showed CPE even after three blind passages (Table-11).

Table 8 : Seroprevalence of reovirus infection in stunted and apparently healthy (control) chickens with counter-immuno-electrophoresis (CIEP)

Area	Number of sera positive amongst stunted chicken population			Number of sera positive amongst apparently healthy (control) chicken population		
	Farm-1	Farm-2	Total	Farm-1	Farm-2	Total
A	1/3 (33.33)	0/3 (0.00)	1/6 (16.67)	0/2 (0.00)	0/2 (0.00)	0/4 (0.00)
B	14/22 (63.64)	10/18 (55.56)	24/40 (60.00)	0/4 (0.00)	0/4 (0.00)	0/8 (0.00)
C	0/3 (0.00)	0/2 (0.00)	0/5 (0.00)	0/2 (0.00)	0/2 (0.00)	0/4 (0.00)
D	0/3 (0.00)	0/2 (0.00)	0/5 (0.00)	0/2 (0.00)	0/2 (0.00)	0/4 (0.00)
Total	15/31 (48.38)	10/25 (40.00)	25/56 (44.64)	0/10 (0.00)	0/10 (0.00)	0/20 (0.00)

Figures in parentheses are in percent.

Table 9 : Seroprevalence of reovirus infection in stunted and apparently healthy (control) chickens with enzyme-linked immunosorbent assay (ELISA)

Area	Number of sera positive amongst stunted chicken population			Number of sera positive amongst apparently healthy (control) chicken population		
	Farm-1	Farm-2	Total	Farm-1	Farm-2	Total
A	1/3 (33.33)	1/3 (33.33)	2/6 (33.33)	0/2 (0.00)	0/2 (0.00)	0/4 (0.00)
B	18/22 (81.82)	14/18 (77.77)	32/40 (80.00)	2/4 (50.00)	0/4 (0.00)	2/8 (25.00)
C	1/3 (33.33)	0/2 (0.00)	1/5 (20.00)	0/2 (0.00)	0/2 (0.00)	0/4 (0.00)
D	0/3 (0.00)	0/2 (0.00)	0/5 (0.00)	0/2 (0.00)	0/2 (0.00)	0/4 (0.00)
Total	20/31 (64.52)	15/25 (60.00)	35/56 (62.50)	2/10 (20.00)	0/10 (0.00)	2/20 (10.00)

Figures in parentheses are in percent.

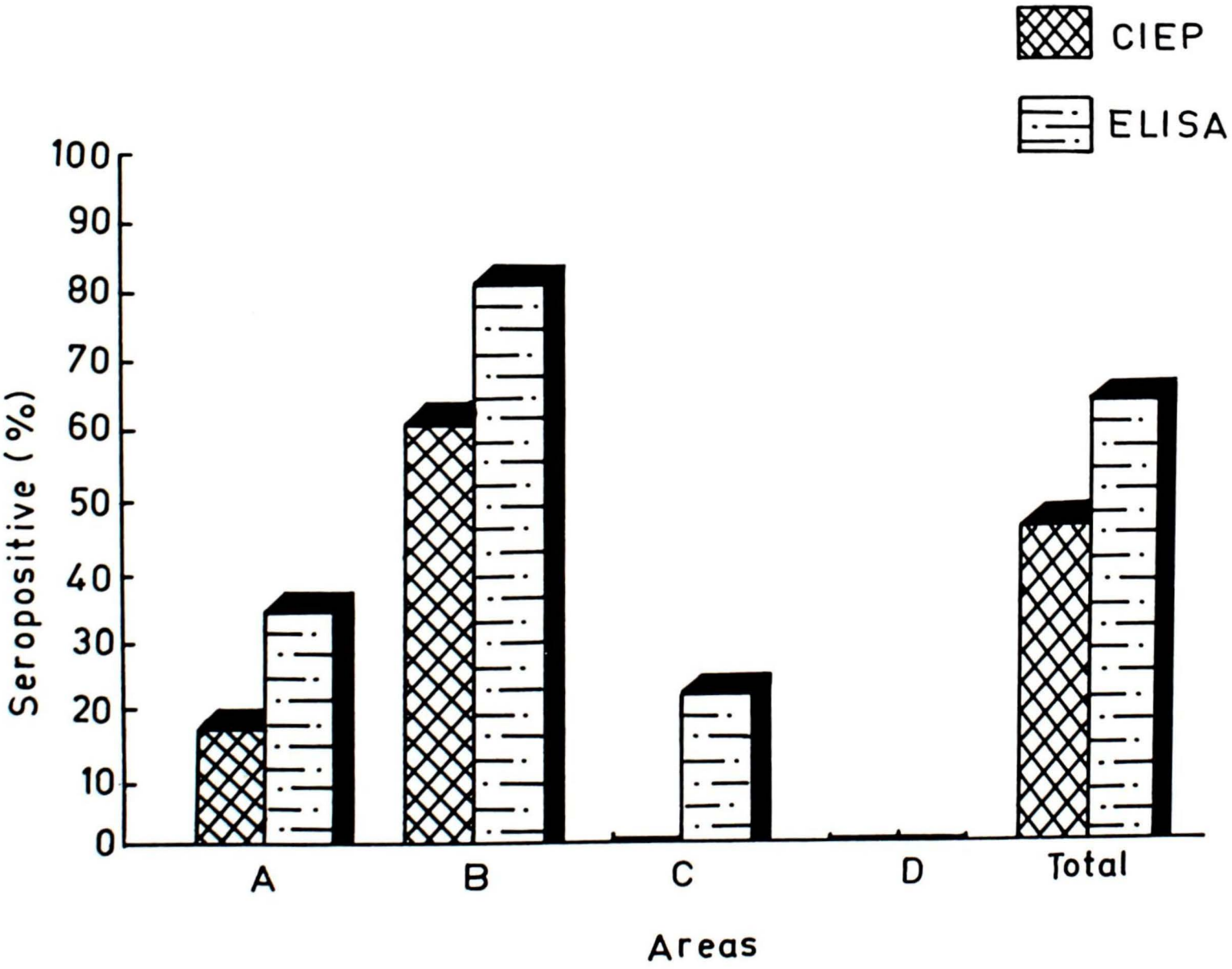


FIG. 8. SEROPREVALENCE OF REOVIRUS INFECTION IN STUNTED CHICKENS WITH CIEP AND ELISA

Table 10 : Total number of serum samples detected positive with CIEP and ELISA

Type of chicken tested	Number tested	Positive with CIEP	Positive with ELISA
Apparently healthy	973	39 (4.00)	55 (5.65)
Stunted	56	25 (44.64)	35 (62.50)
Total	1029	64 (6.22)*	90 (8.75)

* Sera positive with ELISA also.

Figures in parentheses are in percent.

Table 11 : Cytopathic effects (CPE) on chorioallantoic membrane (CAM) using inoculum prepared from stunted and apparently healthy procured chickens

Area	Farm number	Samples of stunted chickens showing CPE			Samples of apparently healthy procured chickens showing CPE		
		Int.	Pan.	Total	Int.	Pan.	Total
A	1	0/1*	0/1	0/2	0/1	0/1	0/2
	2	0/1	0/1	0/2	0/1	0/1	0/2
B	1	1/8	1/8	2/16	0/2	0/2	0/4
	2	1/5	0/5	1/10	0/2	0/2	0/4
C	1	0/1	0/1	0/2	0/1	0/1	0/2
	2	ND	ND	-	0/1	0/1	0/2
D	1	ND	ND	-	0/1	0/1	0/2
	2	ND	ND	-	0/1	0/1	0/2
Total		2/16	1/16	3/32	0/10	0/10	0/20

* Number showing CPE/Number inoculated

Int. = Intestine

Pan.= Pancreas

ND = Not done

The cytopathic effects were thickening and pock lesions with one central large necrotic area surrounded by white small necrotic foci and haemorrhages on the CAM (Fig. 9). The embryos of eggs showing CPE on CAM were alive but stunted as compared to those of controls. Besides, the liver of stunted embryos showed characteristic green discolouration as compared to the controls (Figs. 10 and 11).

4.2.1 Titration of the virus

The three samples showing CPE on CAM were further passaged till the 6th passage after which the intestinal and pancreatic inoculum of the same chicken were pooled for titration of the virus. Ten fold dilutions were made upto 10^{-8} and 0.2 ml of each dilution was inoculated in four embryonated eggs. It was observed that all the four eggs inoculated with dilution upto 10^{-4} showed CPE on CAM. Out of the four eggs inoculated with 10^{-5} dilution, three showed CPE and only two out of four inoculated with 10^{-6} showed CPE. None of the four eggs each inoculated with a titre of 10^{-7} and 10^{-8} showed CPE. A dilution of 10^{-6} showed CPE in 50 per cent of the eggs and hence the egg infective dose (EID_{50}) of the isolate was calculated as 10^{6} (Table-12).

