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Studies with reovirus isolated from guinea fowls (*Numida meleagridis*)

Estudos com reovírus isolado de galinha-d'angola (*Numida meleagridis*)

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SUMMARY

This paper describes some properties of a reovirus isolated from the pancreas and intestines of guinea fowls suffering from a transmissible enteritis. Coronavirus was also recovered from kidneys of the same birds. The guinea fowl reovirus is pathogenic for guinea fowl, duck and chicken embryos, but it does not reproduce by itself the field findings when inoculated in day-old guinea poults, nor was it found to be pathogenic for chicks and ducklings on experimental infection.

UNITERMS: Guinea fowls; Reovirus.

INTRODUCTION

Reovirus were reported as the cause of viral arthritis of chickens, and more recently they have been recognized as viral agents widely spread in nature. Avian reoviruses have been associated with a range of morbid conditions, but are also present in normal avian species (Rosenberger; Olson[13]; 1991). Reoviruses were found in clinically affected chickens, ducks, turkeys, pigeons, geese, and psittacines, but their etiological relationships have not always been established. Hence, a number of factors wich relate to the virus as well as to the host have been considered to influence the outcome of reovirus infection in chickens (Robertson; Wilcox[13], 1986). The purpose of this report is to describe the isolation and characterization of a reovirus isolated from young guinea fowls showing a transmissible enteritis-like syndrome, associated with high mortality.

CASE HISTORY

At the end of 1988, a Brazilian guinea fowl farm in Descalvado, SP, experienced considerable financial losses because of high mortality among young guinea fowls. The illness was characterized by depression, dehydration and increasing mortality (about 14%, at 28 days of age). Yellowish-brown kidneys, urates in the ureter, gaseous and watery -- yellowish contents in the ceca were observed, as well as intestines with thin walls and brown contents. Focal necrosis and bubble-like structure in the pancreas were seen in necropsied birds, after histopathologic examination. In the present clinic case, herpes-like particles were found in the intestinal contents while reovirus and coronavirus respectively were isolated from pancreas / intestines and kidneys of the affected birds. The present study was undertaken in order to investigate the possible pathogenic effects of the reovirus in the guinea fowl.

MATERIAL AND METHOD

Birds and eggs: Specified-Pathogen Free (SPF) embryonated chicken eggs and day-old chickens were obtained from Granjas Rezende (Uberlândia, MG, Brazil). Embryonated Pekin duck eggs were supplied from a controlled breeding stock of BioVet Laboratories (Vargem Grande Paulista, SP, Brazil). Guinea fowl fertile eggs were provided by Emape (Fortaleza, CE, Brazil) wich has a commercial breeding stock of the heavy type of guinea fowl, imported from France and considered to be free of this kind of disease according to the field data.

Reovirus-like isolate: The primary isolation was obtained by inoculation of 10% suspensions of pancreas and duodenum (previously centrifuged and passed through 0.22 µm Millipore filters) in embryonated specific pathogen free (SPF) chicken eggs, respectively with 5 and 10 days of incubation, via the yolk sac (YS) and chorioallantoic membrane (CAM) routes. A whole-embryo suspension was prepared from YS-inoculated eggs as a virus stock and stored at -70°C until used in the experiments. This virus strain was designated 2370/89.

Embryo pathogenicity: The virus suspension was titrated for infectivity in SPF chicken embryos inoculated by either CAM, allantoic cavity (AC) or YS routes. YS inoculation was performed in 5-day old embryos, AC and CAM inoculations were respectively done in 8 and 10 day old embryos. Guinea fowl and duck embryos with 7 day of age were also inoculated by YS route.

Cell cultures: Whole chicken embryo fibroblast (CEF), chicken embryo liver cell culture (CELi) and day-old chicken kidney (CK) cell cultures were prepared according to classical methods described by Purchase *et al.*[12] (1989), inoculated with virus stock suspension and then examined daily development of cytopathic effect. Virus stock was diluted at 10^{-3} in Tryptose Phosphate Broth (TPB)(Difco), clarified by centrifugation (3 minutes at 3,000 rpm) and passed through Millipore filters (0,45 µm) before absorption in cell culture. Cell cultures were washed one time after absorption for 30 minutes at 37°C.

