Arch. Inst. RAZI (1992) **42/43**, 103-116

Isolation and Characterization of Avian Reoviruses from the Cases of Malabsorption Syndrome and Arthritis / Tenosynovitis in Chickens.

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Summary: Four strains of avian reoviruses were isolated from the cases of arthritis/tenosynovitis and malabsorption syndrome in chickens. The isolates were initially adapted to grow in embryonated eggs and subsequently to chick embryo cell culture where a characteristic cytopathic effect was produced. Two selected strains proved to possess an RNA genome. They were resistant to a lipid solvent and were shown to be relatively heat stable.

In electron microscopic examination of the isolates, particles with typical reovirus morphology were observed. Cross neutralization tests revealed close serological relationship between one of the virus isolates, strain R 61/1 and the reference strain U.Conn.S1133, thus placing the former in the same serotype as the latter. This relation was also shown with the other isolates, although in one-way tests.

Experimental infection in SPF chicks with the strain isolated from the joint tissues caused microscopic lesions of tenosynovitis.

Avian reovirus infections appear to be widespread in our poultry flocks and the significance of their role as potential pathogens in some conditions needs to be further evaluated.

Keywords: *Reoviruses / Arthritis / Tenosynovitis / Malabsorption syndrome / Chickens / Diseases*

Introduction

Reovirus infections are ubiquitous in poultry on a world wide basis and the virus can be recovered from a variety of tissues of both diseased and

apparently healthy birds (Kawamura et al. 1965; Olson 1984). Avian reoviruses have been recognized as the aetiology of viral arthritis/tenosynovitis (VA) in chickens and turkeys (Van der Heide 1977; Olson 1984). In this respect, they may act alone or in combination with other pathogenes i.e.Staphylococcus aureus and/or Mycoplasma synoviae. VA is perhaps the best defined of all the conditions attributed to these agents. Avian reoviruses have also been incriminated as the aetiological agents of a number of other conditions in chickens including acute or chronic respiratory diseases, enteritis, cloacal pasting in very young chicks, pericarditis, hydropericardium, myocarditis, hepatitis, atrophy of the bursa of Fabricius and thymus, and the more recently described stunting/runting or malabsorption syndrome in chickens (Van der Heide 1977; Kouwenhoven et al. 1978 a; Kouwenhoven et al. 1978 b; Olson 1984).

The increased pathogenicity of reoviruses for chickens following exposure to some other infectious agents such as infectious bursal disease virus has been reported and in turn reoviruses may exacerbate the severity of disease caused by other pathogens such as chicken anaemia agent or E.coli (Springer et al. 1983; Engstrom et al 1988; Rosenberger et al 1985).

The present communication describes the isolation and characterization of four strains of reoviruses from the cases of arthritis/tenosynovitis and malabsorption syndrome in chickens.

Materials and Methods

Embryonated eggs

Specific Pathogen Free (SPF) fertile eggs (Valo, Lohmann, Germany) were used in this study. They were inoculated either by yolk sac (Y/S)route on 6 th.day of incubation or onto chorioallantoic membrane (CAM)on 11 th. day. Death of the embryo or presence of pocks by the 5 th.day of inoculation were the criteria for virus replication by Y/S and CAM routes respectively.

Cell culture

Chick embryo fibroblast cell cultures (CEF) were prepared from 11-day-old SPF chick embryos by conventional methods. Cells were grown in Hanks solution + yeastolate and lactalbumine hydrolysate (LYH)supplemented with 10% calf serum+ antibiotics, and maintained in the same medium but with 2% calf serum unless otherwise stated.

Viruses

Cell culture adapted U.Conn.S.1133 strain was employed as a reference avian reovirus. No further cloning of the virus was attempted at this laboratory. It was passaged once in CEF cell before a stock was prepared. Strains R 61/1,R63/2, R 63/3 and R 63/4 were at the 14th. egg passage and 8th. CEF cell culture passage when they were given three passes at limiting dilution and virus stocks were grown in cell culture. Herts 33 strain of Newcastle disease virus (NDV) and a cell culture adapted fowl pox virus, strain 92, were also used in this study.