4.2.2 Identification of virus

4.2.2.1 Electron microscopy

The electron microscopic examination of CAM revealed the presence of virus like particles resembling reovirus. The size of the virion was about 50-60 nm. The virion appeared spherical in shape although some particles showed a hexagonal outline on higher magnification (Fig. 12). The double capsid structure with central electron dense and outer coat less dense was quite appreciable (Fig. 13). The virus particles were found in the cytoplasm of the cell in the vicinity of the nucleus and grouped together to give a honey comb

Fig. 9 Photograph of reovirus infected chorioallantoic membrane (72 h p.i.) showing thickening and pock lesions.

Fig. 10 Photograph of chicken embryo (control) showing normal embryo size and colour of liver.

Fig. 11 Photograph of reovirus infected embryo showing stunted growth and greenish discolouration of liver.

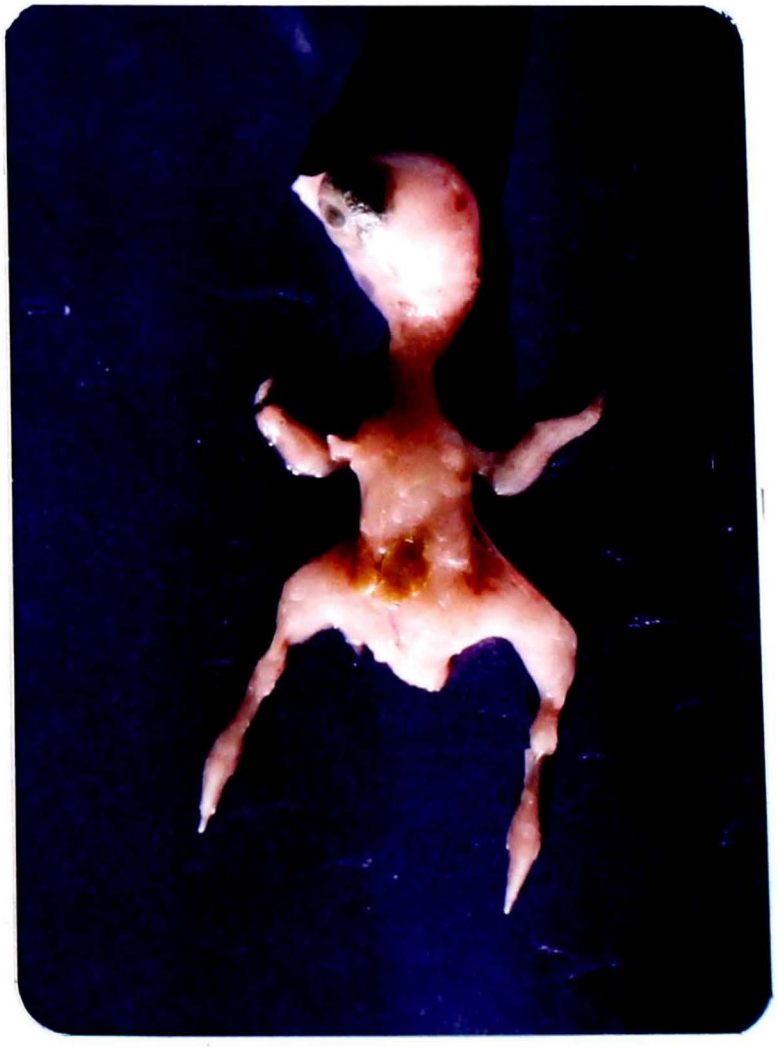


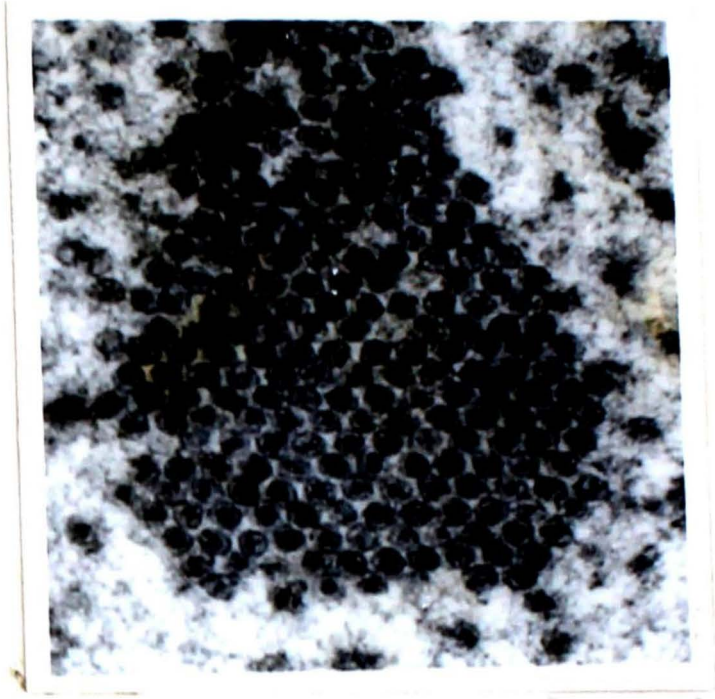
Table 12 : Titration of the virus

Dilution	Number of eggs inoculated	Number of eggs showing CPE on CAM
10^{-1}	4	4
10^{-2}	4	4
10^{-3}	4	4
10^{-4}	4	4
10^{-5}	4	3
10^{-6}	4	2
10^{-7}	4	0
10^{-8}	4	0

Egg infective dose (EID_{50}) = 10^{6} .

Fig. 12 Electron micrograph of chorioallantoic membrane (72 h p.i.) showing virus particles with spherical to hexagonal outline with a central electron dense area and less dense outer coat (55,000x).

Fig. 13 Electron micrograph of chorioallantoic membrane (72 h p.i.) showing viral aggregates forming a honey comb mat like structure (33,000x).



mat like appearance (Figs. 14 and 15). Besides these virus particles, vacuolations in the cytoplasm of the cells were also observed (Fig. 16).

4.2.2.2 Dot immunobinding assay

Dot immunobinding assay (DIA) was applied to confirm the isolates to be reovirus using both positive reovirus sera received from IVRI as well as from Holland. It was found that both the isolates as well as the positive control gave a dark brown spot on the strip with both the positive sera which confirmed the isolated virus to be an avian reovirus. No such brown dot was visible on the negative control strip, hence indicating a negative reaction (Fig. 17).

4.3 Pathology

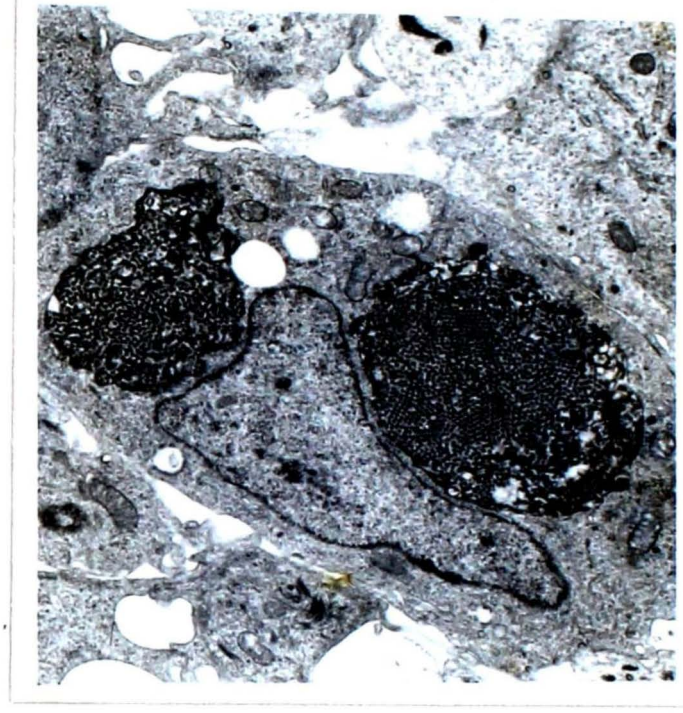
4.3.1 Necropsy

Apparently, the affected stunted chickens were very weak and their size was reduced in comparison to the healthy chickens of the same hatch. Such stunted chickens invariably had ruffled feathers and pasted vents.

On post mortem examination, undigested food material in the intestine of stunted chickens was a common finding. The intestines were distended with accumulation of gas while in some cases, the intestines were congested. Fibrosis of the pancreas and distention of gall bladder was a consistent finding in stunted chickens. However, hepatomegaly and discolouration of the liver to pale was observed in some of the affected birds.

Fig. 14 Electron micrograph of chorioallantoic membrane (72 h p.i.) showing viral aggregates in the cytoplasm in the vicinity of the nucleus (10,000x).

Fig. 15 Electron micrograph of chorioallantoic membrane (72 h p.i.) showing aggregates of virus (20,000x).



