AN INVESTIGATION ON
THE AETIOLOGY OF PLAGUE-LIKE DISEASE IN DUCKS IN KERALA

BY

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THESIS

Submitted in partial fulfilment of the requirement for the degree

MASTER OF VETERINARY SCIENCE

Faculty of Veterinary and Animal Sciences
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COLLEGE OF VETERINARY AND ANIMAL SCIENCES
MANNUTHY - TRICHUR

1978

D E C L A R A T I O N

I hereby declare that this thesis entitled "AN INVESTIGATION ON THE AETIOLOGY OF PLAGUE-LIKE DISEASE IN LOCKS IN KERALA" is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship, or other similar title or any other University or Society.

Mannuthy,
29---7---1978.

G. Krishnan Nair.

C E R T I F I C A T E

Certified that this thesis, entitled "AN INVESTIGATION ON THE AETIOLOGY OF PLAGUE-LIKE DISEASE IN DUCKS IN KERALA" is a record of research work done independently by Sri. G. Krishnan Nair, under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to him.

Mannuthy,
29-7-1978.

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A C K N O W L E D G E M E N T S

I wish to record my most sincere gratitude to Dr. (Mrs.) S. Sulochana, Ph.D. (Guelph), Associate Professor, Department of Microbiology, under whose guidance this work was carried out.

I express my sincere thanks to the members of the Advisory Committee, Dr. K.T. Punnoose, Associate Professor, Department of Microbiology, Dr. M. Krishnan Nair, Professor of Pathology and Dr.A. . KochuGovindan Unni, Professor of Poultry Science, for their valuable suggestions and continued interest rendered throughout this study.

I am gratefully indebted to Dr. P.K. Aboulla, Professor of Microbiology, Dr. V. Jayaprakasan, Assistant Professor, Dr. Sebastian Joseph and all the members of the Department of Microbiology, for their esteemed help and assistance.

Grateful acknowledgement is made to Dr. P.J. Nair, Ph.D., Dean, Faculty of Veterinary and Animal Sciences, for the facilities provided for the study.

I am thankful to The Superintendent, Veterinary Biological Institute, Mannuthy and to Dr. A. Jalaludeen, Instructor, University Poultry Farm, Mannuthy, for providing the materials necessary for the study.

My thanks are due to Shri. C.R. Ravindran for typing this manuscript.

The financial assistance in the form of Merit Scholarship and study leave given by the Kerala Agricultural University is gratefully acknowledged.

G. KRISHNAN NAIR.

C O N T E N T S

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I N T R O D U C T I O N

INTRODUCTION

Ducks occupy second place to chicken for the production of table eggs in India. There are about 96 lakhs of ducks, forming about seven percent of the country's total poultry population. They are mainly concentrated in the eastern and southern states. The total duck egg production is about 400 million per year, constituting about five percent of the total egg production in the country. West Bengal leads in duck population, followed by Assam, Tamil Nadu, Andhra Pradesh, Kerala, Bihar, Orissa, Jammu-Kashmir, and Haryana (Bulbulo, 1977).

In India, ducks are mainly raised for production of eggs and excess drakes and spent ducks are used for meat purposes. Rearing of ducks is limited to watershed regions and is very popular among the villagers of these regions as a profitable backyard enterprise, because the average egg production from ducks is higher than that from desi fowls. Even indigenous ducks lay about 90 to 100 eggs per year as against 50 to 55 eggs laid by desi hens. Moreover, eggs from ducks are obtained with practically no cost to the owners (National Commission on Agriculture, 1976).

Duck plague, a viral disease of ducks which brings about heavy economic loss in duck producing areas, was first reported in India by Mukerji et al. (1963). They found that

the highly infectious disease, mainly of adult ducks, which occurred in Gobardanga Government duck farm in West Bengal, was caused by an agent indistinguishable from that of duck plague. Since this first report, duck plague is known to exist in Andhra Pradesh and Tamil Nadu.

In Kerala, during the period from April 1976 to January 1977, heavy mortality in ducks was reported due to a disease which showed characteristic symptoms and lesions of duck plague. The disease outbreak was first observed in Kuttanad area in Alleppey district. The mortality commenced during the middle of April. Then the disease quickly spread to the other districts, causing heavy mortality. As per 1972 livestock census, there were 3.62 lakhs of ducks in Kerala. The Director of Animal Husbandry had assessed the overall duck mortality in the State as between fifty thousand to one lakh (Nair, 1976).

Preliminary studies conducted in the Department of Microbiology, College of Veterinary and Animal Sciences, Kerala Agricultural University, Mannuthy, had suggested that the causative agent of the outbreak might be the virus of duck plague. But no systematic study had so far been carried out to isolate and characterize the aetiological agent. Furthermore, it is being reported by the duck breeders that vaccination with duck plague vaccine does not give the expected protection against the disease.

In the light of the above circumstances it was felt that it would be worthwhile to take up a study on the isolation and characterization of the aetiological agent associated with the disease condition as encountered in the ducks in Kerala, so as to have a better understanding about the probelm, which would help to adopt better control measures.

The investigation was directed towards the isolation of the actual aetiological agent responsible for the outbreak of the duck plague-like disease and characterization of the agent and its ability to reproduce the disease.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Duck plague (D.P.) or duck virus enteritis is an acute, generalized viremic disease of ducks, geese and swans (Leibovitz, 1971a).

History

In the Netherlands, Baudet (1923) reported an unrecognized viral infection of domestic ducks. He considered the disease to be caused by a duck adapted strain of fowl plague virus, which failed to infect chickens. But the disease had the symptoms and lesions similar to that of fowl plague (Leibovitz, 1972). De Zeeuw (1930) also believed that the agent which specifically infected ducks was a duck adapted strain of fowl plague. He found that besides chickens, pigeons and rabbits were also refractory to this virus. He suspected that waterfowl might act as a carrier of this disease.

Bos (1942) reexamined the findings of the above workers and came to the conclusion that this duck disease had a distinct viral aetiology and was not due to fowl plague virus. It was he who coined the term 'duck plague' and differentiated it from Newcastle disease. This was later on confirmed by Jansen and Kunst (1949a; 1949b). Subsequent to this, extensive studies had been made on various aspects of this disease and its aetiological agent.

Incidence and Distribution

Since its first report from the Netherlands, duck plague was suspected to occur in France (Lucam, 1949), Switzerland (Kunst, 1958) and China (Jansen and Kunst, 1964a). The occurrence of this disease in India was recognized by Mukerji et al. (1963) and was first reported from the state of West Bengal. Devos et al. (1964) confirmed the existence of this disease in Belgium. In North America, the first outbreak was reported from a commercial domestic duck flock of Long Island (Leibovitz and Hwang, 1967). Subsequently, it was recognized from various parts of U.S.A. (Urban, 1968; Leibovitz and Hwang, 1968a; Walker et al., 1969; Asplin, 1970; Hall and Summons, 1972; Snyder et al., 1973; Proctor et al., 1975; Hwang et al., 1975; Montali et al., 1976). Leibovitz (1968), Leibovitz and Hwang (1968b), Asplin (1970), Snyder et al. (1973), Proctor et al. (1975) and Montali et al. (1976) have shown that D.P. virus not only affected domestic ducks, but also other wild free-flying waterfowls. The first report of this disease in Canada came from a resident population of small Muscovy ducks (Hanson and Willis, 1976).

The disease appeared to have seasonal influence. In the Netherlands, the incidence was higher during spring (Jansen, 1963). Jansen (1964b) found maximum number of cases from January to July and none from August to December.

Mukerji et al. (1963) and Hwang et al. (1975) reported that the outbreaks occurred from March to June in India and Pennsylvania respectively. However, no marked seasonal influence was observed in the concentrated White Pekin duck producing areas of Long Island, where a higher incidence was seen in the fall of 1967 among the wild, free-flying Anseriformes (Leibovitz, 1971a).

Transmission

Jansen (1963; 1964b) reported that the incidence of D.P. was greater in places where the ducks had access to swimming water and that the disease was rare in farms with drinking water troughs. Duck to duck transmission was believed to occur as a consequence of close contact and by ingestion of infected materials. Farm to farm transmission by free-flying waterfowls had also been recognized (Newcomb, 1968).

New outbreaks usually occurred as a result of movement of infected birds onto virus-free waters. Population densities in concentrated duck producing areas also aided the rapid spread of the disease. Unlike in market ducklings, the infection in breeder ducks was self-limiting as the latter were maintained in a defined area. In market ducklings, the infection was continuous and recycling, as the susceptible birds were moved into the recently contaminated environment (Leibovitz, 1972).

Although the virus had been isolated from the cloaca of infected ducks, there was no evidence that the eggs laid by infected birds could transmit the disease (Jansen, 1964b). Similarly, the possible role of blood-sucking arthropods in disease transmission and the suspected carrier state of wild ducks (De Zeeuw, 1930; Van Dorssen and Kunst, 1955), have not been confirmed (Leibovitz, 1972).

The disease could be experimentally produced by oral, intranasal, intravenous, intraperitoneal, intramuscular or cloacal administration of infectious material (Leibovitz, 1972). Proctor et al. (1976) produced a fatal disease in gnotobiotic ducks by oral administration of D.P. virus.

Hosts affected

The natural susceptibility of D.P. was found to be restricted to the members of the family Anatidae (ducks, geese and swans), of the order Anseriformes. Van Dorssen and Kunst (1955) studied the susceptibility of various species of Anseriformes to D.P. They found that Khaki Campbell, Indian Runner, and Pekin ducks were very susceptible, as were most species of wild ducks, wild geese and mute swans. European Teals, Pintails, Mallard and Grey Call ducks were resistant to fatal infection; but they produced antibodies following exposure. On the other hand, Herring gulls and black headed gulls were not only resistant to infection, but also failed to produce antibodies. The

D.P. virus was also isolated from some cross-breds of common duck and related wild ducks (Jansen, 1964b) and geese (Jansen and Hendrikove, 1965; Leibovitz, 1972). Similar observations were made with wild waterfowl (Leibovitz and Hwang, 1968a; Leibovitz, 1971a) and swan (Proctor et al., 1975).

Although fowls were resistant to D.P., day-old chicks could be infected with the virus (Kunst, 1953; Mukerji et al., 1963). Jansen (1964b) was able to infect chicks upto two weeks of age, after serial passage of the virus in day-old chicks. He also found that adult pigeons, rabbits, guinea pigs, rats and mouse were refractory to the infection.

Characteristics of the disease

Natural infection was usually seen in ages ranging from seven day-old ducklings to mature breeder ducks (Leibovitz, 1971a). In domestic ducks, the incubation period varied from three to seven days and once the symptoms of the disease appeared, death usually followed in one to five days. In some instances, the affected ducks showed evidence of apparent recovery, followed by symptoms of increasing severity before death (Jansen, 1964b).