Virus isolation, adaptation and assays

Virus isolation and initial adaptation were carried out in eggs inoculated by Y/S route and serial passages of the yolk sac materials diluted 1:5 in LYH medium. Yolk sac materials were also used for the initial passage in CEF cell culture. For further passages, the cultures were frozen and thawed three times and centrifuged at low speed before the supernatants were inoculated into fresh cell cultures. Virus titrations were carried out in CEF cells using four culture tubes per dilution.

Serology

Virus neutralization was performed by mixing equal volumes of doubling dilutions of serum with 200 $TCID_{50}$ of virus; following one hour at 37°C, four CEF cell culture tubes were inoculated per serum dilution.

For agar gel precipitation (AGP) test the antigen was perepared by homogenizing infected CAM, followed by freezing and thawing once and centrifugation at low speed. The supernatant was used as antigen in the test. The gel consisted of 1% Noble agar and 8% sodium chloride.

Antisera

Rabbits were inoculated with 2 ml. of equal volumes of the stock virus and incomplete Freund's adjuvant intramuscularly. An intravenous boost of 1 ml. of virus was given after 3 weeks and the animals were bled 10 days later.

Two-day-old SPF chicks were inoculated into both foot pads with 0.1 ml. of the stock virus and bled four weeks later.

Histopathology

Joint tissues and heart muscle specimens were fixed in 10% neutral formalin and processed by conventional procedures.

Characterization of the isolates

Scheme for classification and characterization of viruses proposed by Hamparian et al (1963) was followed in this study:

Nucleic acid determination

CEF tube cultures were washed free of serum and inoculated with $10^{3.5}$ $TCID_{50}$ of the respective virus in medium without serum. The virus was adsorbed at 37°C for 1 h.after which the cultures were washed five times with 3 ml. of the maintenance medium and the tubes were then divided into three groups. One group was refed 1.5 ml of the maintenance medium containing 5-iodo-2-deoxyuridine (IUDR) at the concentration of 10^{-4}M, the second group with the same medium which also contained thymidine in an equivalent molar concentration as IUDR, and the third group with normal medium. Uninoculated cultures with IUDR medium were set up to control the possible toxic effect of the drug on the cells. After 3 days, when the control (normal medium)cultures were showing distinct CPE, five tubes from each of the three groups were removed and the whole culture (cells and fluid) were frozen and thawed three times and the product of each set of five tubes was pooled. This was centrifuged at low speed and the supernatants were titrated with a single batch of cells.

Stability to chloroform

The test was conducted according to the method described by Feldman and Wang (1962). Analytical grade chloroform was added in 0.05 ml amounts to 1.0 ml of virus and the mixture was shaken for 10 mins. by hand at room temperature (RT). Chloroform was removed by centrifugation at low speed for 5 mins. and supernatant fluid was titrated for virus infectivity. As controls virus materials were treated likewise with maintenance medium and NDV was treated with chloroform in the same manner as described.

Stability at PH 3.0

The procedure outlined by Ketler et al (1963) was followed. HEPES buffered maintenance medium prepared at PH 7.2 was adjusted to PH 3.0 by adding N/5 HCl. Stock virus was diluted 1:10 in the medium and held at RT. Samples, 0.1 ml, were withdrawn after 1 h. incubation and were diluted 1:10 in the medium at PH 7.2 and titrated. As control, virus was diluted in maintenance medium PH 7.2 and treated likewise.

Thermostability

Stock virus was sealed in 0.7 ml amounts in thin glass ampoules wich were

then submerged in a water bath at 60°C (± 0.1°C). After 1h. and 3h. incubation, ampules were removed from water bath and plunged into an ice cold bath for 15 mins. following wich the contents of the ampoules were transferred to bijou bottles and stored at-50°C until tested for viability. Herts 33 strain of NDV was treated in the same manner and used as a control.

Electron microscopy

Preparation of the grids was conducted as described by McFerran et al (1971). The medium of cell cultures showing advanced CPE was poured off and 3 drops of distilled water added to the monolayer in tubes. These were rotated for 30 mins. following which the fluid was applied to formvar-carbon coated grids and stained with 4 per cent sodium phosphotungstate PH 6.5. The preparations were examined in a Philips 300 electron microscope at an instrumental magnification of X 70000.

Haemagglutination

The test was performed by conventional techniques in microtitre plates using 1% chicken erythrocytes and incubating at RT.