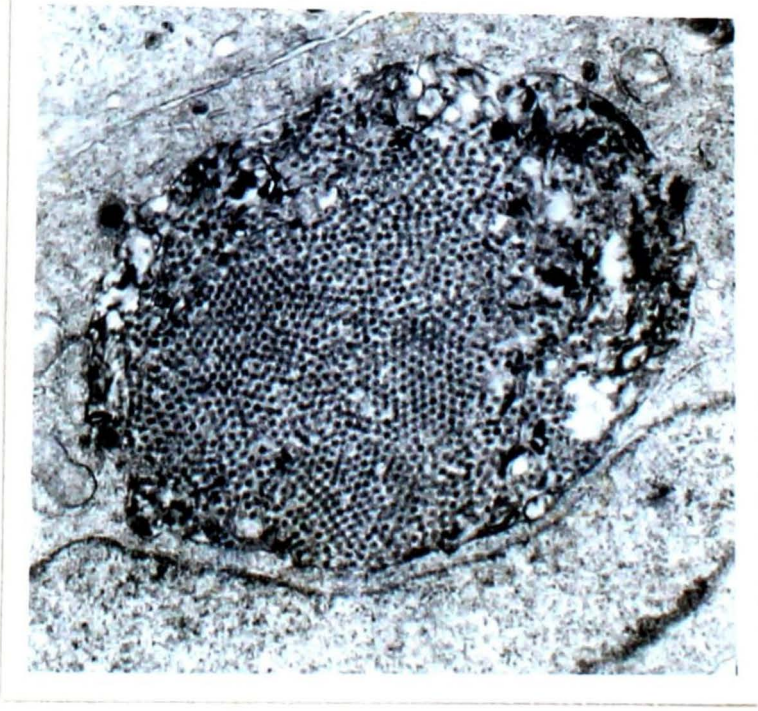


Fig. 16 Electron micrograph of chorioallantoic membrane (72 h p.i.) showing virus particles and vacuolation in the cytoplasm (27,000x).

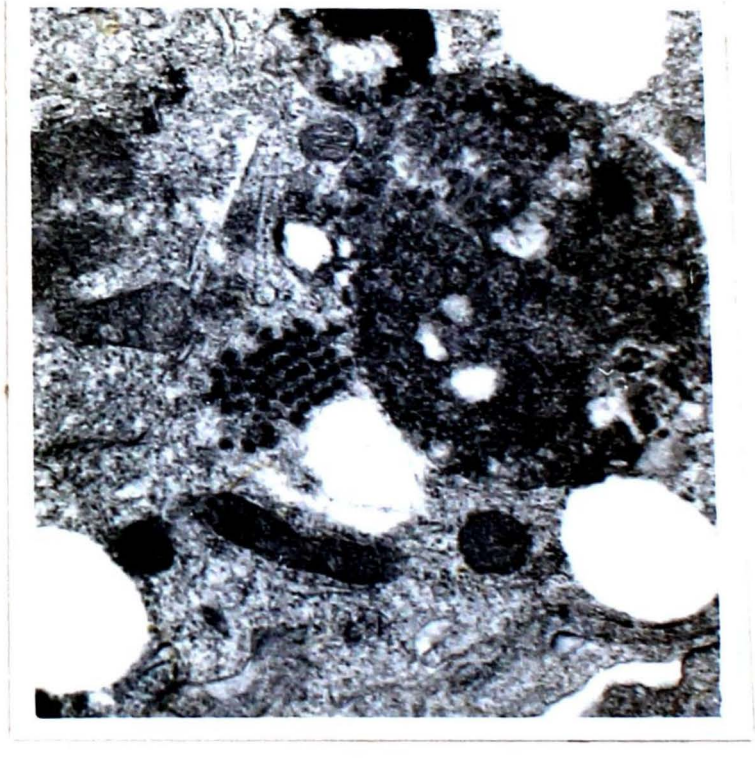


Fig. 17 Photograph of dot immunobinding assay showing a dark dot due to avian reovirus isolates.

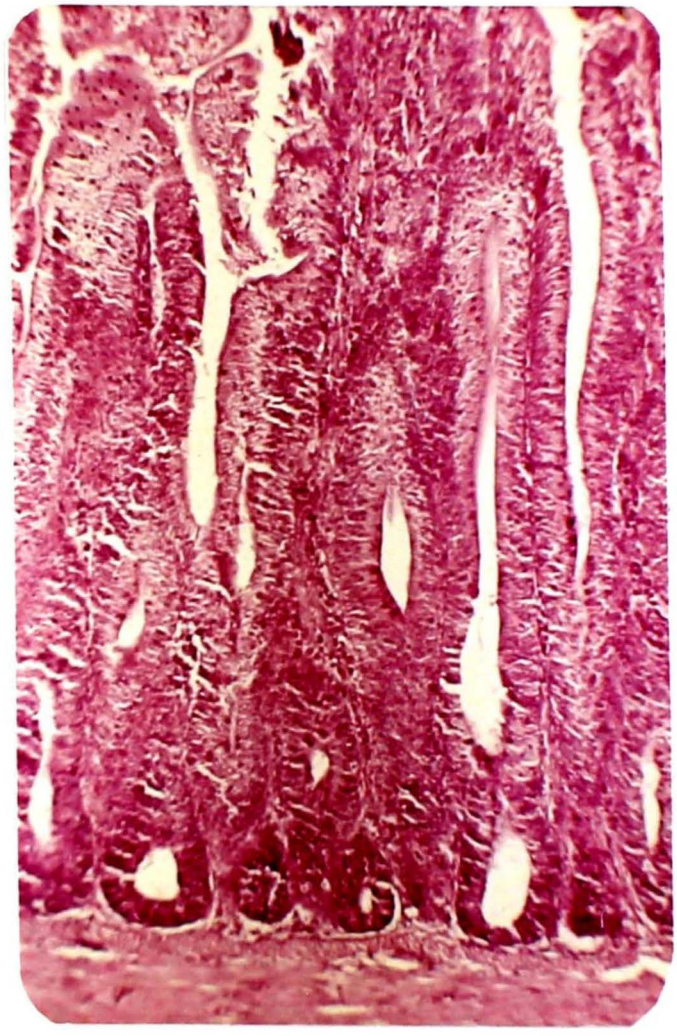

POSITIVE
NEGATIVE

4.3.2 Histopathology

On microscopic examination, the intestines showed necrosis and desquamation of the villus epithelium; the changes being more pronounced at the tips of the villi in comparison to the intestines of healthy birds (Figs. 18 and 19). The infiltration of mononuclear cells comprising mainly lymphocytes was observed at places in the lamina propria. There was hyperplasia of the epithelial cells of crypts of Lieberkuhn. The proventriculus of stunted birds showed necrosis of glandular epithelium and infiltration of mononuclear cells forming lymphoid follicles at places (Figs. 20 and 21). Histologically, the pancreas was the worst affected organ in stunted chickens. The atrophy and necrosis of the acinar cells alongwith infiltration of mononuclear cells were the more pronounced early changes (Figs. 22 and 23) while the infiltration of mononuclear cells and fibrous tissue proliferation (Figs. 24 and 25) along with severe fibrosis in the wall of pancreatic duct (Fig. 26) were observed in the stunted chickens. The bursa of the affected birds showed capsular fibrosis, oedema and mild depletion of lymphoid tissue (Figs. 27 and 28).

Fig. 18 Photo micrograph of intestine of apparently healthy chicken showing normal structure (H&E, 100x).

Fig. 19 Photo micrograph of intestine of chicken with stunting syndrome showing hyperplasia of crypts of Lieberkuhn, desquamation of villus epithelium and mononuclear cell infiltration (H&E, 100x).