Chemical and physical properties: The stock virus preparation of 2370/89 was assayed for sensivity to chloroform treatment, storage at different temperatures (-20°C, 4°C, 37°C, 56°C), and sensivity at pH's 3.0 and 12.0, in accordance with methodology described Nersessian *et al.*[9] (1985).

Hemagglutination test: Virus hemagglutination tests were done by microtitre standard methods (Purchase *et al.*[12], 1989) on fluids from infected eggs and cell cultures using chicken red blood cell suspension at 0.5% V/V.

Filtrability: Stock virus was diluted (10-2) in TPB and passed through 0.45, 0.22, 0.05 µm average pore diameters Millipore filters before titration in embryonated chicken eggs, via YS route.

Electron microscopy: Infected CAM and stock YS-virus suspensions were submitted to electron microscopy examination. CAM portions were fixed in cold 1% glutaraldehyde, postfixed with 1% OsO_4 and embedded in Epoxy. Ultrathin sections were stained with uranyl acetate and lead citrate and observed using a Phillips EM 300 electron microscope. The embryo suspension was centrifuged at 10.000 rpm (L5 SPINCO ULTRACENTIFUGE) for 30 minutes and then pelleted at 40.000 rpm for 1 hour. The concentrated suspension was submitted to direct electron microscopic examination according to McNulty *et al.*[8] (1979).

Agar gel precipitation test: Stock virus wich had been passed 7 times in CEF monolayers and then inoculated by the CAM route in chicken embryo was used to prepare the precipitating antigen according to Purchase *et al.*[12] (1989). Antiserum against S1133 strain of reovirus, obtained through Solvay laboratories (Campinas, SP, Brazil), was used to search for the presence of reovirus common antigens.

Polyacrylamide gel electrophoresis (SDS-PAGE): Virus suspension obtained after 5 passages by YS route in chicken embryos and then to 7 passages in CK or 5 passages in CEli was concentrated by centrifugation at 40.000 rpm (L5 SPINCO ULTRACENTIFUGE) at 4°C and submitted to RNA extraction using SDS 10% (Sodium dodecyl sulphate). CEF passage level 7 was also inoculated into the CAM of chicken embryonated eggs. This virus suspension at CEF/CAM passage level 1 was processed by grinding the CAM, clarifying by centrifugation at 10,000 rpm for 30 minutes and concentration at 40,000 rpm. The S1133 reovirus reference strain, CAM high passaged sample was used to compare the genomic fractions. Chicken gut isolate reovirus 5264/84 (ITO, unpublished), presented as a CAM suspension, was prepared in the same way. The PAGE procedure was performed according to the method initially described by Pereira *et al.*[11] (1983) and modified by Jerez *et al.*[6] (1989).

Experimental infection: Stock virus was inoculated by oral, foot pad and subcutaneous routes in SPF chicks and commercial guinea fowl at one and three days of age, respectively. Virus suspension at 10^4 Embryo Lethal Dose 50% (ELD_{50}) was inoculated, respectively at the volumes of 0.5, 0.2 and 0.3 ml. Each group, including controls, was composed of ten birds and was kept in isolation for two weeks. Four days after inoculation, two birds from each group were necropsied and examined for macro and microscopic lesions.

