

Diagnosis of Avian Reovirus Infection in Local Egyptian Chicks

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Abstract: Avian Reovirus (ARV) infection attacks poultry farms and induces clinical symptoms which may be unapparent or fatal. Affected birds manifested chronic gastroenteritis together with respiratory symptoms in few cases. The current work was carried out to throw light on ARV infection in local chicken. Samples of intestinal content from infected birds showing above mentioned symptoms were taken. The virus was propagated on chicken embryo rough (CER) cells and examined for syncytia formation. These syncytia were typical to that formed by ARV. The isolated virus was subjected to serum neutralization test (SNT), whereas the virus infectivity decreased 2.98 (log₁₀). Fluorescent antibody technique (FAT) showed that Qalubia governorate had high virus incidence followed by El-Sharkia then El-Gharbia (with a titer reciprocal of 512, 422 and 265, respectively). Moreover, electron microscopy showed featureless viral particles which contain outer and inner membrane of 75-80nm. In conclusion, this study showed that Avian Reovirus could be isolated, propagated and identified by simple testes such as EM, FAT.

Key words: ILTV · Fingerprinting · RE · Protein analysis · EM · PCR

INTRODUCTION

Reovirus (*Orthoreovirus*) is one of the six genera of the family *Reoviridae*, characterized by a genome comprising 10 segments of double-stranded RNA enclosed within a double protein capsid shell 80 nm in diameter [1]. These agents have been classified into two groups according to their natural hosts: mammalian reoviruses and avian reoviruses [2, 3]. Although the structures and molecular compositions of avian and mammalian reovirions are often considered to be very similar [4], specific differences in the interaction with their respective host cells have been reported. Thus, only mammalian reoviruses possess hemagglutination activity [5-7] and only avian reoviruses are able to induce fusion of cultured cells [8]. Extensive biochemical and morphological studies have led to understanding of the polypeptide composition of mammalian and avian reovirions and of the protein architecture of the viral particle [4, 9].

Several avian viruses are known to induce formation of syncytia. Avian reoviruses (ARV) are an important cause of disease, especially problems related to leg weakness, in poultry [10-13]. Over 77 independent avian reovirus isolates have been described, all of which can

induce syncytium formation in cultured cells [10, 14]. Syncytium formation is one type of cytopathic effect and several studies have reported the direct cause of this biological phenomenon. The syncytium contains many nuclei within its cytoplasm.

Serum neutralization test (SNT), Fluorescent antibody technique (FAT) and electron microscopy (EM) in clinical obtained from these investigations specimen are very sensitive tests and results are consider reliable [10].

Sudden and repeated outbreaks of mild to severe diarrhea in small aged broiler and breeders chicken were experienced by a number of commercial chicken producers in Qalubia, El-Sharkia and El-Gharbia Governorates. This investigation was undertaken in order to find possible causative viral agents of these outbreaks using simple non expensive and sensitive techniques.

MATERIALS AND METHODS

The present investigations were carried out at The National Research Centre, Dokki, Egypt.

Samples: Intestinal content, from 450 slaughtered and died infected 2-weeks old chicks in Qalubia, El-Sharkia and El-Gharbia Governorates, were clarified by

centrifugation at 3000 rpm for 15 min in cooling centrifuge and the supernatant was collected.

Virus: Reference ARV was kindly supplied by Veterinary Serum and Vaccine Research Institute, Abbassia, Cairo, Egypt.

Cells: Chicken embryo rough cells (CER) were obtained from VACSERA Agoza Cairo, Egypt.

Antisera: ARV Hyperimmune sera was kindly supplied by Veterinary Serum and Vaccine Research Institute, Abbassia, Cairo, Egypt.

Conjugate Serum: AntiRabbit antichickan gamma globulin with fluorescein isothiocyanate was purchased (Sigma Diagnostics Inc. USA).

Experimental Protocol

Virus Isolation and Propagation: Three milliliters of chicken embryo Rough (CER) cells at a concentration of 2×10^6 cells/ml was seeded on 25ml³ prescriptions and then grown in Eagle's medium containing 10% fetal bovine serum (FBS Sigma Diagnostics Inc. USA). At the semi-confluent stage, the ARV was inoculated onto the cultures at multiplicity of infection (moi) of 1 (TCID₅₀/cell) for 2 h at 37°C. After washing with sterile phosphate-buffered saline (PBS), the cells were cultured in fresh Eagle's medium containing 2% FBS. For cytological examination, the cells were examined at 6, 12, 18, 24, 30 and 36 hrs post infection [15].