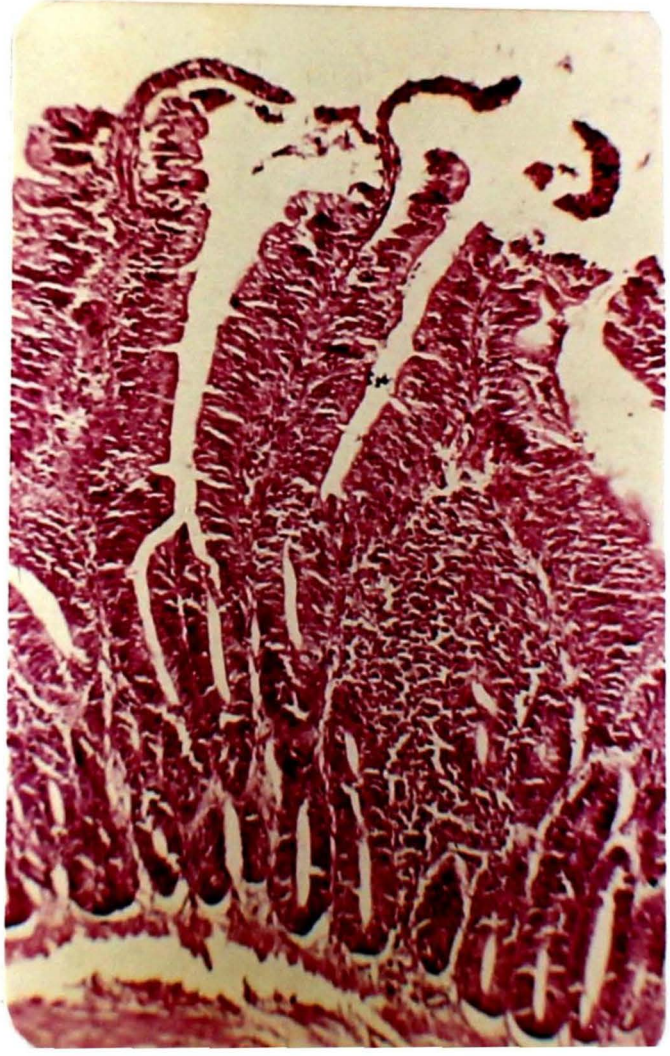
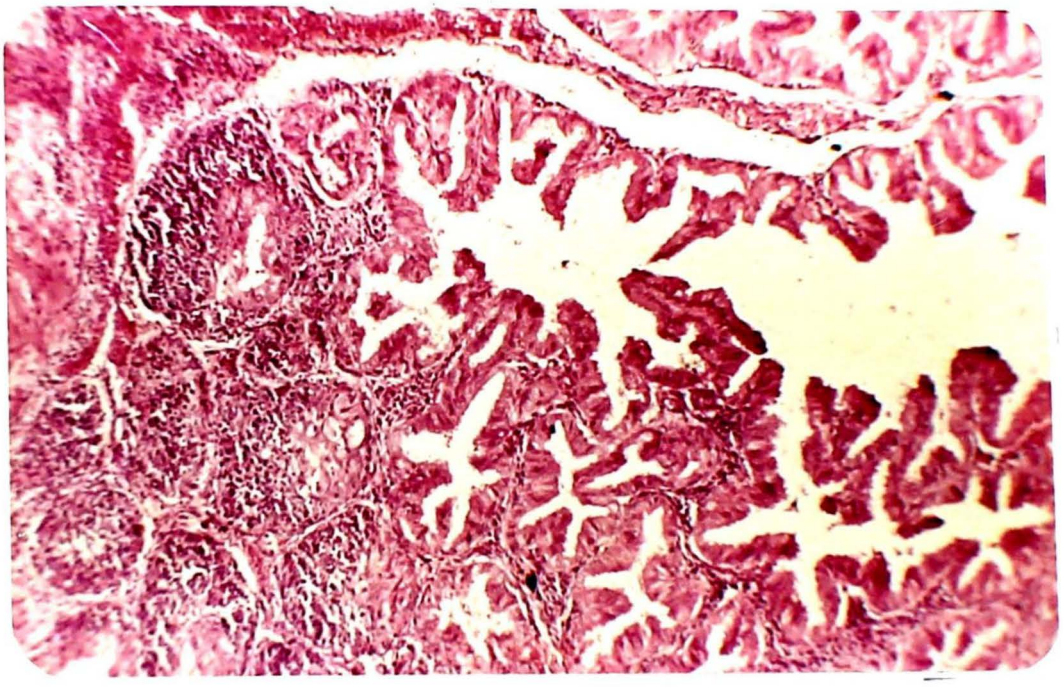


Fig. 20 Photo micrograph of proventriculus of apparently healthy chicken (H&E, 100x).

Fig. 21 Photo micrograph of proventriculus of stunted chicken showing necrosis of glandular epithelium and mononuclear cell infiltration (H&E, 100x).



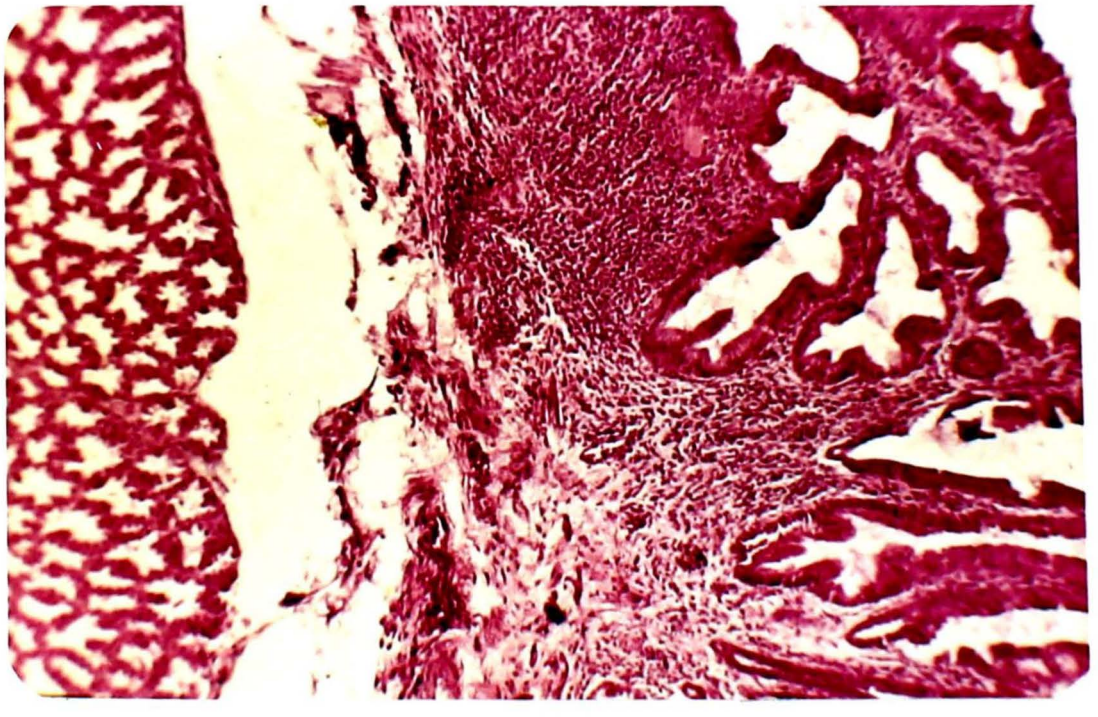
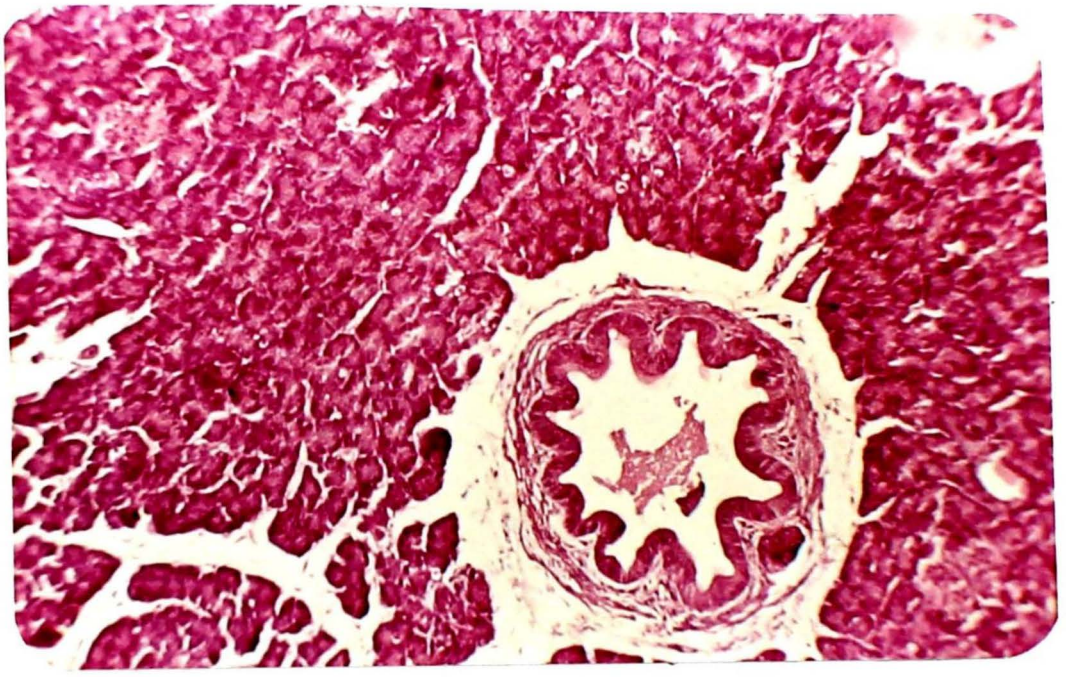


Fig. 21 Photo micrograph of pancreas of apparently healthy chicken showing normal structures (H&E, 100x).

Fig. 22 Photo micrograph of pancreas of stunted chicken showing atrophy of the acini (H&E, 100x).