Sampling and preparation of Specimens

a) Arthritis/tenosynovitis-Metatarsal extensor and digital flexor tendons and tendon sheaths were removed from birds showing clinical signs of lameness and lesions of arthritis/tenosynovitis. Tissues were homogenized as a 10% (W/V) suspension in the maintenance medium. A portion of the suspension was taken for bacterial cultures before antibiotic treatment. Following centrifugation at low speed, the supernatant was harvested and mixed with an equal volume of a fluorocarbon compound and homogenized for 20 sec. in a power driven mixer. Following centrifugation at low speed, the supernatant was withdrawn and used for the isolation purpose. Samples of the heart muscle and joint tissues were fixed in 10% neutral formalin and processed for histopathological study. Blood samples were obtained from live arthritic birds and sera employed for detection of agglutinins to Mycoplasma synoviae (M.s) and Mycoplasma gallisepticum (M.g) by rapid slide agglutination tests using stained antigens.

b) Malabsorption Syndrome - Composite samples of proximal to mid-jejunum, distal duodenum and proximal ileum, and their contents were removed and processed as described except that no attempt was made to examine the specimens for bacteria.

Results

Clinical aspects and gross lesions

a - Arthritis/tenosynovitis - Three - month - old broiler breeder replacement chickens, both live and dead, were submitted to the diagnostic laboratory. Lameness was noticed in some birds while swelling of the metatarsal extensor and digital flexor tendons were pronounced in the majority of them. At autopsy, oedema in the tendons, tendon sheaths and the hock joint tissues was evident. Excess amount of yellowish fluid was present in most of the joints examined.

b - Malabsorption syndrome - Two to four - week - old live chickens were received from three broiler farms. A common noticeable sign was the uneven growth rate and the presence of chicks obviously stunted as compared to the weight of those considered normal for the age. Abnormal feathering in the form of retention of yellow dawn and existance of the primary feathers projecting from the body was also very prominent. At autopsy, the small intestine was markedly distended and contained poorly digested food. The bursa and thymus were atrophied and necrosis of the femoral epiphyse was evident in some specimens.

Isolation of the agents and pathogencity for egg embryo

A relatively high embryo death rate was already established by the 5 th. or 6 th. egg passage. Further passages were undertaken to achieve higher levels of adaptation and more consistant death patterns. Embryo mortality occurred mostly during the 3 rd. and 4 th. days after inoculation. Low dilutions of the infected yolk sac materials caused haemorrhages in the viscera and under the skin, hence a dark red discoloration of the embryo. Mortality of the embryos following inoculation onto CAM was considerably delayed compared to Y/S route. The embryos were usually stunted with enlarged mottled liver and spleen. Pocks were produced as small white necrotic foci on CAMs which also had oedematous appearances.

Growth in CEF cell cultures

CPE was not discernible in the first passage in CEF cells. A rather poor CPE was initially observed in 2 nd.or 3 rd. passes, depending on the strain, before

a distinct CPE could be obtained in further passages. Cytopathology in this cell system inoculated with high doses of all the strains under study was first detected 24h. after infection but it took about 48h. until became pronounced. It was characterized by appearance of small rounded or irregular refractile cells and also the formation of syncytia. Large number of small dead cells and giant cells were detached from the glass, floating in the medium and leaving empty areas in the cell sheet at this time. It was common to observe detached giant cells still connected to the glass surface by long cytoplasmic processes. Destruction of the monolayers was almost complete by the 3 rd. or 4th. days after inoculation. The 8th.cell culture passages of the isolates failed to produce plaques in CEF cells under agar overlay medium.

The infectivity titres in this cell system ranged from $10^{6.75}$ to $10^{7.25}$ TCID 50/ml, depending on the isolate. In haematoxylin-eosin stained infected cell cultures grown on cover slips, many small dead cells and also multinucleated cells were present but no inclusion could be observed clearly.

Nucleic acid determination

IUDR did not affect the replication of R 61/1 and R 63/2 but it markedly reduced the titre of a fowl pox virus strain (Table 1).

Table 1. Effect of 5-iodo-2 deoxyuridine on the replication of R 61/1, R 63/2 and a fowl pox virus strain.