RESULTS

Virus isolation: The original 10% suspensions of pancreas and duodenum that yielded reovirus-like isolate were inoculated in chicken embryos by allantoic sac route, and did not yield virus isolation after five serial passages. When the suspensions were inoculated in chicken embryos by YS route, swollen liver, hemorragic and oedematous embryos were found in the dead embryos between 4 to 7 days after

Table 1
Comparative pathogenicity of avian reovirus isolate 2370/89 in SPF chicken embryos depending on inoculation route. São Paulo - SP, 1990

Route	ELD50	Embryo lesions observed
CAM	<10-1	CAM oedema and local necrosis Dwarfing Liver necrosis and/or green liver
AC	<10-1	Pale embryo Green liver
YS	10-6.3	Oedema Hemorrages

ELD50 = 50% Embryo Lethal doses

Table 2
Comparative pathogenicity of avian reovirus isolate 2370/89 in embryos of avian species inoculated by yolk sac route. São Paulo - SP, 1990

SPF chicken	guinea fowl	Pekin duck
$10^{4.5*}$	$10^{5.5}$	$10^{4.0}$

* 50% embryo lethal doses

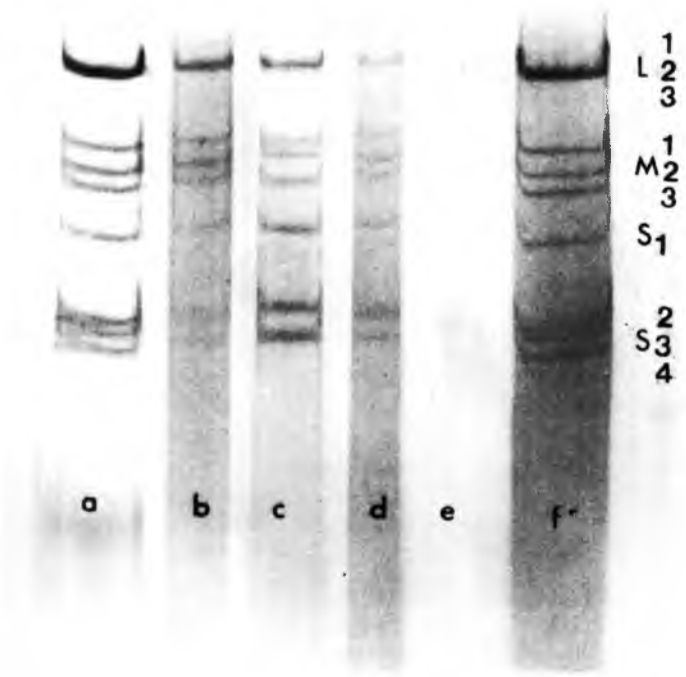

Figure 1
Fifteen-day old guinea fowl embryos infected via YS with 2370/89 isolate. Dwarfed embryos in the left had increased urate in the amnion

inoculation. Embryo mortality was not observed when the suspensions were inoculated by CAM route, but embryo lesions were seen in the infected embryos.

Embryo pathogenicity: The 2370/89 strain of guinea fowl-origin reovirus was found to be of the highest pathogenicity when inoculated by the YS route (Tab. 1). A similar level of sensivity was observed for guinea fowl and duck embryos. Comparatively, the isolate titre found in guinea fowl embryos inoculated by YS route was higher than in chicken and duck embryos (Tab. 2). Guinea fowl embryos which died 10 days after inoculation were dwarfed and had increased urate deposition into the amnion (Fig.1), as well as biliary lesions in the liver. Early dead chicken embryos were hemorrhagic and oedematous.

Cytopathic effect (CPE): The isolate was able to multiply in CEF, CEli and CK. In all those cell cultures the virus produced syncytia and cell degeneration. Syncytia formation was observed at 18 hours after inoculation in CELi (Fig. 2), while in CK syncytia became aparent after 72 hours post inoculation. While the virus appeared to replicate most rapidly in liver cell cultures, the infectivity in CEli was the same as those found for virus cultured in whole embryo and kidney cell cultures. After 7 passages in CK the isolate produced CPE in CEF monolayers. CEF and CK - passaged stock viruses were able to produce minute lesions in the CAM of chicken embryos. During all the passages of the isolate, it was not possible to detect any haemagglutinating activity to chicken red blood cells.