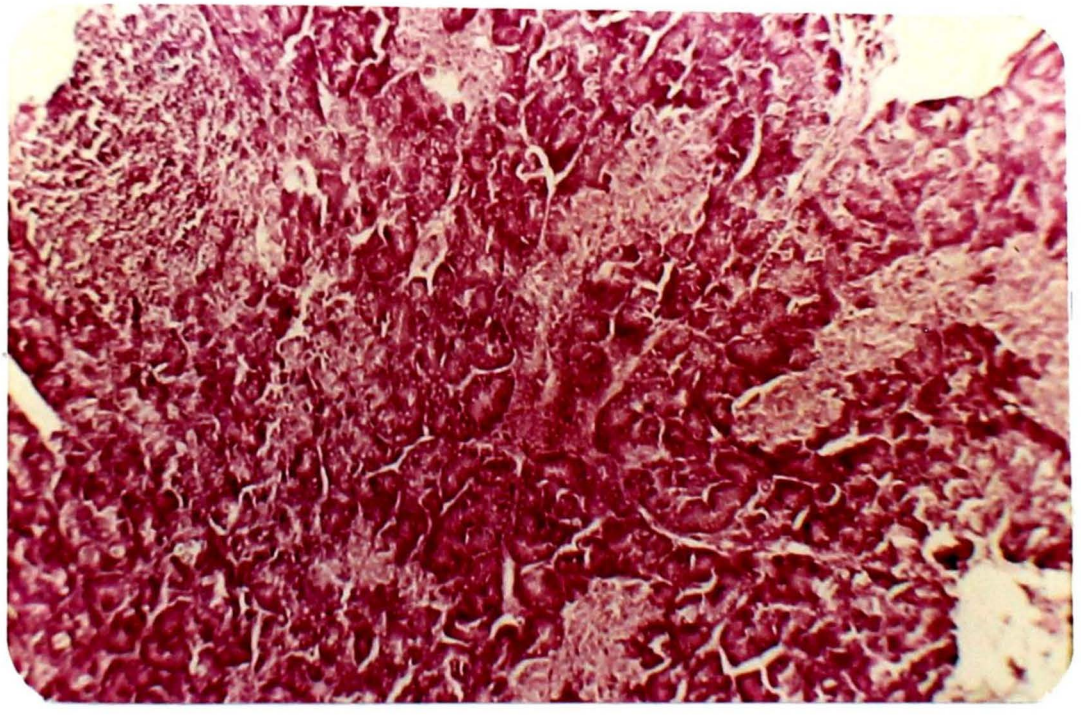
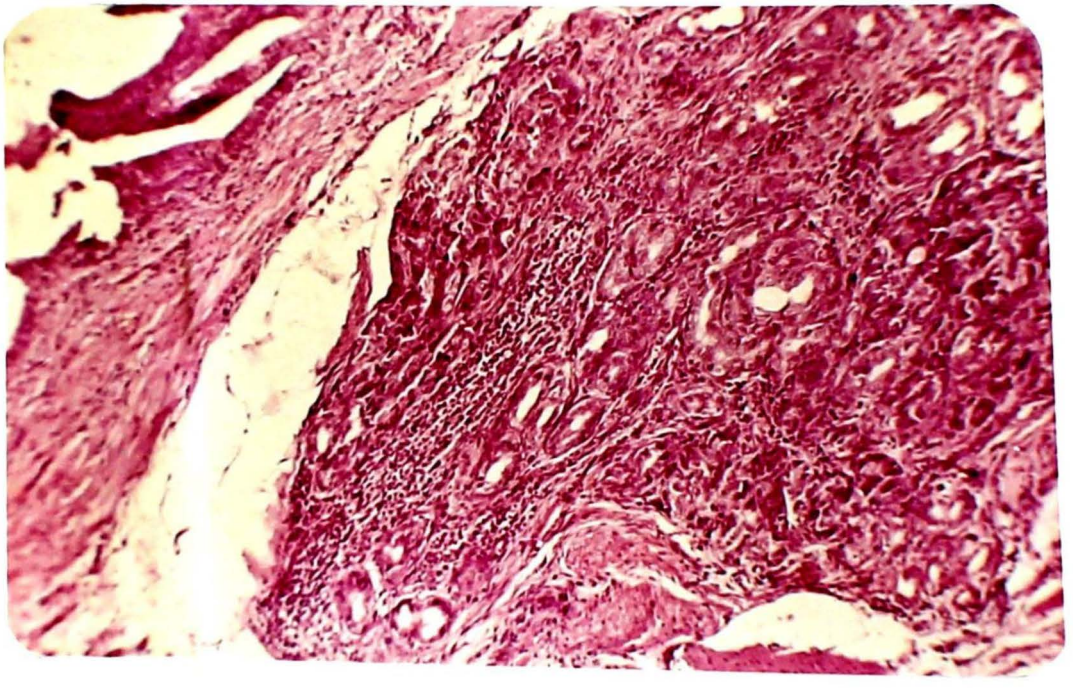


Fig. 24 Photo micrograph of pancreas of stunted chicken showing necrosis of acinar epithelial cells and mononuclear cell infiltration (H&E, 100x).

Fig. 25 Photo micrograph of pancreas of stunted chicken showing proliferation of fibroblasts with mononuclear cell infiltration (H&E, 100x).



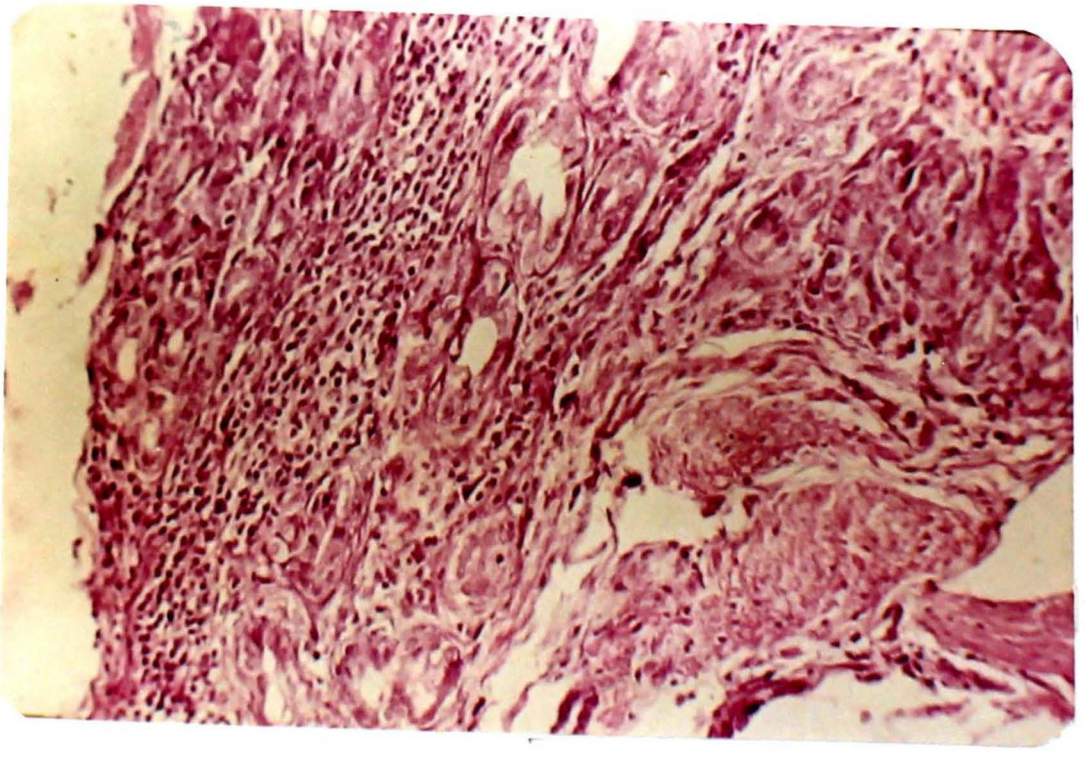


Fig. 28 Photo micrograph of pancreas of stunted chicken showing fibrous tissue proliferation around the pancreatic duct (H&E, 100x).