Except some minor differences, the clinical symptoms described by most of the workers were the same. The early signs of illness were nasal and lachrymal discharges,

drooping wings and profuse greenish-white diarrhoea with marked fouling of the cloaca and the surrounding area. Later on, majority of them showed swollen, sticky eyelids, leading to partial or complete closure of the eyelids (Mukerji et al., 1963; Jansen, 1964b; Hall and Simmons, 1972). Nervous symptoms, characterized by paralysis of the legs and drooping wings with typical posture of the breast touching the ground, were also reported. These nervous symptoms were responsible for the inability of the birds to swim in water (Mukerji et al., 1963; Jansen, 1961; 1964b; Leibovitz, 1971a; Proctor et al., 1975; Montali et al. 1976). It was not unusual that some of the affected birds showed blue beaks and blood-stained vent, preceding sudden death, (Leibovitz, 1971a; Proctor et al., 1975; Hanson and Willis, 1976). In some acute cases, a fully formed egg was found in the cloaca (Jansen, 1964b).

In domestic breeder ducks, the disease did occur, without any premonitory symptoms, with characteristic sudden, high and persistent flock mortality. The affected birds showed a drop in egg production by 25-40 percent (Leibovitz, 1971a). Similarly, Snyder et al. (1973) could not find any clinical signs during an outbreak of D.P. in California except that some birds had depression and diarrhoea prior to death. Absence of any premonitory symptoms was also reported by Montali et al. (1976).

The duration of the disease was variable, usually from one to three days and, in some cases, all the symptoms were not present (Snyder et al., 1973; Montali et al., 1976). Mortality in domestic ducks ranged from 5-100 percent. Adult breeder ducks tended to experience greater percentage of mortality than young ducks (Leibovitz, 1971a). During the 1963 outbreak in West Bengal, Mukerji et al., observed that it was the adult ducks that were mainly affected and that 98 percent of them died in two to four days following the onset of the disease. Stress due to infectious diseases and egg production was attributed to this high mortality in breeder ducks (Newcomb, 1968). Mortality might be higher in cases of dual infection of D.P. virus and latent bacterial infections (Dardiri, 1970). Although newly exposed, susceptible, concentrated populations of wild or domestic waterfowls showed explosive outbreaks with high, persistent mortality and a drop in egg production, chronically infected and partially immune populations of captive or domestic waterfowl showed an insidious, intermittent mortality (Leibovitz, 1975).

Macroscopic observations

In cases where the disease was too acute to cause emaciation, the carcase was usually in good condition (Jansen, 1961; 1964b; Montali et al., 1976). The most striking lesions described were petechiae throughout the

body, particularly on the heart, serous membranes and oesophageal mucosa (Baudet, 1923; Bos, 1942; Jansen, 1961; 1964b; Mukerji et al., 1963; Jansen and Wemmenhove, 1965; Leibovitz, 1971a). The congested ovarian follicles in laying females (Montali et al., 1976) sometimes ruptured, leading to peritonitis (Bos, 1942; Jansen, 1961; 1964b; Mukerji et al., 1963). Presence of a fully formed egg in the oviduct was also not uncommon (Jansen, 1964b). Haemorrhagic enteritis, often with free blood throughout the intestinal lumen, was observed by Jansen (1961, 1964b), Leibovitz (1971a) and Hall and Simmons (1972). During their studies on an outbreak of D.P. in Muscovy ducks, Snyder et al. (1973) found similar symptoms, where the mucosal ulcerations of the small intestine were concentrated throughout the cranial two-thirds of its length. Hanson and Willis (1976) also reported similar ulcerations with pseudomembranes. In an isolated case of D.P. in an immature Canada goose, Leibovitz (1969) observed button-like ulcers on the intestinal lymphoid discs. Occasionally, the lumen of the trachea and bronchi contained free blood (Leibovitz, 1971b).

Depending on the duration of symptoms, diphtheritic changes occurred on the mucosa of the oesophagus, rectum and cloaca (Jansen, 1961; 1964b; Leibovitz, 1971a). The main changes reported in the oesophagus were the presence of patches of diphtheritic membranes (Leibovitz, 1971b),

necrotic membranes (Hall and Simmons, 1972), extensive red ulcerations of the middle third (Snyder et al., 1973; Hanson and Willis, 1976), and haemorrhagic spots arranged in longitudinal rows covered with grey pseudomembranes, sometimes extending to the proventricular-oesophageal junction (Dardiri, 1975; Montali et al., 1976). Ulceration of the proventriculus and red spots on the mucosal surface and blood stained contents in the gizzard were also sometimes observed (Leibovitz, 1971b; Snyder et al., 1973). In caeca, the lesions were confined to the portion between the folds of the mucosal surface (Leibovitz, 1971a) and the rectal lesions were confined to the posterior portion. The densely packed macular lesions of the cloaca often extended into the caudal portion of the colon and salpinx (Leibovitz, 1971a; Hall and Simmons, 1972; Montali et al., 1976).

The lymphoid organs were also affected. The spleen was either normal or smaller in size, dark in colour and mottled (Leibovitz, 1971a; Montali et al., 1976). The thymus and bursa of Fabricius were also involved; the former was characterised by multiple, pin-point haemorrhages and the latter by atrophic changes (Leibovitz, 1971a).

Duck plague virus-infected liver was usually friable (Mukerji et al., 1963; Jansen, 1964b). Leibovitz (1971a) reported that during the early stages of infection, the liver was pale, with irregularly distributed pin-point

haemorrhages and white spots. Later on, the liver became dark bronze or bile stained, without the pin-point haemorrhages, with large and more distinct surface white spots.

The specific response to D.P. virus was found to be dependent on the species affected (Leibovitz, 1971a). Leibovitz and Hwang (1968a; 1968b) have shown that the age, sex, and susceptibility of the host, stage of infection, virulence and intensity of exposure would also affect the lesions produced. In young ducklings, individual lesions of the oesophagus were less frequent, but the sloughing of the entire mucosa, with yellowish-white membrane into the lumen, did occur. Tissue haemorrhages were also less. However, the lymphoid lesions were greater (Leibovitz, 1971a). Cardiac and mesenteric haemorrhages, frequently observed in mature breeder ducks, were also uncommon in young market ducklings (Leibovitz, 1972).

In older ducks, regression of the lymphoid organs such as the thymus and the bursa had already occurred and hence lesions were absent in these organs. In adult domestic ducks and mallards, tissue haemorrhages and changes of reproductive organs were more pronounced (Leibovitz, 1971a).

Duck plague virus

The identity of duck plague virus as a herpes virus was made by Breese and Dardiri (1963), from an electron

microscopic study on thin sections of infected cell cultures. At present this virus is classified as one of the members of the genus herpes virus of the family Herpetoviridae (Western Hemisphere Committee on Animal virus characterization, 1975).

Herpes viruses are large (180-250 nm in diameter), icosahedral, double stranded D.N.A. viruses with an outer envelope. They are sensitive to ether and chloroform and multiply in the nuclei of infected cells. The envelope is acquired when the virus particles are released from the nucleus (Watson, 1973).

Skalinskii and Borisovich (1969) studied the morphology of duck plague virus. They found that D.P. virus was spherical in shape, and 80-220 nm in diameter. It contained an inner core of coiled filament of about 17 nm in diameter, a central hole of 4-5 nm across, and radial projections on the outer membrane. Using filtration technique, Hess and Dardiri (1968) demonstrated that the size of D.P. virus lay between 220 nm and 100 nm.

From its sensitivity to deoxyribonuclease and insensitivity to ribonuclease, Breese and Dardiri (1968) considered the D.P. viral nucleic acid as D.N.A. type. Acridine orange staining of infected cell cultures also showed characteristic nuclear fluorescence indicative of D.N.A. (Hess and Dardiri, 1968).

Physico-chemical Characteristics

Kunst (1968) observed that D.P. virus was inactivated at 56°C. in 30 minutes, but in 30 minutes only 90 percent inactivation occurred when the temperature was brought down to 50°C. However, Hess and Dardiri (1968) reported complete inactivation of the virus in ten minutes at 56°C. and in 90-120 minutes at 50°C. At 22°C. the infectivity was lost only after 30 days. When stored at -20°C. all the activity of the virus was preserved and the virulence remained unaltered for many years, when kept in the freeze-dried form (Jansen, 1964b).

Duck plague virus was stable between pH 7-9, for a period of six hours, but got inactivated at pH below 6 and above 10. At pH 3 and 11, the rate of inactivation was very rapid (Hess and Dardiri, 1968). A total inactivation of the virus was observed by Kunst (1968), in three hours, when exposed to pH 3 and kept at 4°C. He also reported a 90 percent inactivation at pH 10, under the same circumstances. There was a marked difference in the stability of D.P. virus between pH 10 and 10.5 (Hess and Dardiri, 1968).

Propagation

Duck plague virus could best be cultivated on the chorio-allantoic membrane (C.A.M.) of 10-14 day-old embryonated duck eggs (Jansen, 1961; 1964b; Mukerji et al., 1963).

Then death of the embryo occurred in four days, due to extensive haemorrnage. Skalinskii and Borisovich (1969) observed that the virus grew well in the allantoic sac of developing duck and chick embryos. The distribution and concentration of attenuated D.P. virus inoculated through the chorio-allantoic sac of embryonated chicken eggs have shown that C.A.M. and allantoic fluid yielded a better virus harvest. With virulent virus, better yield could be obtained if the inoculation was through the allantoic or yolk sac (Butterfield et al., 1969). Although Skalinskii and Borisovich (1969) have shown that the virus grew readily in the allantoic sac of duck and chick embryos, the latter was unsatisfactory for primary isolation (Jansen, 1961; 1964b). However, it could be adapted to chicken embryos (Mukerji et al., 1965).

Various cell cultures such as duck (Kunst, 1967; Dardiri and Hess, 1968; Dardiri, 1969), chick (Dardiri and Hess, 1968; Dardiri, 1969) and muscovy (Kocan, 1976) embryo fibroblasts and duck embryo fibroblast cell line CC-141 were suggested for the primary isolation and propagation of D.P. virus. However, Hanson and Willis (1976) reported that their attempts to isolate the causative agent of an outbreak of D.P. in Alberta, in embryonated duck eggs and, in primary duck and chicken kidney cells, were unsuccessful.

Following 24-36 hours after infection of CCL-141 cells with D.P. virus, there was marked pyknotic rounding of affected cells, which later on aggregated to form small, grape-like clusters. The foci then enlarged, became necrotic, and sloughed off from the glass surface, leaving small holes or plaques in the monolayer, (Wolf et al., 1974; 1976). By 72 hours, most of the cells had fallen leaving cellular debris, (Wolf et al., 1976).

In stained preparations, there was an increased basophilia which started to appear as early as six hours and increased markedly during the following 36 hours (Wolf et al., 1974). He also reported that the ability to induce marked basophilia in cell cultures was a striking feature of D.P. virus. Hess and Dardiri (1968) and Dardiri (1969) demonstrated eosinophilic, granular, intranuclear inclusion bodies which appeared as early as twelve hours following virus infection.