	R 61/1	R 63/2	Fowl Poxvirus
IUDR	6.8 *	6.75	1.5
IUDR + thymidine	6.9	6.5	4.4
Normal medium	7.2	6.8	4.6

* Log_{10} TCID50/ml

Stability to choroform - R 61/1 and R 63/2 were insensitive to the lipid solivent. The infectivity of the NDV strain on the other hand, was completely abolished by treatment in the same manner (Table 2).

Table 2. Effect of chloroform on the infectivity of R 61/1 and R 63/2

Virus	treated	untreated
R 61/1	6.7 *	7.0
R 63/2	6.5	6.7
NDV	0	8.1

* Log_{10} TCID50/ml

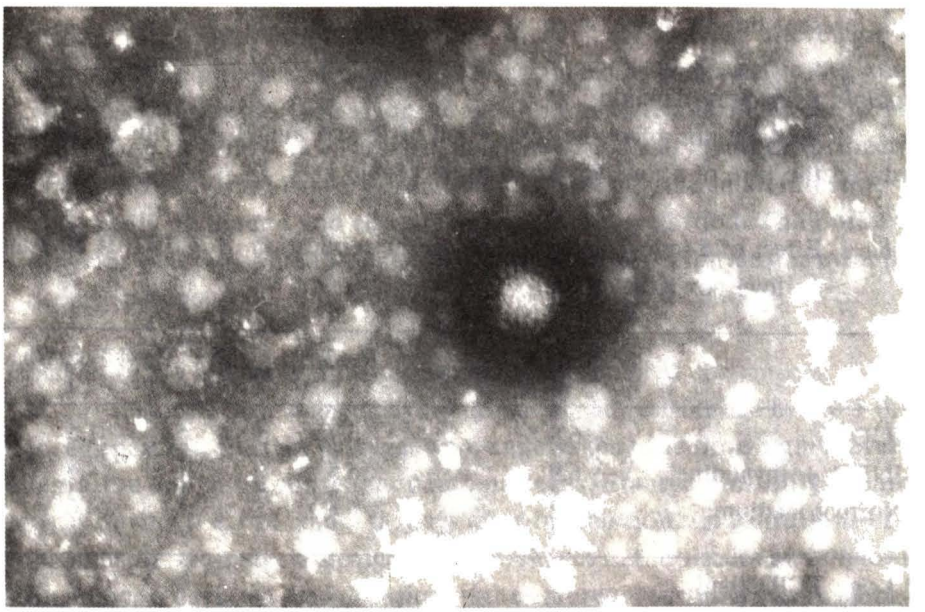

Fig 1-1- *Negative contrast electron micrograph of the strain R61/1 x190,000*

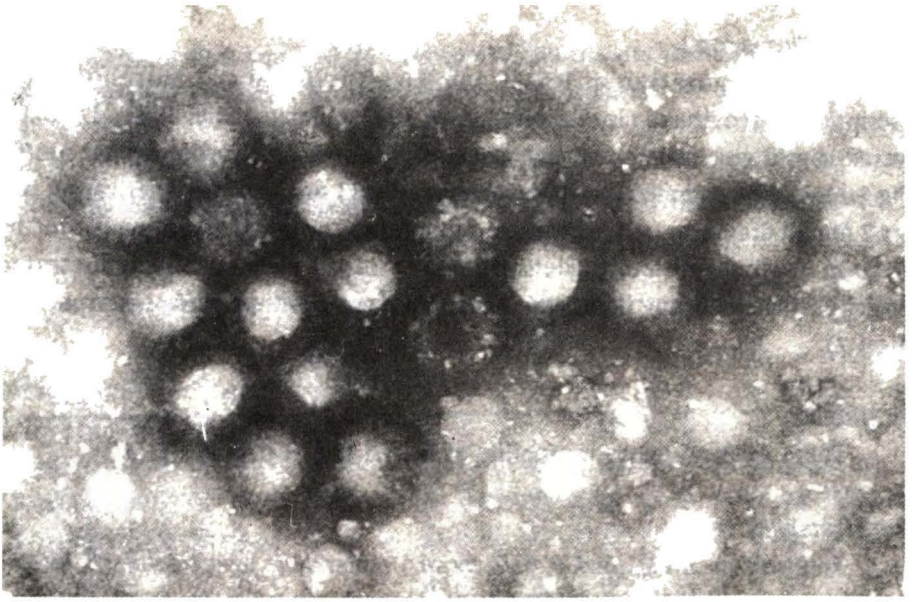

Fig 1-2- *Electron micrograph of the strain R 63/2 (pelleted) containing both penetrated andunpenetrated complete particles. X 250,000*