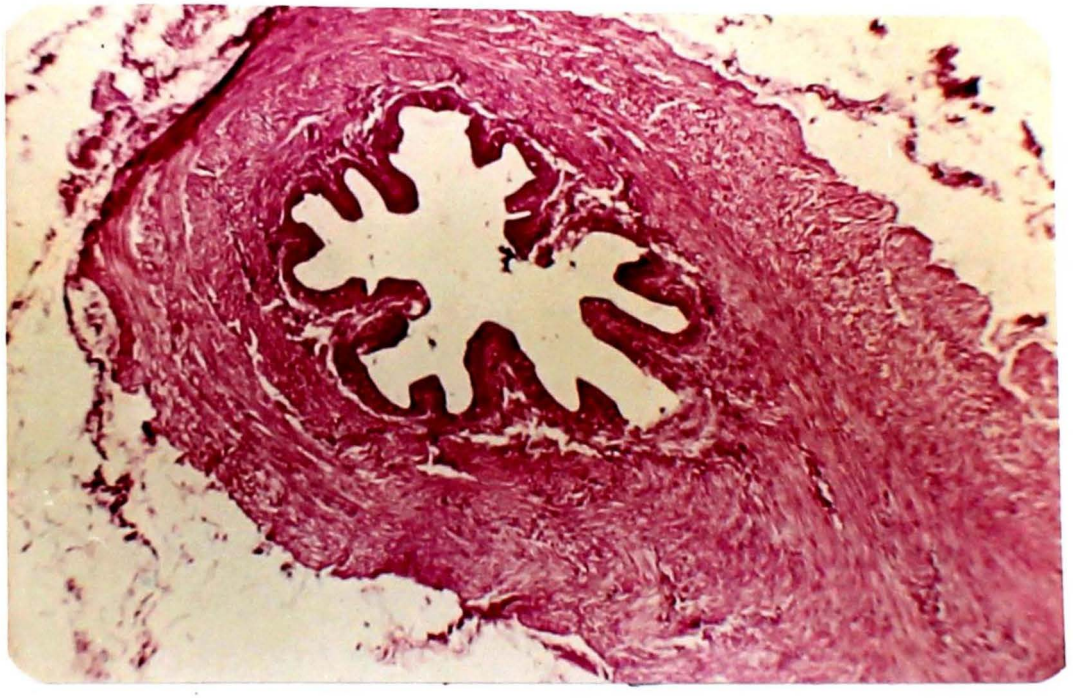
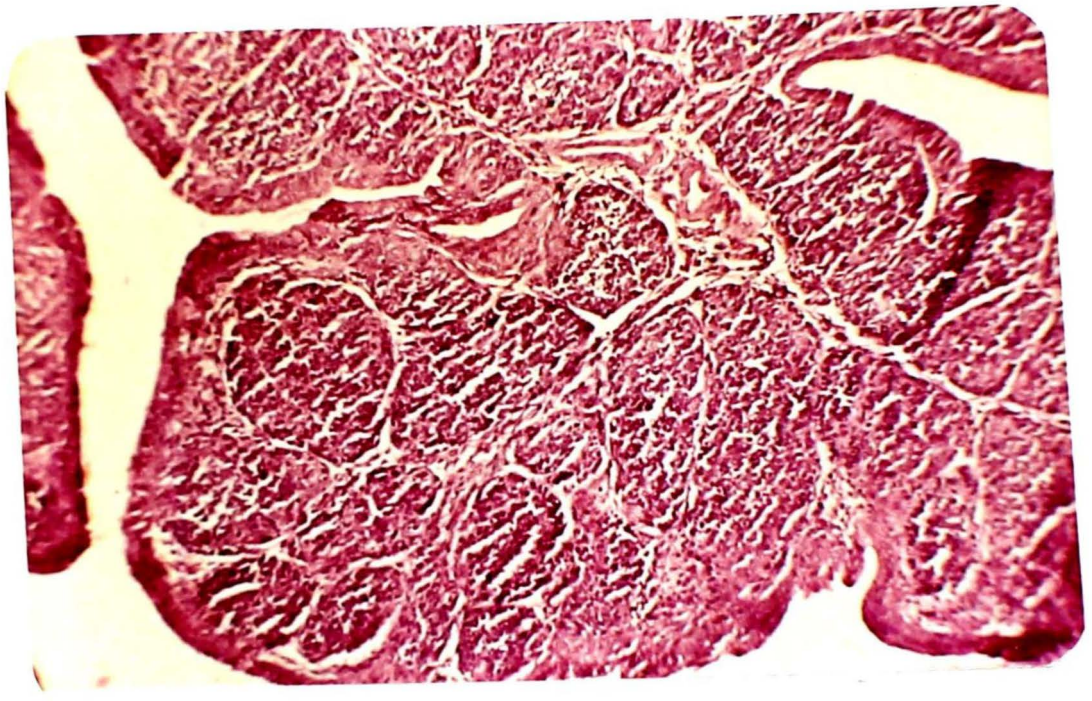
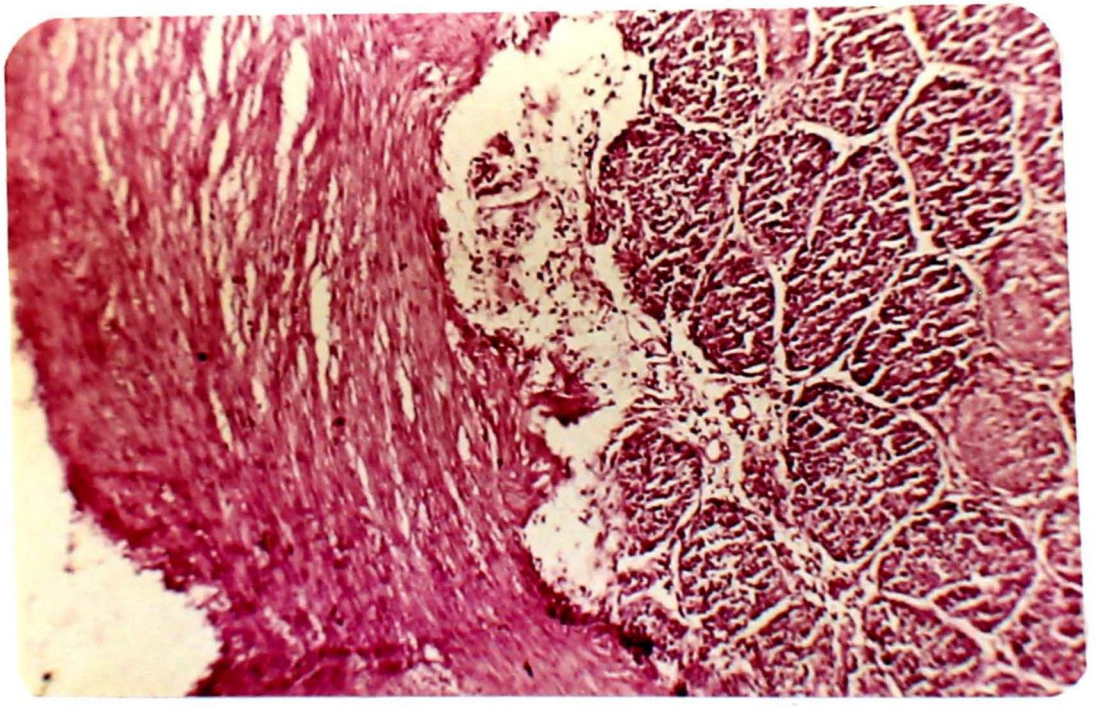


Fig. 27 Photo micrograph of bursa of fabricius of stunted chicken showing capsular fibrosis and depletion of lymphoid tissue (H&E, 100x).

Fig. 28 Photo micrograph of bursa of fabricius of apparently healthy chicken showing intact lymphoid tissue with normal structures (H&E, 100x).





Discussion

5. DISCUSSION

Tremendous efforts have been made and are still in progress to understand the stunting syndrome all over the world. The disease poses a serious threat to the poultry industry in terms of reduced weight gain in broilers and mortality. Although the aetiology of the condition remains obscure till date but reovirus has been frequently isolated from affected chickens (Kouwenhoven et al., 1978a, Pass et al., 1982). Stunting syndrome is one of the emerging problems of poultry industry in India. The importance of the infection can be adjudged from the fact that the demand of vaccine against reovirus has increased steadily. In India, the seroprevalence of reovirus infection has been reported (Kataria et al., 1983, Verma and Verma, 1986) but there seems to be no report on the isolation and identification of reovirus from stunted chickens although retarded growth of chickens has been observed at various farms. The present study was undertaken to assess the problem of stunting syndrome at eight broiler farms around Hisar city to find its association with reovirus.

On survey, it was found that at two farms,the stunting in chickens was a more severe problem as compared to other farms. Further inquiries revealed that the source of day old chicks at the severely affected farms was different from that of other farms. There appears to be an association between stunting and the parent flock of chicken. Bracewell (1982) reported similar observations about the stunted birds and established their association with the source of infection from the hatchery. A marked difference in the average weight of stunted and apparently healthy chickens was observed; the affected birds weighing only one fourth of the weight of apparently healthy chickens at certain farms. Besides weight loss, poor feathering, pasted vent and weakness

were the common findings. Similar findings in stunted chickens have been reported by Bracewell and Randall (1984) who observed uneveness in body size, pasted vent and diarrhoea with increased mortality in affected birds. Bracewell and Wyeth (1981) also found that such birds failed to develop full mature feathering.

To rule out the possibility of aflatoxin being involved in causing stunting in chickens, samples of feed from all the farms were analysed. No relation of aflatoxin with stunting syndrome was observed as the aflatoxin levels were found below the permissible limits (30 ppb) at farms with high prevalence of stunted chickens whereas the less severely affected farms had slightly higher levels (30 ppb to 60 ppb) of aflatoxin. This is concordant with the findings of Bracewell and Randall (1984) who did not find the association of mycotoxins and stunting syndrome in chickens.