The plaque forming ability of this virus was employed for its titration by plaque assay and for titrating antisera by plaque reduction test (Dardiri and Hess, 1968). Kocan (1976) recommended muscovy embryo fibroblasts for diagnostic purposes due to their better susceptibility and plaque forming ability. Wolf et al. (1976) made a comparative study on the suitability of primary cells and a line of fibroblast-like cells from the Pekin duck

(CCL-141) for the propagation of D.P. virus. They found that both the systems were equally good for quantifying virus upon isolation. The time taken for the appearance of progeny virions and attainment of peak titres were also the same. However, the primary cells yielded as much as five to six times more virus as did the cell line, although the plaques were more easily discernible in the latter.

Electron microscopy of thin sections of infected cell cultures have shown that the developmental forms which appeared in twelve hours time were seen only in the nucleus. By 24 hours time, in addition to viral forms seen in the nucleus, larger particles with an envelope were also seen in the cytoplasm (Breese and Dardiri, 1963). On the other hand, Leibovitz (1972) observed new cell associated virus, four hours following infection and extracellular forms, two to four hours later.

Haemagglutination

Duck plague virus lacked haemagglutination property and did not agglutinate fowl, duck, sheep, or horse erythrocytes (Mukerji et al., 1963; Jansen, 1961; 1964b). The virus also failed to produce haemadsorption (Dardiri and Hess, 1968). However, Skalinskii and Borisovich (1969)

reported that the vaccine strain of D.P. virus could adsorb onto and agglutinate fowl erythrocytes.

Antigenic character

Although different strains of D.P. virus differed in their virulence, all the strains so far tested had the same antigenic structure and were immunologically identical (Jansen, 1964b; Dardiri and Hess, 1968; Jansen and Kunst, 1967b). Jansen and Kunst (1964a) reported that complete cross immunity existed between Dutch and Indian strains of D.P. virus. Similarly, birds recovered from Dutch strain of D.P. virus were completely immune to American strain and vice versa (Jansen and Kunst, 1967b).

Diagnosis

A tentative diagnosis of D.P. could be made by examination of gross lesions at necropsy and histopathological examination. Virus isolation and identification coupled with neutralization test, making use of a known D.P. virus antiserum, would help to confirm the diagnosis (Leibovitz, 1971a). According to Dardiri and Hess (1967) a neutralization index of 1.75 indicated infection with D.P. These authors also recommended the use of embryo adapted D.P. virus strain for neutralization test, because that was more convenient and safer than the newly isolated

virulent strains. A successful diagnosis of D.P. could also be made by fluorescent antibody staining of infected tissues (Leibovitz, 1975).

Apart from D.P. virus, certain bacteria and fungi have also been isolated from cases of D.P. Snyder et al. (1973) reported the isolation of Escherichia coli and Proteus spp., from heart blood, E. coli from liver, and E. coli, Proteus spp., and Providence spp., from intestinal ulcers. Montali et al. (1976) could isolate Paracolobactrum coliforme and P. intermedium from the heart blood and liver and, Aspergillus fumigatus from air-sacs of ducks with typical lesions of D.P.

Immunity and vaccination

Ducks that survived natural or experimental infection were described to have solid immunity (Jansen, 1964b; Dardiri, 1975). Maternal antibody could protect their offspring only for a short period of one to two weeks (Toth, 1970a).

Artificial immunization against duck plague was practiced both for prevention and control. Jansen (1964b) developed an attenuated D.P. vaccine by serially passaging the virus in developing chick embryos. Mukerji et al. (1965) also observed that after the 25th serial passage in chicken embryos, the virus could be safely used for

active immunization of ducks. Rapid resistance conferred by these vaccines made Jansen (1964a) and Mukerji et al. (1965) to think that its action was by interferon phenomenon. Toth (1968) reported that presence of maternal antibody could interfere with the effectiveness of live virus vaccine, leading to incomplete protection and persistent field infection in vaccinated flocks (Leibovitz, 1971a). These live vaccine strains were not excreted by the vaccinees (Jansen and Kunst, 1964b) and could not be reactivated by serial passage in duck embryos, (Jansen and Kunst, 1967a). But Bhattacharya et al. (1977) have shown that D.P. vaccine strain of much deteriorated embryolethal property could be exalted to a satisfactory level by back passaging it in duck embryos. These workers also found that by this method the property of the seed virus could be improved to confer good protectivity to susceptible ducklings, as revealed by serum neutralization and potency tests.

Studies on the effectiveness of inactivated vaccines have shown that they were not as effective as modified, live virus vaccines. Butterfield and Dardiri (1969) made a comparative study on the immunologic and serologic response of White Pekin ducks to tissue culture modified live attenuated and, inactivated viruses. They found that virus inactivated with 0.05 percent acetylaziridine at

37°C. for six hours, induced serological response and protection as great as the live, attenuated virus. However, when the inactivation was done with 0.4 percent beta propiolactone, it failed to bring about such effects. In another study, Toth (1970a) observed that incomplete inactivation by acetylaziridine gave only less protection than the live virus vaccines, and beta propiolactone-treated virus did not protect the ducks at all.

Jansen and Wemmenhove (1966) observed a lack of positive correlation between the virus neutralizing antibody and the ability of vaccinated birds to withstand challenge. Although only a low percentage of ducks had positive serum titres one year after vaccination with a live virus vaccine, all the vaccinated birds withstood challenge with virulent virus. When modified virus vaccines of duck virus hepatitis and D.P. were given as a combined inoculation, no apparent interference was noticed between the two viruses (Toth, 1970b).

MATERIALS AND METHODS

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1. Specimens for virus isolation

Three different samples were employed during the present investigation.

a) Spleen and liver or spleen alone, from two week-old ducklings experimentally infected with spleen extracts of ducks that had died during the outbreak, and stored at -20°C. in the Department of Microbiology. The viability of these was tested two weeks before the start of the experiment (DPV-M).

b) Spleen extract of an ailing duck brought from Nilambur, from a flock affected with duck plague-like disease. The bird on necropsy showed lesions suggestive of duck plague (DPV-N).

c) Known duck plague virus infected spleen received from Veterinary Biological Institute, Mannuthy (DPV-K).

2. Hank's Balanced salt solution (H.B.S.S. Cunningham, 1966)

The required pH (7.2) was obtained by adding 7.5 percent sodium bicarbonate.

3. Tissue culture growth medium (G.M.)

Hank's balanced salt solution was supplemented with 0.5 percent lactalbumin hydrolysate, 0.2 percent yeast extract, and 10-12 percent calf serum. Antibiotics at the rate of 200 I.U. of penicillin and 200 micrograms of streptomycin per ml along with mycostacin 50 units per ml were also added.

4. Maintenance medium (M.M.)

Same as above except that the serum concentration was brought down to 4-6 percent.

5. Calcium magnesium free buffer (C.M.F-P.B.S.).

Prepared as described by Cunningham (1966).

6. 7.5 percent sodium bicarbonate solution

Prepared as per the method given by Bishai et al. (1974).

7. Citric acid-phosphate buffer (pH 4.7)

Citric acid (0.1 M. solution) 39.8 ml was mixed with 10.2 ml of 0.2 M. solution of dibasic sodium phosphate and diluted to make 100 ml.

8. Phosphate buffer (pH 7.2)

Monobasic sodium phosphate (0.2 M. solution)

28 ml was mixed with 72 ml of 0.2 M. dibasic sodium phosphate and diluted with distilled water to make up the volume to 200 ml.

9. Phosphate buffer (pH 9.1)

To 8.5 ml of phosphate buffered saline, 1.5 ml of 0.1 N sodium hydroxide was added to attain a pH of 9.1.

10. Trypsin

A stock solution of five percent trypsin (1:250 Difco) was prepared in C. M.F-P.B.S. sterilized by filtration through seitz filter pads, distributed in small quantities, and stored at -20°C. When needed, the working solution was prepared by diluting the stock solution with C.M.F-P.B.S. to give a final concentration of 0.25 percent.

11. Antibiotic solution

A stock solution of sodium penicillin and dihydrostreptomycin sulphate, was prepared in sterile distilled water and stored at -20°C. The concentrations of these antibiotics were decided in such a way that when one ml of this mixture was added to 100 ml, a final concentration of 200 I.U. of penicillin and 200 micrograms of streptomycin per ml was obtained.

12. Mycostatin

Stock solution to contain 5,000 units per ml was prepared in sterile distilled water and stored at -20°C.

13. Calf serum

Blood collected from cross-bred bull calves of about one to one and a half years was allowed to clot in a slanting position and refrigerated overnight, for easy separation of serum. The serum thus separated was transferred to a sterile flask, inactivated at 56°C. for half an hour, filtered through seitz filter pads, and stored at -20°C. until used.

14. Duck eggs

These were obtained from a private duck breeder in Trichur.

15. Chicken eggs

Required White Leghorn eggs were obtained from the University Poultry Farm, Mannuthy.

16. Chicken erythrocytes

Blood, collected in Alsever's solution and washed three times in physiological saline, was used in 0.5 percent suspension.

17. Serum samples

a) Sera obtained from birds vaccinated against duck plague, with chicken egg adapted vaccine, and challenged with DPV-K.

b) Sera from birds vaccinated with the above vaccine, and challenged with spleen extracts from field cases.

c) Control sera obtained from uninoculated, normal birds.

18. Experimental ducklings

Ducklings employed in this experiment were obtained from two sources--University Poultry Farm, Mannuthy, and a private duck breeder in Trichur.

Processing of Specimens

The carcase was opened aseptically, and the spleen and liver or spleen alone, were collected in Petri dishes containing H.B.S.S. with two and a half times the concentration of antibiotics used in the G.M. and A.M. (500 I.U. of penicillin and 500 micrograms of streptomycin per ml; 50 microgram per ml of mycostatin) was used (H.B.S.S-1). The tissues were minced properly, transferred to a tissue grinder, and homogenized well. The tissue extract so obtained was further diluted with H.B.S.S-1, to make a

10-15 percent suspension and was incubated at 37°C. for 30 minutes. It was then centrifuged at 500 x g for ten minutes at 4°C. The supernatant so obtained formed the stock inoculum and was stored at -20°C, in small aliquots.

Virus isolation

Virus isolations were attempted in embryonated duck eggs, chicken eggs, and duck embryo fibroblasts (D.E.F.).

a) Duck embryo inoculation

(i) Chorio-allantoic membrane (C.A.M.) method (Betts, 1967).