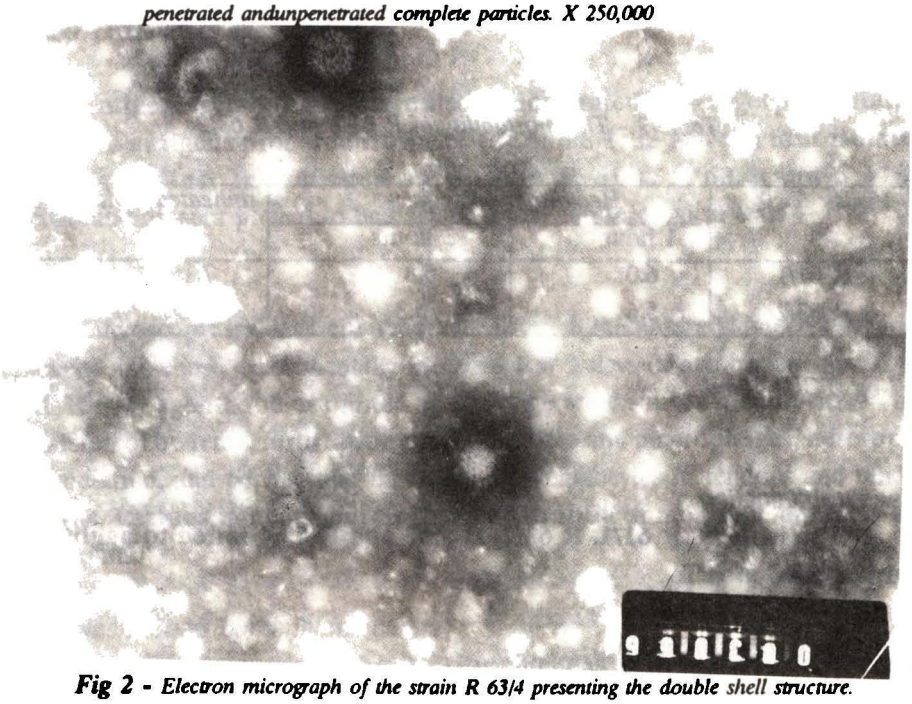

Fig 2 *- Electron micrograph of the strain R 63/4 presenting the double shell structure. x 100,000*

Electron Microscopy

The configuration of R61/1 and R63/2 fulfilled the morphological description of reoviruses (Jordan and Mayor 1962; Gomatos et al 1962). Double - layered capsid was evident and only some particles had a clear hexagonal profile (Fig 1.1&2). The complete particles measured 74±3 nm. and the inner capsid was 43±2 nm. in diameter. All preparations contained both particles penetrated by the stain in which an electron dense inner capsid was visible, and unpenetrated perfect particles showing inner and outer capsids and distinct surface morphology (Fig 1.2).

PH stability

There were no losses in viability of R 61/1 and R 63/2 at PH 3.0.

Thermostability

The isolates R 61/1 and R 63/2 showed remarkable heat resistance, lossing only about twenty and forty folds in titres respectively after incubation at 60° c for 3h. (Table 3).

***Table 3** - Thermostability of R 61/1 and R 63/2*

Virus	*Heating at 60° c for:*		*unheated control*
	1 hour	3 hours	
R 61/1	6.0*	5.8	7.1
R 63/2	5.6	5.3	6.9

* Log_{10} *TCID 50/ml*

Haemagglutination

The isolates R 61/1, R 63/2, R 63/3 and R 63/4 failed to agglutinate chicken RBC at R.T.

Serological classification

Virus neutralization tests, employing both the chicken and rabbit antisera, revealed serological relationship among the virus isolates themselves and between these and the reference strain U.Conn.S 1133. This was shown in cross neutralization tests between the latter strain and R 61/1, but in one-way testing

with R 63/2, R 63/3 and R 63/4 (Table 4). The results should be rectified in further tests by inclusion of the antisera to these isolates.