Seroprevalence of reovirus infection was estimated in stunted and apparently healthy chickens separately with CIEP and ELISA to observe if reovirus was associated with stunted chickens. A high rate of seroprevalence of reovirus infection in apparently healthy chickens (11.29% with CIEP and 14.83% with ELISA) was recorded at two particular farms having more number of stunted chickens (13.04%) than at two other farms where the number of stunted chickens was very less (0.32%) and seroprevalence rate was also very low (1.60% with CIEP and 3.61% with ELISA). No sera were found positive from the remaining four farms (Table 6 and 7). Moreover, the seroprevalence of reovirus infection in stunted chickens was found to be high (60.00% with CIEP and 80.00% with ELISA) at two particular farms having more stunted chickens than at other farms having a few stunted chickens with low seroprevalence of reovirus (Table 8 and 9). The high seroprevalence of reovirus infection

in apparently healthy and stunted chickens at two particular farms with more number of stunted chickens and low or nil seroprevalence at other farms with less number of stunted chickens indicate that reovirus might be associated with stunting syndrome in chickens.

A comparatively low seroprevalence (4.00% with CIEP and 5.65% with ELISA) was observed in broilers of eight weeks than in young chickens of 3 to 4 weeks (stunted) in which seroprevalence was quite high (44.64% with CIEP and 62.50% with ELISA). Young chickens are more susceptible to reovirus infection has earlier been reported by various workers. Kerr and Olson (1964)reported a marked decrease in infectivity to reovirus infection in older chickens . Jones and Georgiou (1984) also found an age related resistance in experimental birds infected with R2 strain of reovirus.

Various diagnostic tests have been employed for detection of avian reovirus antibodies for studying seroprevalence. Agar gel precipitation test (AGPT) has often been used by a number of workers. Kataria et al. (1983) studied seroprevalence of reovirus infection in poultry in different states of India using AGPT. Using the same test, seroprevalence was studied in Manipur (Verma and Verma, 1983), Maghalaya (Verma and Verma, 1986), Arunachal Pradesh (Verma et al., 1987) and Tripura (Verma et al., 1989). Several workers tried to observe the efficacy of AGPT and compared it with other diagnostic tests. Robertson et al. (1984) reported a seroprevalence of 42.00 per cent using AGPT and 66.00 per cent with serum neutralization test in Australia. Schwarzmaier (1983) and Chen et al. (1991) found ELISA to be more sensitive than AGPT in detecting reovirus infection. ELISA was reported to be even more sensitive than immunofluorescence test (Schwarzmaier et al., 1984). Keeping in mind the higher efficacy of ELISA in detecting reovirus

infection, it was therefore preferred to use ELISA in the present study. Counter-immuno-electrophoresis (CIEP) which is reported to be more sensitive than AGPT (Kataria et al., 1989) was also employed in the present study to compare its sensitivity with ELISA.

ELISA was found to be more sensitive than CIEP as it not only detected more samples positive but all samples positive with CIEP were also detected positive with ELISA. Although the reports on the comparison of ELISA with CIEP are not available but ELISA has been reported to be more sensitive than serum neutralization test (Robertson et al., 1984), AGPT (Schwarzmaier, 1983, Chen et al., 1991) and immunofluorescence test (Schwarzmaier et al., 1984).

A total of 56 stunted and 20 apparently healthy chickens were procured for isolation of virus. But isolation was attempted from 16 stunted and 10 apparently healthy chickens only. These stunted chickens from which isolation was attempted were all positive for reovirus infection with ELISA and all the apparently healthy ones chosen for isolation were negative with the above test. Moreover, it was not convenient to check the samples for virus isolation from all stunted and apparently healthy chickens in the time bound period of study. Virus isolation was attempted from the intestine and pancreas of the stunted chickens. These organs were preferred for isolation of reovirus as there are several reports of its isolation from these organs (Van der Hiede et. al, 1981, Page et al., 1982, Pass et al., 1982, Kouwenhoven et al.,1983, Robertson et al., 1984, Carli et al., 1991).

Of the 16 stunted chickens tested, the reovirus was isolated from three samples of two birds including two intestinal filtrates and one pancreatic filtrate. Both the chickens were from the area having high seroprevalence of

reovirus infection. Isolation of reovirus from stunted chickens has earlier been reported by Olsen (1977), Kouwenhoven et al. (1978a), Pass et al. (1982), Kouwenhoven et al. (1983) and Gerganov et al. (1987). This again showed that there was an association between stunting syndrome and reovirus. However, Robertson et al. (1984) isolated reovirus from stunted as well as apparently healthy chickens and suspected a carrier status of reovirus in chickens. According to Martland (1989), either the reovirus present in the healthy chickens was in low number which was unable to initiate the disease or reovirus could not cause stunting unless triggered by other factors like stress, nutrition and environment. In the present study, isolation of virus was carried out from a limited number of samples, therefore, it is difficult to say whether carrier status of reovirus exists or not but three samples from which virus was isolated belonged to stunted chickens and no virus was isolated from the apparently healthy chickens.

Among the cytopathic effects, thickening of CAM and a central large necrotic area surrounded by white small necrotic foci were prominent. The infected embryos were stunted and showed greenish discolouration of the liver. These findings are concordant with the findings of Dutta and Pomeroy (1967a), Mustaffa-Babjee and Spradbrow (1971) and Bains et al. (1974). Olson (1975) also reported the greening of liver in the infected embryos and attributed it to the affection of gall bladder. Guneratne et al. (1982) observed thickening and oedema of infected CAM with dwarfing of embryos and necrotic lesions on the liver.

Various routes have been adopted for inoculation of embryonated chicken eggs. Dutta and Pomeroy (1967a) used yolk sac route for multiplication of the isolate. Glass et al. (1973) reported yolk sac route more sensitive than

allantoic cavity route. Spradbrow and Bains (1974) found that the presence of reovirus could be demonstrated more easily via CAM route than on cell culture. Guneratne et al. (1982) compared the yolk sac route with CAM route and recommended the later for isolation and cultivation of reovirus. However, in the present study, dropped chorioallantoic membrane of 10 day old embryonated chicken eggs was used for observing CPE and multiplication of virus. It was found to be convenient and sensitive for carrying out reovirus isolation studies.

The virus particles were demonstrated in the infected CAM under electron microscope and resembled reovirus. The diameter of the virus measured 50 to 60 nm which is in accordance with the findings of Dutta and Pomeroy (1967b), Koide (1970) and Deshmukh et al. (1971). However, some workers have reported the size of reovirus to be 70 to 82 nm (Kawamura et al.,1965, Mustaffa-Babjee et al., 1973 and Kataria et al., 1985). The virus appeared as a spherical particle, however, some particles showed hexagonal outline on higher magnification. Mustaffa-Babjee et al. (1973) and Farmer and Taylor (1985) also reported the reovirus to be spherical in shape. Kataria et al. (1985) isolated reovirus from tenosynovitis in poultry and reported its shape from round to hexagonal. The virus also showed double capsid structure with spherical outline and honey comb matting appearance. This is considered characteristic of reovirus and has been reported by Mustaffa-Babjee et al. (1973).

The isolates were confirmed as reovirus using the hyperimmune serum of reovirus obtained from IVRI, Izatnagar and Doorn, Holland by dot immunobinding assay (DIA). A positive reaction comparable to the known positive antigen was observed by both the sera which also confirmed the isolate as reovirus. Earlier, the DIA has been used for detection of rotavirus antigen in the faecal and intestinal contents of diarrhoeic calves by Chauhan and Singh (1992). It

involves the direct application of an antigen to pieces of nitrocellulose strip and the detection of an antigen-antibody complex in the form of a coloured dot. The nitrocellulose strip acting as the solid support offers several advantages over other conventional procedures (Hawkes, 1986). The nitrocellulose bound to plastic in the form of dipsticks further simplifies the assay procedure. DIA is simple and can be completed in four hours. The results in the form of a coloured spot in positive cases can be read with the naked eyes. No sophisticated instrument is required and the dipsticks can be stored for a long period without deterioration. The test is cheap and can be used in field for rapid diagnosis of reovirus infection in poultry.

The gross and pathological lesions observed in stunted chickens were found to be more severe in the pancreas. Grossly, the pancreas was found to be fibrosed. Pass et al. (1982) also found the pancreas to be thinner in affected birds. Bracewell and Randall (1984) reported fibrosis and rounding of the fine borders of pancreas near the closed end of duodenum. Histopathologically, there was severe necrosis and atrophy of the acinar cells along with fibrosis of the pancreatic duct. This has earlier been reported by Page et al. (1982). According to Martland and Farmer (1986), pancreatic atrophy is due to fibrosis of the pancreatic duct wall and occlusion of the pancreatic duct causing dysfunctioning of the pancreas. In the intestines, undigested food material with accumulation of gas was found. According to Martland (1989), undigested food in affected birds was probably due to impaired outflow of the digestive enzymes from the pancreas hence inhibiting digestion. Desquamation of the intestinal villus epithelium observed in the present study might have also caused poor absorption leading to stunting syndrome. Atrophy, capsular fibrosis and depletion of lymphocytes observed in bursa have earlier been reported by Page et al.(1982)and

Tang et al. (1987). Necrosis of the glandular epithelium of proventriculus was observed in some stunted chickens. However, Page et al. (1982) observed proventriculitis in affected chickens.