Eleven to twelve day-old embryonated duck eggs were candled, and the air cell, the embryo, and an area on the side, free from large blood vessels, were located. The air cell region and the spot marked on the side, were sterilized with tincture of iodine. With the help of a dental drill, a hole was made at the spot on the side of the shell, taking care not to puncture the shell membrane. Another hole was made at the centre of the air cell, to penetrate both the shell and shell membrane. A drop of sterile saline was placed on the hole on the side of the egg and the fibres of the shell membrane were gently separated apart. Just below

this hole, an artificial air cell was created, by applying negative pressure with a rubber teat, onto the hole made in the air cell. After checking the false air cell by candling, 0.2 ml of the inoculum was dropped onto the C.A. 4. Both the openings were sealed with melted paraffin. The eggs were gently rotated for a couple of times, to spread the inoculum over the entire C.A.M. under the false air cell. Control eggs were similarly treated except that 0.2 ml of sterile saline was used in place of the inoculum. All the eggs were incubated at 36-37°C. in a horizontal position, with the false air cell upwards. The eggs were candled every morning and evening. Embryos that died before 24 hours were discarded. The embryos that died after 24 hours, and those alive after seven days, were kept at 4°C. before the C.A.M. was harvested.

(ii) Allantoic cavity inoculation (Bishai et al., 1974)

The position of the air cell and the embryo, of 11-12 day embryonated eggs, was marked. After disinfecting the air cell region with tincture of iodine, a hole was drilled at this position, and 0.2 ml of the inoculum was introduced into the allantoic cavity, with the help of a tuberculin

syringe and a 22 gauge needle. The hole was then sealed with melted paraffin, and the eggs were incubated at 36-37°C. in an upright position. Control eggs were also similarly treated with 0.2 ml of sterile normal saline. All the eggs were examined daily. The embryos that died after 24 hours, and those alive after seven days, were transferred to the refrigerator.

b) Chick embryo inoculation

Ten to eleven day-old chick embryos were inoculated, both by C.A.M. and allantoic cavity methods. The procedures adopted for inoculation were the same as that described for duck embryos.

Collection of C.A.M. and allantoic fluid

Eggs, prechilled for a minimum of four hours at 4°C. were disinfected at the air cell region. The shell at this portion was cut, flipped open, and the contents of the eggs were poured out, taking care not to remove the C.A.M. Using sterile forceps, the C.A.M. which was attached to the shell membrane, was separated out; transferred to a Petri dish containing saline, and spread out for examination.

After removing the shell over the air cell, the shell membrane and the C.A.M. were sheared off with a

forceps, and the fluid was collected using a Pasteur pipette. The allantoic fluid thus collected was stored in small vials, at -20°C. for further studies.

c) Duck embryo fibroblast cultures

Twelve to fourteen day-old embryonated duck eggs, after disinfection of the air cell region, were cut open with care, to remove the embryo, leaving all the contents back within the shell. The embryos so removed were transferred to a Petri dish containing C.M.F-P.B.S. with 200 I.U. of penicillin, 200 micrograms of streptomycin and 50 units of mycostatin per ml. The head, limbs and internal organs of the embryos were removed with utmost care. The rest was minced well with scissors, and washed twice with C.M.F-P.B.S., followed by two changes of C.M.F-P.B.S. containing 0.25 percent trypsin. The minced tissues were then transferred to a trypsinization flask. Prewarmed (37°C) 0.25 percent trypsin solution was added at the rate of 25 ml per embryo. A teflon coated magnetic stirring bar was added and stirred on a magnetic stirrer for five minutes. Following this period, the supernatant was poured off, and washed with fresh, prewarmed trypsin to remove any cytotoxic factors if present. Fresh trypsin was again added and stirred for 30-45 minutes.

This dispersed cell suspension was filtered through a double layered sterile muslin cloth. The filtrate was centrifuged at 100 x g for ten minutes, discarded the supernatant, and resuspended in growth medium. The process of washing was repeated twice. At the end, the pelletted cells were resuspended in growth medium, at the rate of 15 ml per embryo.

The above cell suspension was seeded into sterile test tubes at the rate of one ml per tube and incubated at 37°C. A satisfactory monolayer was usually obtained the next day and was used for virus isolation, titration, or neutralization test.

Inoculation

Tubes with satisfactory monolayers were selected, poured off the growth medium, and the cell layer was washed with maintenance medium. To this, the stock inoculum of either DPV-N or DPV-K was added at the rate of 0.2 ml per tube and incubated at 37°C. for one hour, to facilitate adsorption. Following this adsorption period, the inoculum was poured off, washed again with maintenance medium, replaced with one ml of fresh M.M. and incubated at 37°C. Control tubes were similarly treated except that instead of the inoculum, 0.2 ml M.M. was used. At 24 hour intervals, the tubes were examined under the

microscope for the evidence of cytopathic changes (C.P.E.).

The C.P.E. was studied, both in stained and unstained preparations. Coverslip cultures inoculated with the isolates were stained either by Giemsa or haematoxylin-eosin staining technique.

Staining techniques

Coverslip culture tubes selected at various intervals were washed thrice with serum-free H.B.S.S. fixed in methanol for about 24-48 hours, and stained with Giemsa stain for 30 minutes. After this period, the coverslips were washed for a couple of times in distilled water, dried, mounted in D.P.X. and examined under the microscope.

Another set of coverslip cultures were fixed in ten percent formol saline for ten minutes, and stained in Mayer's haematoxylin and 0.5 percent eosin.

Titration of infectivity

Serial, ten-fold dilutions of the isolate were prepared in the maintenance medium. To previously washed monolayer cultures, 0.2 ml of each dilution was added, using three tubes per dilution. After an adsorption period of one hour at 37°C. 0.8 ml of M.M. was added to

make up the volume to one ml and again incubated. The tubes were examined every 24 hours for cytopathic changes. The tissue culture infective dose 50 was calculated after 96 hours, by the method of Reed and Muench (1938).

pH stability

The pH stability of Nilambur isolate (DPV-N) and known duck plague virus (DPV-K), was studied according to the method described by Rasmussen (1969).

Ten percent dilutions of DPV-N and DPV-K were prepared in citric acid-phosphate buffer (pH 4.7), P.B.S. (pH 7.2), and P.B.S. (pH 9.1). These mixtures were kept at room temperature for four hours and their pH rechecked. The samples were then neutralized either with 1 N sodium hydroxide or 1 N hydrochloric acid. The degree of inactivation was determined by inoculation of these samples onto D.E.F. cell cultures, at the rate of 0.2 ml per tube.

Thermostability

One in ten dilutions of the isolates DPV-N and DPV-K were heated at 56°C. for 30 minutes, and rapidly cooled to -20°C. Their infectivity was determined by

inoculation onto D.E.F. cell cultures, at the rate of 0.2 ml per tube.

Chloroform sensitivity

The infected tissue culture fluid was mixed well with chloroform in the ratio 1:05 (5%) and was shaken intermittently for ten minutes. At the end of this period, the mixture was centrifuged at 100 x g for ten minutes to separate the chloroform. The clear supernatant fluid was collected and inoculated onto D.E.F. cell cultures, to determine its infectivity.

Sensitivity to 5-Iodo-2 deoxyuridine (IUdR)

The method described by Nath et al. (1971) was followed in this case. The regular medium of D.E.F. cultures was poured off, the monolayer was washed and replaced with medium containing 100 micrograms per ml of IUdR. Control tubes were also kept with the same medium, but without the drug. All the tubes were incubated at 37°C. for four hours, and then inoculated with 0.2 ml of either DPV-N or DPV-K. A known R.N.A. virus, Newcastle disease virus, was also inoculated in the same manner. The tubes were examined daily for cytopathic changes. Absence of C.P.E. in the presence of the drug, compared to that of control tubes, was taken as an indication of

inhibition of viral multiplication.

Haemagglutination

Two-fold dilutions of the test samples (tissue culture passaged and original samples of DPV-N and DPV-K) were made in physiological saline, in Perspex haemagglutination plates. To 0.5 ml of these dilutions in each well, added 0.5 ml each of 0.5 percent chicken R.B.C. They were mixed well and left at room temperature. Readings were made after the controls had settled, usually after 45 to 60 minutes.

Serum neutralization

Two separate test sera along with one control serum, were tested against both DPV-N and DPV-K strains.

Serial, two-fold dilutions of the sera in maintenance medium, were mixed with equal quantities of either DPV-N or DPV-K samples, each containing 100 $TCID_{50}$ per 0.1 ml. These serum-virus mixtures were incubated at 37°C. for one hour. The residual infectivity of these mixtures was detected in D.E.F. cell cultures, by inoculating three tubes per dilution. The virus control tubes received 100 $TCID_{50}$ of the respective samples. All the tubes were incubated at 37°C. and examined daily for the

evidence of cytopathic changes. Readings were made when the virus control had shown specific, distinct C.P.E.

Gel diffusion test

Agar gel was prepared in double distilled water, with one percent Difco Noble agar and 3.5 percent sodium chloride. The test was carried out on ordinary microscope slides, which were cleaned with methanol and coated with one percent Noble agar in distilled water. The slides were placed on a plain surface and poured two and a half ml of melted agar, with the help of a five ml pipette. After about three to five minutes, the slides were gently transferred to 4°C. for proper setting of agar. After about ten minutes time, wells were cut on the agar, with a diameter of three mm and a diffusion distance of five mm between the antigen and antiserum. For each antigen, three wells were cut, two surrounding a central one. The agar from the wells was removed by vacuum sucking. The central well was filled with either DPV-N or DPV-K (both cell culture passaged and original samples were tested), and the peripheral wells with the two different sera. While filling the well, care was taken not to trap air bubbles at its bottom, so as not to inhibit diffusion of antigen and antiserum into the agar. These

slides were incubated in a moist chamber at 37°C. and examined at intervals, until 96 hours, for the presence of any precipitin lines.

Pathogenicity studies

Duck plague-like virus strain from Nilambur (DPV-N) and known strain of duck plague virus (DPV-K) were examined for their pathogenicity to ducklings aged one to six weeks, in two separate trials (Table VIIa and b).

Experiment-I

A total of twenty ducklings were divided into three groups, groups A and B containing eight each, and group C four. Group A was given strain DPV-N, both by intramuscular and oral routes, 0.5 ml each, while group B received DPV-K at the same dose and through the same routes. The control group C was inoculated with spleen extract from normal duck. The three different groups were housed separately. Strict attention was given not to cross contaminate, while attending the birds.

Experiment-II

Twentyfive ducklings that were employed in this experiment, were divided into three groups-A, B and C. Groups A and B contained ten ducklings each, and group C

contained five. Groups A and B received DPV-N and DPV-K respectively, through the same routes as mentioned previously, but in double dose.

In both the trials, the birds were observed daily for development of duck plague-like disease. All dead and survived birds were necropsied and examined.

R E S U M E S

RESULTS

Virus Isolation

a) Duck embryo inoculation

Eleven to twelve day embryonated duck eggs inoculated with extracts of spleen and liver or spleen alone, from two week old ducklings died of experimental infection with duck plague-like disease, did not show any specific lesions either on the C.A.M. or in the embryo. Although some of the embryos died within five days following infection, no specific lesions could be detected. The allantoic fluid and C.A.M. extracts were also haemagglutination negative. Four blind passages were done with the C.A.M. extracts into fresh, eleven day old embryos, but no specific lesions could be detected.