Treatment of the virus: The isolate was stable after treatment with chloroform. Neither storage at -20°C, 4°C / 60 minutes, 37°C / 1 hour, 56°C / 2 hours, nor exposure to pH 3.0 or pH 12.0 affected the stability of the virus. Loss of titre was found when the virus was submitted to 56°C / 5 hours.

Filtrability: The virus suspension passed through 0.22 µm filters underwent a slight diminution of its titre when compared to the results of using a 0.45 µm filter. The virus was not filtrable at 0.05 and 0.025 µm (Tab.3).

Virus particle: Virus particles compatible with reovirus morphology measuring aproximately 60 mm were detected in the YS suspension and in the CAM cells (Fig.3).

Precipitant antigen: The 2370/89 isolated shared a common group-specific antigen with S1133 chicken reovirus.

Genome: RNA migration pattern concerning L, M (M_1, M_2 and M_3) and S (S_1, S_2, S_3 and S_4) segments were similar to S1133 and 2370/89 isolate at the 7th CEF passage and at the 1st CAM passage. After 7 passages in CEF CK and CELi passaged virus suspensions had some variation of M_2, S_3 and S_4 motilities (Fig. 4).

Pathogenicity for chicken and guinea fowl: Mortality and clinical symptoms were not observed in either of the groups of experimentally inoculated chicks and guinea fowl.

DISCUSSION

The Reovirus isolated from guinea fowl pancreas shared

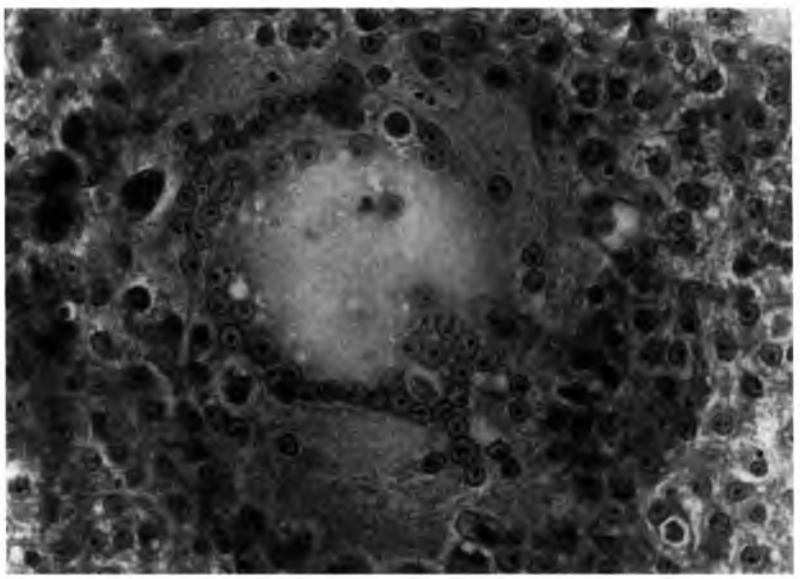

Figure 2
Coverslips with confluent chicken embryo liver cell harvested 18 hours after inoculation. Haematoxylin-eosin, 660 X magnification

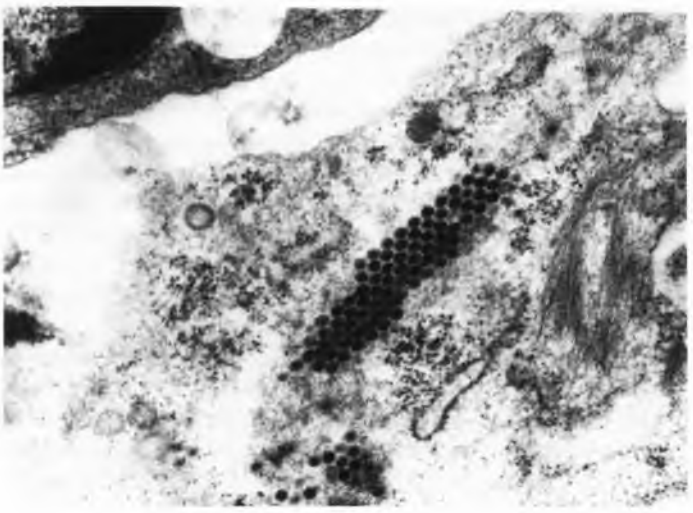

Figure 3
Virus particles in the CAM cells. Magnification of 34 000 X, 1 cm = 295 nm.