The present findings strongly suggest the association of reovirus with stunting syndrome in chickens based on the following observations :

1- The high seroprevalence of reovirus infection in apparently healthy and stunted chickens at the farms with more number of affected chickens and very low or nil seroprevalence at farms with a few number of stunted chickens.

2- Isolation and identification of reovirus from stunted chickens and no isolation of reovirus from the apparently healthy chickens.

3- It is further supported by the history that the source of purchase of chicks was different at farms where more number of stunted chickens were recorded.

4- Characteristic clinical manifestations and post mortem lesions were observed which simulated reovirus associated stunting syndrome.

The present study is an attempt in this direction on field cases. Reovirus has been isolated from only two out of sixteen stunted chickens. It will be worthwhile if experimental studies are carried out to understand the stunting syndrome in a better way.

Summary

6. SUMMARY

There are reports from abroad that stunting syndrome in chickens is associated with reovirus infection but there is no such report from India. Therefore, it was planned to study the prevalence of reovirus infection in India during 1991-92. For the present study, eight broiler farms situated around Hisar city (Haryana) were selected and arbitrarily placed in four areas A , B, C and D. The cases of stunted chickens were recorded by observing weight loss and clinical manifestations while seroprevalence was studied by using antigen obtained from IVRI, Izatnagar. Counter-immuno-electrophoresis (CIEP)and enzyme-linked immunosorbent assay (ELISA) were used as diagnostic tests to study the seroprevalence in apparently healthy and stunted chickens. Virus isolation was attempted from sick (stunted) as well as apparently healthy procured chickens to establish the association of stunting syndrome with reovirus.

A total of 8100 chickens were examined out of which 327 (4.04%) were found to be stunted. Of these, 8 (0.32%) were at the farms in area A, 300 (13.04%) at farms in area B, 10 (0.66%) at farms in area C and 9 (0.50%) in area D. The stunted chickens showed clinical manifestations like poor and ruffled feathers, weakness and pasted vent. These symptoms were prominent among chickens in area B. A significant loss of body weight ranging from 53.84 to 75.00 per cent was recorded in stunted chickens, loss being highest in stunted chickens of area B.

A total of 1029 serum samples were tested for the presence of reovirus infection. Out of these, 973 were from apparently healthy chickens and 56 from the stunted chickens.

Of the 973 sera of apparently healthy chickens (including 20 control) tested with CIEP and ELISA, 39 (4.00%) were found positive with CIEP for

reovirus infection. Out of these, 4 (1.60%) were from area A and 35 (11.29%) from area B. With ELISA, an overall seroprevalence of 5.65 per cent was recorded with 9 (3.61%) and 46 (14.83%) samples from farms in area A and B respectively, giving a positive reaction. Sera collected from chickens in area C and D did not reveal the presence of reovirus antibodies both with CIEP and ELISA. Sera of 20 control chickens was found negative with CIEP but two serum samples among control chickens were found positive when tested with ELISA.

Fifty six sera from stunted chickens were also tested with CIEP and ELISA for reovirus infection. Out of these, 25 (44.64%) were found positive with CIEP. Of these , 1 (16.67%) belonged to area A and 24 (60.00%) to area B. ELISA however, detected 35 (62.50%) sera positive for reovirus infection out of 56 tested. Of these, 2 (33.33%) were from area A, 32(80.00%) from area B and 1 (20.00%) from area C.

ELISA therefore, appeared more sensitive as it detected more sera positive for reovirus in both apparently healthy and stunted chickens. It not only detected more sera positive but all samples detected positive with CIEP were also found positive with ELISA.

Virus isolation was attempted in 10 day old embryonated chicken eggs through the chorioallantoic membrane (CAM) route using inoculum prepared from intestine and pancreas of 16 stunted and 10 apparently healthy procured chickens. Only three samples from two birds showed cytopathic effects (CPE). The data revealed that these chickens belonged to area B. All samples from procured healthy chickens did not reveal CPE even after three blind passages.

The CPE on CAM included thickening, pock lesions with one large central necrotic area surrounded by small white necrotic foci and haemorrhage.

The infected embryos were found stunted with greenish discolouration of the liver.

The CAM showing CPE were checked for the presence of virus particles using electron microscope. The virus particles were demonstrated having spherical to hexagonal outline, measuring 55 to 60 nm in diameter in the cytoplasm of the cell. The particles were grouped together giving a honey comb mat like appearance characteristic of reovirus. The aggregates of virus particles caused vacuolation in the cytoplasm of the CAM cells. The isolates were confirmed as avian reovirus by dot immunobinding assay (DIA) using avian reovirus hyperimmune serum obtained from IVRI, Izatnagar and the Poultry Health Institute, Doorn (Holland).

Observations on the stunted chickens also revealed that the affected chickens had lost half to three-fourth weight as compared to the apparently healthy chickens. In addition, symptoms of ruffled feathers and pasted vent were prominent. The gross pathological changes included undigested material in the intestine with accumulation of gas. Fibrosis of pancreas and distension of gall bladder were commonly observed. Hepatomegaly and discolouration of liver were also seen in some cases.

The histopathological examination of pancreas revealed degenerative and necrotic changes with fibrosis of the wall of pancreatic duct. The bursa showed capsular fibrosis with lymphoid tissue depletion. The intestine showed necrosis and desquamation at the tips of villi with mononuclear cell infiltration in lamina propria. Proventriculus showed necrosis and mononuclear cells infiltration.

A high seroprevalence rate of reovirus infection in chickens at the two broiler farms having more number of stunted chickens and a very low or

nil seroprevalence at other farms with a few number of stunted chickens suggest the association of reovirus with stunting syndrome. Isolation and identification of reovirus from stunted chickens and no isolation of reovirus from apparently healthy chickens further confirms the above findings. Application of dot immunobinding assay in detection of avian reovirus antigen in the present study will go a long way to carry out epidemiological and immunological studies of reovirus infection in poultry.

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Appendix

Deionized double glass distilled water (DGDW) was used to prepare all the reagents required in this study.

1- Phosphate buffered saline (PBS)

a) PBS (pH 7.2)

Sodium chloride (NaCl)	:	20.2 gm
Disodium hydrogen phosphate ($Na_2HPO_4.2H_2O$)	:	1.15 gm
Potassium dihydrogen phosphate (KH_2PO_4)	:	0.2 gm

DGDW to make 1000 ml

b) PBS (0.01M, pH 7.5)

Two solutions A and B were added to make 0.01M phosphate buffer.

Solution A : Added 1.56 gm of sodium dihydrogen phosphate ($NaH_2PO_4.2H_2O$) to 1000 ml DGDW.

Solution B : Added 1.42 gm disodium hydrogen phosphate (Na_2HPO_4) to 1000 ml DGDW.

Added 16 ml of solution A and 84 ml of solution B to make 200 ml with DGDW.

2- Citric acid phosphate buffer (pH 5.0)

Disodium hydrogen phosphate ($Na_2HPO_4.2H_2O$)	:	11.86 gm
Citric acid	:	7.30 gm

DGDW to male 1000 ml

3- Veronal buffer (0.2 M, pH 8.6)

Two solutions A and B were added to make veronal buffer (pH 8.6)

Solution A : Added 20.62 gm sodium barbitone to 500 ml DGDW.

Solution B : DGDW was added to 1.679 ml concentrated hydrocloric acid to make 100 ml.

Added 50 ml of solution A and 6 ml of solution B and diluted to 200 ml with DGDW.

4- Washing solution

Sodium chloride (NaCl)	:	20.2 gm
Disodium hydrogen phosphate ($Na_2HPO_4.2H_2O$)	:	1.15 gm
Potassium dihydrogen phosphate (KH_2PO_4)	:	0.2 gm
Tween-20	:	0.5 ml
DGDW to make 1000 ml		

5- Buffered formalin (10%)

Formaldehyde	:	100 ml
Disodium hydrogen phosphate (anhydrous)	:	6.5 gm
Sodium dihydrogen phosphate ($NaH_2PO_4.H_2O$)	:	4.0 gm
DGDW to make 1000 ml		

VERIFIED
Manjeet Singh
Signature

6- Orthophenylenediamine dihydrochloride (OPD)

One 10 mg OPD tablet (Sigma Chemical Co., USA) was dissolved in 10 ml citric acid phosphate buffer and 10 ul of 3% H_2O_2 was added. Freshly prepared substrate was used.

7- Diaminobenzidine tetrahydrochloride (DAB)

The substrate solution consisted of DAB (5 mg), PBS (pH 7.2) 10 ml and 3% H_2O_2 10 ul. Freshly prepared substrate was used.