However, when DPV-N spleen extract was inoculated, the embryos and the C.A.M. were highly congested, five days following inoculation. When extracts of these membranes were inoculated onto duck embryo fibroblast cells, characteristic cytopathic changes were produced (Table I).

All the embryos inoculated by the allantoic cavity route, either with DPV-M or DPV-N, remained normal.

b) Chick embryo inoculation

Ten to eleven day old chick embryos inoculated either by C.A.M. or allantoic route, with the same inoculum as above (DPV-M and DPV-N), did not show any specific lesions or haemagglutination.

c) Use of cell cultures

Primary duck embryo fibroblasts grown in Hank's medium containing 0.5 percent lactalbumin hydrolysate, 0.2 percent yeast extract, and 12 percent calf serum, had the morphological features of fibroblasts. The cells were more or less spindle-shaped, with acidophilic cytoplasm that sometimes contained small vacuoles and few granules. The nuclei were centrally placed, more or less oval in shape, and stained light blue with haematoxylin. They had one or two, sometimes more, nucleoli. A complete, usually dense monolayer was formed within 24 hours (Fig. 1).

In infected cells, the C.P.E. produced by DPV-M and DPV-K, were more or less the same.

In unstained preparations, there was rounding of individual cells, which were distributed at random. As the neighbouring cells were also involved, there were aggregations of the rounded cells (Fig. 2). These

changes were visible, 24-36 hours following infection and first appeared along the periphery of the monolayer. By about 72 to 96 hours, these aggregated cells had sloughed off from the glass surface, leaving holes on the remaining monolayer.

Haematoxylin and eosin stained preparations, of both DPV-N and DPV-K had marked basophilia (Fig. 3). Syncytium formation was less frequent. There was necrosis of the affected rounded cells, which on detachment, left only cellular debris. Heavy cytoplasmic granulation was seen in most cases (Fig. 4). Some of those cells showed both eosinophilic and basophilic structures, with a halo around, in their nucleus and cytoplasm. In some cases, elongation of the cells to form bridges across the holes, were also seen (Fig. 5).

Titration of virus

The virus samples DPV-N and DPV-K, were quantitated on duck embryo fibroblasts. The titres of both the samples were calculated according to the method described by Reed and Muench (1938). The initial titres of these samples were 10^5 and $10^{6.25}$ respectively, for DPV-N and DPV-K. On further passages in this same system, an increase in titre was observed for both, being $10^{7.5}$ and $10^{8.25}$.

Resistance to physical and chemical agents

1. pH stability

The stability of DPV-N and DPV-K strains was tested at three different pH values, namely, pH 4.7, 7.2 and 9.1 (Table II).

The results obtained showed that DPV-N was less susceptible to pH 4.7 than DPV-K, where the titre of the former was 7 or more, while that of the latter was reduced to below 5. Complete inactivation of both the samples was observed, when they were exposed to pH 9.1 for a period of four hours. However, both the strains were unaffected at pH 7.2 (Table II and Fig. 6). The pH sensitivity was evidenced from the failure of these samples to produce specific C.P.E. in D.E.F. cultures, compared to untreated control samples.

2. Thermostability

Although a marked reduction in titer was noticed, there was no complete inactivation when a 1:10 dilution of tissue culture passaged DPV-N was subjected to 56°C. for 30 minutes. Under the same circumstances, DPV-K was completely inactivated (Table III).

3. Chloroform sensitivity

Sensitivity to lipid solvents and thereby the presence of an envelope, of these two strains, was studied by exposing them to five percent chloroform for a period of ten minutes. The results shown in table IV indicate that there was complete inactivation of both the strains. All the D.E.F. cell cultures inoculated with chloroform treated samples, remained as normal monolayers, while, untreated viral samples produced C.P.E.

4. Nucleic acid type

Pretreatment of D.E.F. cell cultures with 100 micrograms of 5-iodo-2-deoxyuridine (IUdR) inhibited the replication of both DPV-N and DPV-K. Such an inhibitory effect was not observed when they were grown in untreated control cells. A known R.N.A. virus--Newcastle disease virus--inoculated into IUdR treated cells, multiplied as it was in untreated cells, indicating that the isolate DPV-N, like duck plague virus DPV-K, also contained D.N.A. as its nucleic acid (Table IV).

Haemagglutination

There was no haemagglutination when DPV-N and DPV-K, either in tissue culture passaged or original form,

were tested with 0.5 percent chicken erythrocytes in normal saline (Table V).

Serum neutralization

The readings of the serum neutralization tests were made when the virus control tubes had shown marked C.P.E.--usually 96 hours following infection. Neutralization was indicated by the absence of C.P.E. in tubes inoculated with virus-serum mixtures.

Neutralization test with serum-a (collected from ducks vaccinated with chicken embryo adapted D.P. vaccine and then challenged with DPV-K), and DPV-N, had a serum titre of 22. (The antiserum titers were expressed as the reciprocals of the serum dilutions, representing the 50 percent end points against 100 $TCID_{50}$ of virus). On the other hand, when the serum was tested against DPV-K, the titer was 45 (Table VI).

On treatment of DPV-N and DPV-K with serum-b (collected from ducklings vaccinated with D.P. live virus vaccine, and challenged with spleen extract from field cases), the serum titers were 11 and 45, respectively (Table VI).

Gel diffusion test

Gel diffusion test in one percent Noble agar,

employing sera obtained from vaccinated ducks that resisted challenge with DPV-K, did not produce any precipitin reaction when tested against either DPV-N or DPV-K.

Experimental infection

Experiment-I

This experiment was designed to determine whether the materials received in the laboratory did contain viable agent that could produce disease with symptoms and lesions similar to those produced in the field. The symptoms and lesions produced by the field strain DPV-N, was compared with those produced by a known duck plague virus strain, DPV-K.

Group A All the eight ducklings of this group were given the original inoculum of DPV-N, both by oral and intramuscular routes, at the rate of 0.5 ml per each route (Table VIIa). Unlike the controls (Fig. 7) all the eight inoculated birds became sluggish from 24 hours onwards. They showed weakness of the limbs, drooping wings, and greenish diarrhoea, with soiling of the cloacal region. The birds preferred to sit with their breast touching the ground (Fig. 8). When forced to move, they did so only with hesitation. Within 96 hours, all except one duckling had died. The duckling that survived day five, was sacrificed.

On post-mortem examination, one of the ducklings had characteristic intestinal petechiation. Keratinization of gizzard mucosa was seen in most cases. The liver had mottled appearance. Petechiation of the liver, sometimes with white streaks or white spots, were also observed. In some birds, it was dark red in colour, or had dark red patches. The spleen also had dark-reddish patches.

Virus isolation trials, from spleen extracts of some of these birds, were successful.

Group B

The eight ducklings in this group had received the original inoculum of DPV-K, at the same dose and by the same routes, as in group A. They showed more or less similar symptoms and lesions as shown by birds in the above group. The symptoms were seen from 24 hours after inoculation. Sluggishness, weakness of the limbs, drooping wings and greenish diarrhoea, were characteristic (Fig. 9). In this case also the birds preferred to sit with their breast touching the ground. All the birds died within 48 to 96 hours.

At necropsy, the lesions seen were similar to those of birds in group A. Here also, keratinization of gizzard mucosa was seen, along with mottling of liver and

dark red spleen (Fig. 10).

Group C

Birds in this group were inoculated with normal duck spleen extract. One of them died by fifth day. This dead one and the remaining live birds on necropsy, did not show any specific lesions.

Experiment II

The infectivity of the strains after passage in coll cultures, was investigated. Here again the birds were grouped into three--A, B and C, where A and B received strains DPV-N and DPV-K respectively. The third group that received only uninfected tissue culture medium, formed the control. The dose and routes of inoculation were as given in table VIfb.

Group A

Of this group of ten birds that had received DPV-N, nine died within five days, while one survived day five. Among those that died within five days, three did not show any marked symptoms; the other six showed symptoms such as greenish diarrhoea and weakness of the limbs, followed by death. The one duckling that had survived, remained symptomless upto day 14th, when it developed greenish diarrhoea and weakness of limbs, and died by day 17th.

At necropsy, mostly the liver was dark red in colour, with faint grey patches. One of the birds had ulcers in the gizzard mucosa. Another bird showed slightly raised, irregular shaped white plaques on the anterior two-thirds of oesophageal mucosa. The duckling that was sacrificed on day 17th showed mottling of the liver and whitish foci on the gizzard musculature (Fig. 11).

Group B

Birds of this group started to show symptoms of the disease, two days following infection. They became inactive and began to show greenish diarrhoea. By day five, all except two had died. The two ducklings that had survived, continued to be normal upto day 13th, when symptoms such as greenish diarrhoea and weakness of the limbs developed, followed by death on day 15th.

On post-mortem examination, there were white streaks, and slight petechiation on the right lobe of the liver. In some birds, the liver was pale and the heart showed pin-point haemorrhages on the myocardium. Ulcers were present on the mucosa of the gizzard. No other specific lesions were observed in rest of the organs.

Group C

Of the five control birds, one died by day five, showing slight petechiation on liver. No specific lesions

were observed in rest of the organs. All the other controls that survived until day 17th, did not show any specific lesions, when they were sacrificed.

DISCUSSION

DISCUSSION

The highly contagious disease of ducks that occurred in Kerala, from April 1976 to January 1977, was suspected to have some viral aetiology, because vaccines against bacterial organisms such as Pasteurella Spp. isolated from these cases did not protect the birds from contracting the disease. Moreover, the affected birds had the symptoms and lesions suggestive of duck plague and the birds vaccinated with D.P. vaccine resisted challenge with spleen extract from field cases, which killed unprotected ducklings. In addition, experimentally infected chickens failed to produce any disease, (Nair, 1976). A specific viral aetiology for disease conditions in domestic ducks and other waterfowls with the symptoms and lesions similar to that observed during the 1976-'77 outbreak was described by Baudet (1923); DeZeeuw (1930) and Bos (1942). Bos (1942) described a distinct viral aetiology for these conditions and named the disease 'duck plague' and the agent, duck plague virus. He also demonstrated that this agent was antigenically distinct from Newcastle disease virus. Jansen (1961; 1964b) and Mukerji et al. (1963) found that the agent responsible for these conditions in ducks could best be isolated from

the spleen and liver extracts of affected birds, by inoculation onto the C.A.M. of 10-14 day old embryonated duck eggs. They found that the infected embryos would die with characteristic lesions such as extensive haemorrhage, 4-10 days following inoculation.