Table 4 - Results of neutralization tests using the virus isolates and the reference strain of avian reovirus.

	Antisera			
	Chicken		*Rabbit*	
Virus	S 1133	63/1	S 1133	63/1
S 1133	512*	256	128	85
R 61/1	512	300	100	110
R 63/2	220	185	85	74
R 63/3	256	185	74	64
R 63/4	185	128	100	85

* Titres expressed as the reciprocal of the final serum dilution which neutralized 200 TCID 50 of the virus.

Antigenic relationships were also demonstrated by agar gel precipitation tests with antigens prepared from the U.Conn.S 1133 and the four isolates, reacting against the chicken antisera to R 61/1, R 63/2 and S 1133. Preinoculation sera proved to be negative in all the serological tests.

Histopathology

Microscopic lesions were almost the same in the naturally infected arthritic chickens and those experimentally inoculated at 2 days of age by foot pad route with R 61/1 agent, and examined at 1,2 and 3 weeks after inoculation. They consisted of oedema, infiltration of lymphocytes and macrophages, heterophil accumulation, and perivascular infiltration of lymphocytes in tendons and tendon sheaths. Lesions in heart muscle were infiltration of mononuclear cells and heterophils.

Mycoplasma and bacterial infections

Joint specimens from the arthritic chickens were negative for bacteria. Rapid slide agglutination tests with the serum samples showed the birds were also M.g.negative but positive for M.s. infection. No attempt was made to isolate mycoplasma in culture.

Serological survey

A total of 530 blood samples were obtained from twelve broiler breeder organizations and the sera employed for the detection of antibodies to avian reoviruses in AGP tests. All the farms contained birds which were positive reactors.39.6% of all the samples were positive. The incidence of antibody ranged from 23.3% to 55% depending on the farm.

Discussion

The agents described in the present study revealed the typical morphology of reoviruses as examined by negative contrast electron microscopy (Jordan and Mayer 1962). This group is also characterized by the lack of sensitivity to chloroform, thermostability and survival at low PH values. Proof that the agents belong to reoviruses was further extended by the results of serological studies, employing U.Conn. S 1133 as an avian reference strain of this group.

Cytopathological aspects of the strains under study differed in detail as compared to the findings of some other workers. Mustaffa-Babjee et al. (1973) reported that syncytia were not commonly observed in CK and CEF cell cultures infected with an Australian avian reovirus strain. Furthermore, they noted CPE in the latter cell system much less clear than in the former. In the present study distinct CPE was produced by all the strains in CEF cells leading to almost complete destruction of the cell sheet, and syncytia formation was a prominent feature.

In view of both egg and contact transmission of avian reoviruses (Van der Heide 1977) and the results of the serological survey in some major breeder organisations, it may be assumed that the infection is widely distributed in our poultry flocks. Embryonated eggs harbouring the virus, cell cultures or chicks derived from such eggs and also chicks infected by their penmates pose serious problems to research workers and vaccine manufacturers. Therefore, the freedom of flocks supplying these biological systems from the infection is imperative.

Reoviruses have been one of the candidate virus groups coined as the possible primary causal agents of malabsorption syndrome. Although, more recently, smaller non enveloped viruses have attracted the attention in this context, the role of reoviruses at least as part of a probable multifactorial aetiology is disputable.

Serological classification of avian reoviruses should not be considered as a matter of academic curiousity. The aetiological role of these as primary or

secondary agents in relation to the most conditions attributed to them has not been definitely established so far. Viral arthritis, at present, is perhaps the only clear cut disease in this respect caused by avian reoviruses. Various types of vaccines against the disease have been in use, albeit not at a worldwide extent and then with varied levels of effectiveness. This may be at least partly due to the lack of adequate information on the relative importance of serotypes in causing the condition, the number of types occurring in any one area, and the degree of possible cross protection existing between them. Should the importance of reoviruses as the causal agents in some other conditions become established, there would be more pressure for the use of different types of vaccine. It has been suggested that there is little or no protection conferred by the group antigens (Springer et al. cited by Olson 1984) and therefore the inclusion in vaccines of all serotypes prevalent in an area would be required for an effective immunization program.

In view of rather limited number of strains studied, further investigation is needed to assess the presence of other serotypes in our poultry population.

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