Table 3
Detection of infectivity of strain 2370/89 after filtration through Millipore filters. São Paulo - SP, 1990.

Filter (µm)	EID_{50}	Mean death time
0.45	$10^{6.3}$	146.40 + 38.07
0.22	$10^{5.2}$	212 + 41.34
0.05	$<10^{1.0}$	
0.025	$<10^{1.0}$	

ELD_{50} = 50% Embryo Lethal doses
Mean death time (hours) observed for embryos

a number of characteristics in common with chicken isolates. Isolation and cultivation features are similar to those reported for chicken isolates (Guneratne *et al.*[4], 1982), and genomic profiles are compatible with avian reovirus (Huang *et al.*[5], 1987). It was found, however, that the 2370/89 isolate needed preliminary passages into CEli and/or CK before it was able to produced a clear CPE in CEF. This strain is not pathogenic for day old chicks, or at significant levels for AC-inoculated chicken embryos Takase *et al.*[15] (1987) have concluded that this kind of characteristics is associated with the low pathogenic strains of chicken reovirus. According to these authors, strains of low pathogenicity are serologicaly different from highly pathogenic strains able to produce severe footpad swellings and tenosynovitis in chicks. As the guinea fowl isolate did not produce retarded growth or foot pad oedema in guinea fowl, it can reasonably be concluded therefore that this virus was not the cause of the high mortality in the field. It would appear that this reovirus would be playing only a secondary or opportunistic role, and other pathogens could be involved in the etiology of this present flock's disease history. The clinic disease resembles the disease recognized in France and named "entérite frilosité", because the poults congregated under the brooder and had yellowish

Figure 4
Pattern of RNA migration in SDS-PAGE. a,b,c,d = 2370/89 isolate replicated respectively in CEF, CAM, CEK and CELi; e = 5264 Reovirus isolate in CAM, and F = S1133 in CAM

diarrhoea. It commences at 7 to 10 days of age causing 5 to 10% mortality and the etiology is unknown (Debaste[3], 1972; Cauchy[1], 1975). In Italy, an apparently similar disease was named transmissible enteritis and attributed to a filtrable agent infection (Corazzola; Zanin[2], 1970; Pascucci *et al.*[10], 1982). Rotavirus was observed in the faeces samples of diseased birds, but did not reproduce the field findings (Pasccuci *et al.*[10], 1982). Given the present clinical history and virus detection data (reovirus, coronavirus and herpes-like virus), further studies should be carried out to clarify the primary cause(s). And also to establish a nomenclature for this disease. In 1988, Kles[7] reported a sporadic outbreak of a disease occurred in France, between 1967 and 1986, which was called "X disease" or Fulminant disease (Maladie foudrayante), because of its subtle onset and high lethality. Adenovirus was present in the disease guinea fowls, as well as a herpesvirus-like agent with which the disease could be reproduced. It appears quite likely that disease observed here in Brazilian guinea fowl is the same as that which has been described as a fulminant disease in France, and will require further investigation in order to clarify the disease-causing potentiating roles of these various agents.

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RESUMO

Este trabalho descreve algumas propriedades de um reovírus que foi isolado de pâncreas e intestino de galinhas-d'angola que padeciam de uma enterite transmissível. Coronavírus foi isolado do rim das mesmas aves. O reovírus de galinhas-d'angola é patogênico para embriões de galinha-d'angola, de pata e de galinha mas não reproduziu os achados de campo, quando inoculado em angolinhas, e nem foi patogênico para pintos e patinhos inoculados experimentalmente.

UNITERMOS: Galinha-d'angola, Reovírus.

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