Duck or chick embryo inoculation

During the present investigation, virus isolation trials were made by inoculating spleen extracts from field cases (DPV-N) onto the C.A.M. and allantoic cavity of both duck and chicken embryos. Embryos which received DPV-N by the C.A.M. route died, showing severed congestion. Although Skalinskii and Borisovich (1969) were successful in propagating D.P. virus in the allantoic sac of both duck and chick embryos, our attempts to propagate the virus by these procedures were unsuccessful. Jansen (1961) and Mukerji et al. (1963) also found that chick embryo was unsuitable for primary isolation. The replication of the virus on the C.A.M. of duck embryos was confirmed by inoculation into the D.E.F. cell cultures.

A reduction in viability, or insufficient concentration of the agent, was suspected for the failure of DPV-M to produce death with specific lesions. DPV-M for C.A.M. inoculation was prepared from a dead duckling kept

in the frozen state at -20°C. The frequent power failures that occurred during this period might have affected the viability of the agent. Although four blind passages were done, it could not either revive the agent or increase its number to a level sufficient enough to produce specific lesions and death of the embryo. Dardiri and Breese (1974) found that suspensions of tissues from ducks suspected to have died of D.P. did not produce any specific lesions or mortality in inoculated duck embryos. These suspensions also failed to produce any death in week old ducklings. However, birds developed antibodies to D.P.V. 21 days after inoculation. These reports by Dardiri and Breese (1974) indicate that specimens containing viable D.P. virus need not always produce lesions or death either in embryos or in the susceptible ducklings. Hanson and Willis (1976) also reported that all their attempts to isolate the causative agent during an outbreak of D.P. in Alberta, in embryoneted duck eggs and primary cultures of duck and chicken kidney, were unsuccessful.

Cytopathic effects

Viruses of the same group usually produce more or less similar changes in the same kind of cells and sometimes even in different cell types (Hoskins, 1967). Wolf et al. (1976) described the cytopathic changes of D.P.

virus as pyknotic rounding of the affected cells and its subsequent aggregation to form small, grape-like clusters. The C.P.E. observed in duck embryo fibroblasts infected with either DPV-N or DPV-K were also similar to those described by Wolf et al. (1976). The changes described by Wolf et al. (1976) and those observed during the present investigation, were the characteristic changes produced by herpes viruses in cell cultures (Jennings, 1967; Darlington and Granoff, 1973) suggesting that, like D.P. virus, the isolate DPV-N could also be a herpes virus.

Marked basophilia of the cytoplasm was one of the characteristics observed in haematoxylin-eosin stained preparations. Wolf et al. (1974) also reported the same observation and suggested that the ability to induce marked basophilia in infected cell cultures was a striking feature of D.P. virus. There was also a marked difference in the granularity of the cytoplasm of control uninfected cells and cells infected with DPV-N or DPV-K. This extreme granularity due to the coagulation of cell colloids (Jennings, 1967) might have resulted from some toxic factors. The possibility of serum factors acting as a toxic substance was ruled out by using various batches of serum at various concentrations. Moreover, no such effect was noticed when the same sera were used for growing cell cultures such as foetal bovine kidney, foetal ovine kidney or chicken embryo

fibroblasts, which were infected with materials suspected for parainfluenza-3 and New castle disease virus (Subbchana, 1977). Hence this increased granularity is thought to be due to the after effect of an interaction between D.E.F. cells and viruses of DPV-N or DPV-K. Hess and Dardiri (1968) and Dardiri (1969) demonstrated eosinophilic granular inclusions in D.P. virus infected chicken and duck embryo cell cultures, as early as 12 hours following infection. These inclusion bodies described for D.P. virus in chicken embryo and duck embryo cell cultures were not detected during the present study. This could probably be due to the failure to select infected cell cultures at shorter intervals. However, inclusion body-like structures were seen in the nucleus of some of the infected cells, although, its identity was not confirmed. No mention about the inclusion bodies was also made by Wolf et al. (1976), in their comparative study on the suitability of primary D.E.F. cells and a duck fibroblast cell line CCL-141 in the propagation of D.P. virus.

Virus titration

During the course of this present investigation, the field isolate DPV-N and the known duck plague virus DPV-K were titrated at various intervals. Early titrations, done following two tissue culture passages, showed tissue

culture infective dose 50 as 10^5 and $10^{6.25}$ for DPV-N and DPV-K respectively. On further passages, an increase in titer was observed with both the strains. This increase in titer could probably be due to the adaptation of the new isolate to D.E.F. cell cultures. The low titers of DPV-K could be attributed to its long term preservation at -20°C.

pH stability

Sensitivity to pH is one of the criteria that could be employed for grouping a newly isolated virus into a particular group. Rapid inactivation of D.P. virus at pH 3 and 11 was reported by Hess and Dardiri (1968). Hunst (1968) also found a total inactivation of this virus when it was exposed to pH 3 for a period of 3 hours at 4°C.

The results presented here on the effect of pH 4.7, 7.2 and 9.1 on DPV-N and DPV-K have shown that the former was unaffected at pH 4.7 and 7.2, but was completely inactivated at pH 9.1. Strain DPV-K differed from DPV-N in that a marked reduction in its titer by about 3 log was observed when it was exposed to pH 4.7 for a period of 4 hours at room temperature. However, pH 7.2 and 9.1 had the same effect on both the strains. Although the results showed that DPV-N behaved more or less like DPV-K and other

strains of duck plague virus (Leibovitz, 1971a), the former was more resistant to pH 4.7, compared to the latter.

Thermostability

Complete inactivation of D.P. virus at 56°C. was reported to occur in ten minutes. This observation of Hess and Dardiri (1968) differed from that of Kunst (1968) in that the latter found that it was necessary to subject the virus to 56°C. for 30 minutes, for its complete inactivation. When a 1:10 dilution of tissue culture passaged samples of DPV-N and DPV-K were exposed to 56°C. for 30 minutes, it was only the DPV-K that was completely inactivated, while there was only a marked reduction in titer of DPV-N.

The nature of the suspending medium was reported to have a considerable influence on the susceptibility of viruses to temperature. Presence of extraneous substances such as proteins could protect them from the effect of temperature. Since the virus suspension used in this study was in the form of tissue culture fluid containing 6 percent calf serum, it was difficult to compare the heat inactivation studies reported by other workers, because there was no description on the nature of virus suspension used in their studies.

Chloroform sensitivity

Chloroform sensitivity of an unknown virus is usually done to find out whether it is enveloped or not. Envelopes, being derived from the host cell membrane, are lipoprotein in nature and are sensitive to lipid solvents such as ether and chloroform, where their infectivity is either completely lost or greatly reduced. Herpes viruses, containing considerable amount of lipids in their envelope, are sensitive to chloroform (Roizman and Roane, 1963).

The chloroform sensitivity of D.P. virus had already been demonstrated by Kunst (1968) and Hess and Dardiri (1968). The complete inactivation of DPV-N and DPV-K when treated with five percent chloroform indicate that like DPV-K, DPV-N is also an enveloped virus.

The chloroform was preferred to ether because of its polarity as a lipid solvent. It is also more heavier than ether and can be easily separated by centrifugation. Moreover, chloroform is as effective as ether (Feldman and Wang, 1961).

Nucleic acid type

Certain halogenated deoxyuridines such as 5 iodo-2 deoxyuridine, could inhibit the replication of D.N.A. viruses, due to their effect on D.N.A. synthesis (Prusoff, 1972). But they do not have any effect on the

multiplication of R.N.A. viruses. Kunst (1968) found that the replication of D.P. virus and the development of C.P.E. could be inhibited by IUdR. Unlike in control untreated cells, DPV-N and DPV-K failed to produce any C.P.E. in cells treated with IUdR at the rate of 100 micrograms/ml. However, no effect on the multiplication of a known R.N.A. virus, Newcastle disease virus, was observed when it was grown in drug treated cells. From these observations, it was concluded that like D.P. virus, DPV-N is also a herpes virus.

Haemagglutination

Similar to the observations made by Mukerji et al. (1963) and Jansen (1961; 1964b), the field isolate DPV-N and the known D.P. virus DPV-K, failed to produce any haemagglutination reaction, when tested with 0.5 percent chicken R.B.C. at room temperature.

Serum neutralization

Leibovitz (1975) recommended serum neutralization test for the serological identification of D.P. virus. Sera from birds that were vaccinated with D.P. vaccine and that had resisted challenge with virulent D.P. virus DPV-K, not only neutralized the homologous strain, but also the field isolate DPV-N. However, the antibody titer

to DPV-N was only 50% of that to the homologous strain. Sera from birds vaccinated with the above vaccine and resisted challenge with the field isolate, also neutralized both DPV-K and DPV-N. But the antibody titer to DPV-N was only 25% of that to DPV-K. Although the neutralization test indicate an antigenic relationship between DPV-N and DPV-K, the difference in serum titers suggest an antigenic difference between these two strains. If this is so, the reports of Jansen and Kunst (1964a; 1967b) that the strains of D.P. virus isolated from various parts of the world are immunologically identical, have to be reevaluated.

The tentative diagnosis made prior to the start of this experiment and results presented in this thesis suggest that DPV-N is indistinguishable from duck plague virus. However, there are often complaints from the duck breeders that the duck plague vaccine now used in the field does not give an expected protection against the so called duck plague-like disease and that they do get the disease in their vaccinated flock, but to a reduced rate. This indicate that the D.P. vaccine is not giving complete protection. This could probably be due to the possible antigenic variations as suggested from the serum

neutralization studies. If it is so, it is a contradiction to the observations made by Jansen (1968) and Daraini and Hess (1960).

Although the results presented here were reproducible, detailed cross-neutralization and cross protection tests have to be made before any definite conclusions are made.

The beta procedure, employing constant virus varying serum concentration, was preferred because this procedure requires only small amounts of serum and can be tested with low titered virus. Moreover, it is also easier to compare sera with low titers (Hitchner, et al., 1975).

Gel diffusion

Although cell culture extracts of avian herpes viruses such as Marek's disease virus and turkey herpes virus were reported to produce precipitin lines in gel precipitation tests, no such observations could be made when duck plague antiserum was tested against DPV-K or DPV-N. The different antigens of Marek's disease virus that bring about such a reaction are, the soluble supernatent antigen; cellular antigen and skin antigen. (Bulow and Biggs, 1975). During this present study it

was only the infected tissue culture fluid without concentration that was used as the antigen. The absence of any precipitin lines either with DPV-K or DPV-N could probably be due to the absence of any soluble antigens or its lack or sufficient concentration. So far there is no report on the use of agar gel precipitation test with D.P. virus.

Experimental infection

Ducklings about the age of 1-6 weeks injected with spleen extracts of either DPV-N or DPV-K, showed some of the typical symptoms of D.P. after an incubation period of 24-48 hours, followed by death in 48-72 hours. In domestic ducks, Jansen (1964b) reported an incubation period of 3-7 days and death in 1-5 days, following the onset of symptoms. The symptoms described by Mukerji et al. (1963), Jansen (1961; 1964b), Hall and Simmons (1972), Leibovitz (1971a) and Proctor et al. (1975), such as profuse greenish diarrhoea, with marked soiling of the cloaca and the surrounding region and nervous symptoms characterized by weakness of the legs and drooping wings with typical posture of the breast touching the ground, were observed during the present experimental infection studies. The ducks that died during the outbreak in Kerala were also described to have the same symptoms

(Nair, 1976).

The survival of a few birds in group A and B beyond day five, might be due to the presence of maternal antibodies, as reported by Toth (1968), where he found that ducks with high titers of neutralizing antibody could provide passive protection to their offspring, from infection with virulent virus for a period of two weeks.

The lesions of D.P. described by various workers such as petechiation throughout the body (Baudet, 1923; Bos, 1942; Jansen, 1961; 1964b; Mukerji et al., 1963; Jansen and Wemmenhove, 1965; Leibovitz, 1971a; Hall and Simmons, 1972), were also reported among birds that died during the 1976-'77 outbreak in Kerala (Nair, 1976). Although experimental infection of ducklings did not show all the symptoms in all the birds, some of the birds did show petechiae in the liver and heart musculature. Except in one case, there was no petechial haemorrhage and ulcers in the intestine. The diphtheritic changes on the oesophageal mucosa, described by Jansen (1961; 1964b) and Leibovitz (1971a), were present only in birds infected with DPV-N. Moreover, lesions which specifically appeared during the 1976-'77 outbreak in Kerala, such as thickening of the gizzard mucosa, sometimes with necrosis of the

gizzard musculature, were also a feature in some of the ducklings infected with DPV-N.

Leibovitz (1971a) and Leibovitz and Hwang (1968a; 1968b) reported the influence of species, age, sex, susceptibility and stage of infection on the lesions produced by D.p. virus. Leibovitz (1971a) found that although in young ducklings individual lesions of the oesophagus were less frequent, sloughing of the entire mucosa was not uncommon. It was also reported by him that tissue haemorrhages were less in young birds and in those below five weeks of age, cardiac and mesentric haemorrhages were uncommon. This would probably explain why such symptoms were rare in the experimental birds, which were only 1-6 weeks of age.

The propagation of DPV-N and DPV-K in D.E.F. cell cultures was confirmed by infecting ducklings, with the infected tissue culture fluid after freezing and thawing it for three times. All the birds that received either DPV-N or DPV-K, died with typical symptoms of D.P. except three from group B. Although these three birds died without showing symptoms of D.P. they had the lesions suggesting the acute nature of the disease. Moreover, it is not necessary that in all cases of D.P. all the symptoms need be present (Snyder et al., 1973; Montali et al., 1976).

DPV-N and DPV-K

From the observations made during the present investigation, DPV-N is found to be indistinguishable from the known duck plague virus DPV-K in various characteristics (Table V). Both produced similar C.P.E. in D.E.F. cell cultures, were inactivated with chloroform, multiplication was inhibited by IUdR and produced similar symptoms and lesions in experimentally infected ducklings. In addition, DPV-N was neutralized with antiserum to DPV-K.

Leibovitz (1971a) reported that an isolate which failed to multiply in chick embryos, but did so on the C.A.M. of 10-14 day old duck embryos; produced characteristic C.P.E. in D.E.F. cell cultures; got inactivated with chloroform; the multiplication of which was inhibited with IUdR; and produced characteristic symptoms and lesions of duck plague in experimental ducklings, could be identified as duck plague virus. Since DPV-N fullfils these requirements, it strongly suggests that DPV-N is duck plague virus.

The differences observed with pH and thermostability in comparison with DPV-K and the low serum titers of DPV-N, might be due to some strain variation. If this is so, it will explain the incomplete protection afforded

by the D.P. vaccine now used in the iield. This coula be overcome if vaccines are prepared from the field isolate.

Besides vaccination, other measures sucn as restriction of the movement of infectec birds and keeping the unaffected susceptible population away from the contaminated environment, will help to attain complete protection.

SUMMARY

SUMMARY

In Kerala, during the period from April 1976 to January 1977, there occurred heavy mortality in ducks due to a disease with characteristic symptoms and lesions of duck plague (D.P.). The following is a summary of the results of an investigation carried out on the isolation and characterization of the aetiological agent.

Three different samples--from a duckling died of experimental infection and kept frozen at -20°C. (DPV-M); from an ailing adult duck brought from an affected flock in Nilambur (DPV-N) and a known virulent duck plague virus strain (DPV-K) received from the Veterinary Biological Institute, Mannuthy, were included in the present study.

Attempts for virus isolation were made in 10-14 day-old duck embryos-either by the chorio-allantoic membrane (C.A.M.) or allantoic route of inoculation-and in 10-11 day-old chicken embryos, employing the same routes of inoculation. Only duck embryos inoculated by the C.A.M. route showed signs of virus multiplication, as evidenced by the death of the embryo with typical haemorrhagic lesions.

In duck embryo fibroblast (D.E.F.) cell cultures, DPV-N and DPV-K produced similar changes, characteristic of those described for duck plague virus. The important changes observed in the infected monolayers were the rounding and clumping of cells, marked basophilia, cytoplasmic granulations and later on, destruction and sloughing of the monolayer. On titration in this system, although the initial titers of both DPV-N and DPV-K were only 10^5 and $10^{6.25}$ respectively, on later passages it was found to increase and reached upto $10^{7.5}$ and $10^{8.25}$.

Studies on the physico-chemical characteristics also suggested the close resemblance of DPV-N to the duck plague virus DPV-K. Observations were made with chloroform sensitivity, inhibition with 5-iodo-2 deoxyuridine and haemagglutination. They were completely inactivated with 5% chloroform: multiplication was inhibited with 100 micrograms of IUdR and they did not show any haemagglutination reaction with 0.5% chicken R.B.C.

However, differences were observed between the field isolate, DPV-N and known duck plague virus DPV-K. On exposure to 56°C. for 30 minutes, a marked reduction in titer was only seen with DPV-N, while DPV-K was completely inactivated. Similarly, although the effect of pH 7.2 and 9.1 on both the strains was the same, DPV-N was

more resistant to pH 4.7 than DPV-K.

Neutralization tests employing sera, from birds vaccinated with D.P. vaccine, and resisted challenge with either DPV-K (a) or spleen extract from field cases (b) have shown that although both the strains DPV-N and DPV-K were neutralized, the serum titers obtained with DPV-N was about 50% to 25% of that shown by DPV-K.

Attempts to detect any soluble antigens by gel precipitation test were not successful.

Experimental infection of 1-6 week-old ducklings, either with the spleen extract or tissue culture passaged materials of DPV-N and DPV-K, procuced similar symptoms and lesions, that were characteristic of duck plague and those described for the duck plague-like disease outbreak in Kerala. From these observations, it is strongly suggested that the outbreak of a duck plague-like disease which occurred in Kerala during April 1976 to January 1977, was due to a virus indistinguishable from that of duck plague.

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ГЛАВА 3

Table I. *In vitro* propagation of field isolates DPV-M; DPV-N and known duck plague virus DPV-K.

Systems used	DPV-M	DPV-N	DPV-K
DE-CAM[1]	-	+	NT
DD-A[2]	-	-	NT
CE-CAM[3]	-	-	NT
CD-A[4]	-	-	NT
DDb[5]	+	+	+

1. Duck embryo - chorioallantoic membrane; 2. Duck embryo - allantoic cavity; 3. Chick embryo - chorioallantoic membrane; 4. Chick embryo - allantoic cavity; 5. Duck embryo fibroblasts.

+ = Positive; - = Negative; NT = Not tested.

Table II. Effect of pH on the viability of field isolate DPV-N and known duck plague virus DPV-K.

pH	DPV-N	DPV-K
4.7	7.25*	<5
7.2	7.50	8
9.1	--	--
Control	7.5	8.25

* Log $TCID_{50}$/0.2 ml. -- = Complete inactivation

Table III. Effect of temperature on the viability of field isolate DPV-N and known duck plague virus strain DPV-K.

Treatment	DPV-N	DPV-K
56°C for 30 minutes	5*	-
Untreated	5.5	6.25

* $\log TCID_{50}/0.2$ ml

- = Complete inactivation.

Table IV. Chloroform and IUdR sensitivity of field isolate DPV-N and known duck plague virus DPV-K.

Treatment	DPV-N	DPV-K
5% Chloroform	-	-
100 micrograms of IUdR	-	-
Untreated control	7.25*	8

* Log $TCID_{50}$/0.2 ml

\- = Complete inactivation.

Table V. *In vitro* characteristics of field isolate DPV-N and known duck plague virus DPV-K.

Characteristics	DPV-N	DPV-K
Thermostability (56°C. for 30 minutes)	No complete inactivation	Complete inactivation
Sensitivity to pH		
pH 4.7	No reduction in titer	A reduction in titer by 3 log.
pH 7.2	Unaffected	Unaffected
pH 9.1	Complete inactivation	Complete inactivation
Chloroform sensitivity	Sensitive	Sensitive
Haemagglutination	Negative	Negative
Type of nucleic acid (Based on the effect of IUdR on multiplication)	DNA	DNA

Table VI. Serologic comparison of field isolate DPV-N and known duck plague virus DPV-K.

Virus (100 $TCID_{50}$)	Sera from birds vaccinated and challenged with		Control Serum
	DPV-M	DPV-K	
DPV-N	22*	11	-
DPV-K	45	45	-

* Antiserum titers were expressed as the reciprocals of the serum dilutions, representing the 50% end points against 100 $TCID_{50}$.

- = Negative.

Table VIIa. Pathogenicity of spleen extracts of DPV-N and DPV-K.

Virus	No. of birds inoculated	Route and dose of inoculation	Incubation period	No. of birds died	No. with clinical disease	No. with D.P. lesions	Symptoms & lesions
Group-A (DPV-N)	8	I/M* and oral 0.5 ml each	24-48 hours	7	8	8	Sluggishness, weakness of limbs, greenish diarrhoea, petechiation and white spots on liver
Group-B (DPV-K)	8	"	"	8	8	8	Weakness of limbs, greenish diarrhoea, mottled liver and dark red spleen
Group-C (Control)	4	I/M and oral 0.5 ml each (normal spleen extract)	-	1	-	-	-

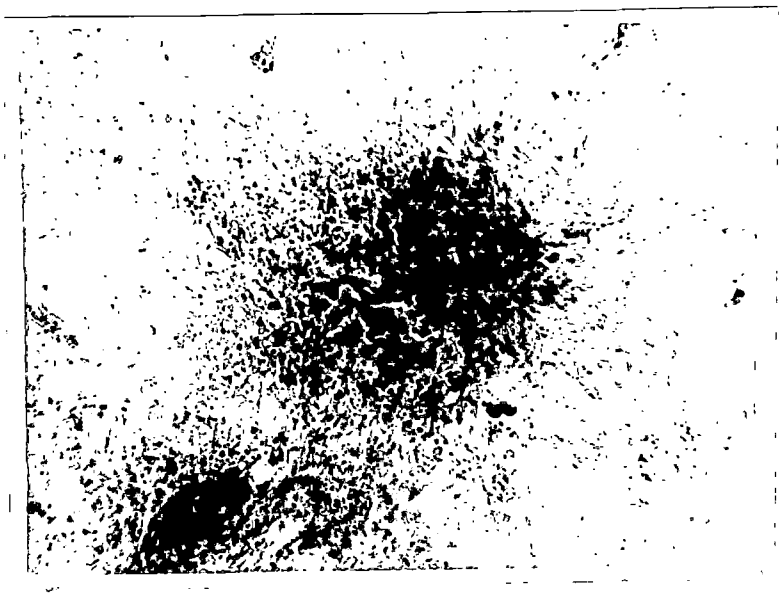
* Intramuscular.

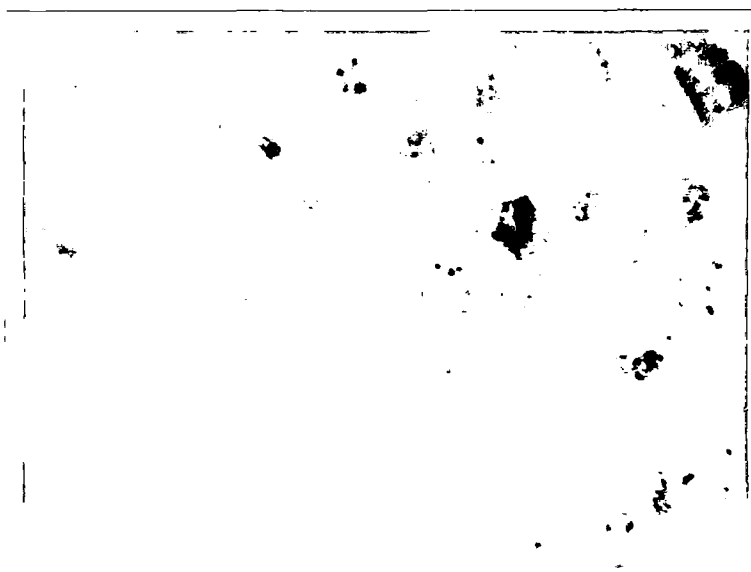
Table VIIb. Pathogenicity of duck embryo fibroblast passaged DPV-N and DPV-K.

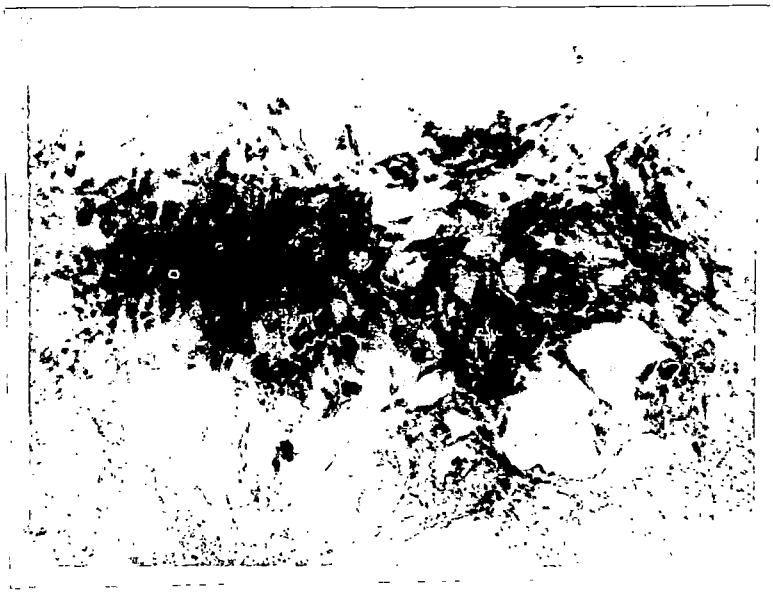
Virus	No. of birds inoculated	Route and dose of inoculation	Incubation period	No. of birds died	No. with clinical disease	No. with D.P. lesions	Symptoms & lesions
Group-A (DPV-N)	10	I/M* and oral, 1 ml each	24-48 hours	10 (nine birds died by day five and the remaining one by day 17).	7	10	Weekness of limbs, greenish diarrhoea, dark red liver with faint grey patches, plaques in anterior 2/3 of oesophageal mucosa, whitish patch on gizzard musculature.
Group-B (DPV-K)	10	I/M and oral, 1 ml each	24-48 hours	10 (eight birds died by day five and the other two by day 15).	10	10	Weakness of limbs, greenish diarrhoea, petechiation on liver and, pin-point haemorrhages on myocardium
Group-C (Control)	5	I/M and oral, 1 ml each (uninfected tissue culture fluid)	-	1	-	-	-

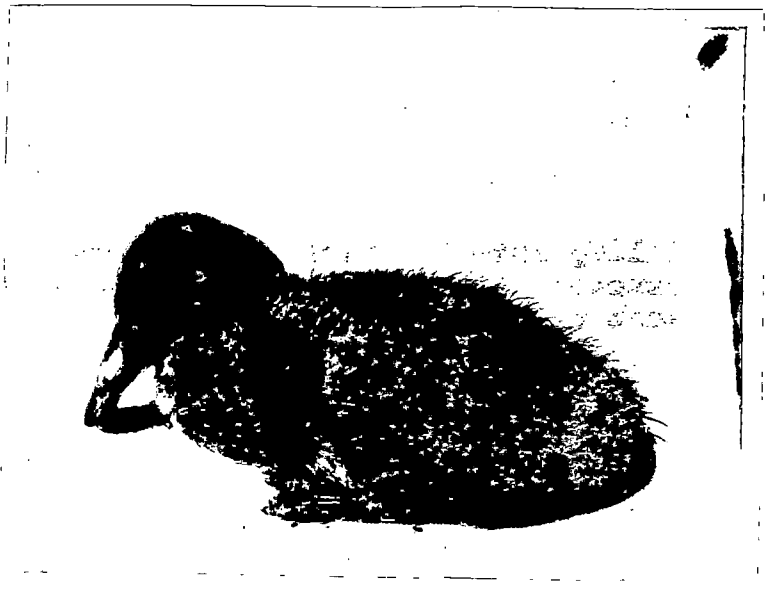
* Intramuscular.

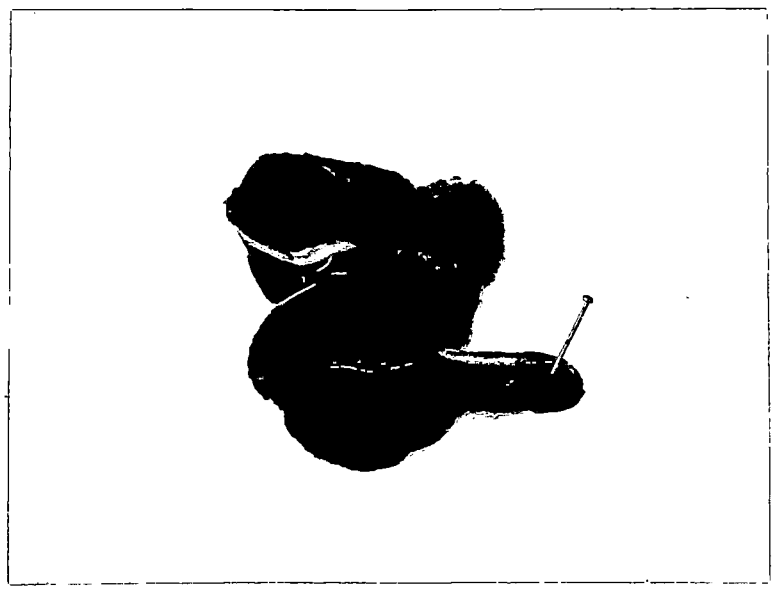
PLATES











AN INVESTIGATION ON
THE AETIOLOGY OF PLAGUE-LIKE DISEASE IN DUCKS IN KERALA

BY

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ABSTRACT OF A THESIS

Submitted in partial fulfilment of the
requirement for the degree

MASTER OF VETERINARY SCIENCE

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1978

ABSTRACT

An investigation was carried out to isolate, characterize and identify the agent responsible for the outbreak of duck plague-like disease in ducks in Kerala.

Specimens (liver and spleen) from field cases, were processed for virus isolation and were inoculated into either developing duck or chick embryos, by chorio-allantoic (C.A.M.) or allantoic cavity method. Virus isolation was possible only by C.A.M. inoculation of duck embryos and was confirmed by inoculation of the C.A.M. extracts into duck embryo fibroblast (D.E.F.) cell cultures.

The cytopathic changes produced by the field isolate DPV-N; its physico-chemical characteristics such as sensitivity to chloroform and 5-iodo-2 deoxyuridine; and the effect of exposure to various pH values such as 4.7, 7.2 and 9.1, were compared with that of a known duck plague virus DPV-K, received from the Veterinary Biological Institute, Mannuthy.

In D.E.F. cell cultures, the cytopathic changes produced by DPV-N and DPV-K were rounding and clumping of cells, with characteristic basophilia and granulation

of the cytoplasm. Although the initial titers of both DPV-N and DPV-K were only 10^5 and $10^{6.25}$, they increased to $10^{7.5}$ and $10^{8.25}$ respectively, on further passages.

The field isolate DPV-N and the known duck plague virus DPV-K were sensitive to 5% chloroform, with complete inactivation in ten minutes. Similarly, both the strains failed to multiply and produce cytopathic changes in cells treated with IUdR, at the rate of 100 micrograms per ml. However, differences were observed in their thermostability and pH sensitivity. Although DPV-K was inactivated completely at 56°C. in 30 minutes, DPV-N was only partially reduced in titer. DPV-N was also found to be resistant, when both the strains were exposed to pH 4.7, for a period of four hours at room temperature. But both were unaffected at pH 7.2 and got inactivated at pH 9.1. Both the strains also failed to produce any haemagglutination reaction with chicken R.B.C. or precipitation reaction in agar gels.

Although duck plague specific antiserum neutralized homologous strain DPV-K and the newly isolated strain DPV-N, the serum titers obtained with the latter was only less.

Experimental infection studies have shown that

one to six week-old ducklings were equally susceptible to DPV-N and DPV-K, either with the spleen extract or with tissue culture passaged sample. The symptoms and lesions produced in both cases, were similar to those described for duck plague and also to those seen during the disease outbreak in Kerala.

The virus that caused an outbreak of duck plague-like disease in Kerala is found to be indistinguishable from that of duck plague. It is also strongly felt that the lack of complete protection of birds vaccinated with duck plague vaccine is due to a possible strain variation between the classical duck plague virus DPV-K and the virus as it occurred during this outbreak. However, it needs thorough in vitro cross neutralization and in vivo cross protection tests before any definite conclusions can be made on the strain variation of duck plague virus.