Serum Neutralization Test (SNT): 0.5ml of 1000TCID₅₀ of the virus suspension was mixed with the same volume of anti sera. The mixture was incubated at room temp for 30 min and then 0.2ml from this mixture was inoculated in 96-well tissue culture plate containing CER cells the test was performed according to Madbouly *et al.* [15].

Fluorescent Antibody Technique (FAT): Monolayer CER cells were trypsinized, suspended in growth medium at a concentration of 3×10^5 cells/mL and mixed with virus suspension to give a ratio of cells to virus (pfu) of 3:1. The technique was made according to Ide [16]. After the final blotting, plates were examined, using a fluorescence microscope. Plate wells were examined for fluorescence at a final magnification of 100 X. Clarity was improved if 10 µL of 10% glycerol-PBS solution pH 7.2 was added to each well. Fluorescence was graded from 1+ to 4+ to indicate a range from faint to strong intensity and

incidence. Only grades of 2+ or higher were recorded as positive. A 50% end point was calculated (where 50% of the wells at that serum dilution show the presence of virus). It was calculated according to Sendecar and Cochran [17].

Virus Purification: It was done according to Woolcocka and Shivaprasad [18]. Positive FAT samples were ultracentrifuged at 100,000 x g (Beckman L5-65 ultracentrifuge, SW50 rotor, Beckman, Palo Alto, California) for 2.5 h at 4°C through a I-2 ml cushion of 30% sucrose (w/v). The pellets were stored in -70°C.

Electron Microscopy: Pellets were resuspended in 0.1 ml of distilled water and one drop of this suspension was placed on 200-mesh formvar carbon-coated grid. The material was stained with a 2% solution of phosphotungstic acid, pH 6.5, as described previously electron microscopic examination [18]. The examination was at the Electron Microscope Unite, National Research Centre, Cairo, Egypt using apparatus model 10 Zesiss, West Germany with 60 kv and resolution of 10°A.

RESULTS

Post Mortem Examination: Intestine was very inflamed and hemorrhagic and their lumen was bloody and granular (Fig. 1).

Virus Isolation and Propagation: Many syncytia appeared in the cultures by 12 hrs post-infection. All syncytia contained several inclusion bodies in the cytoplasm (Fig. 2a). Mitotic figures were occasionally observed within the syncytia (Fig. 2a). By 36 hrs post-infection, most syncytia were necrotic. The number of syncytia containing at least 3 nuclei and inclusion bodies increased rapidly by 18 hrs, reached a maximum at 24 hrs and then decreased from 30 hrs post-infection (Fig. 2b). The number of nuclei per syncytium increased between 12 and 24 hrs post infection (Fig. 2b).

Serum Neutralization Test (SNT): The infectivity titer of prepared virus was decreased 2.98 (log₁₀) when treated with specific anti ARV hyperimmune serums (Table 1).

Fluorescent Antibody Technique (FAT): Both infected and noninfected CER cells formed a complete monolayer within 24 hrs after seeding onto the microculture plates. Although no cytopathic effect (CPE) was seen in infected

Table 1: Neutralizing index of isolated virus

Virus titer alone/ml(log10)	Titer with 4NU of anti ARV antibodies	Neutralizing index
7.74	4.76	2.98

Table 2: Results of FAT in different Governorates

The mean reciprocal of serum dilution end point	Governorates		
	Qalubia	El-Sharkia	El-Gharbia
	512	422	265



Fig. 1: Show very inflamed and hemorrhagic (a). The intestine lumen was bloody and granular (b)

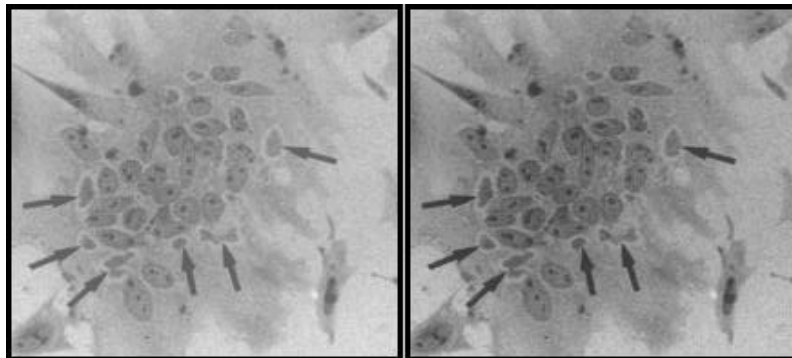


Fig. 2: Syncytia induced by ARV infection. This giant cell has many nuclei and inclusion bodies (arrows) in its cytoplasm, 100X

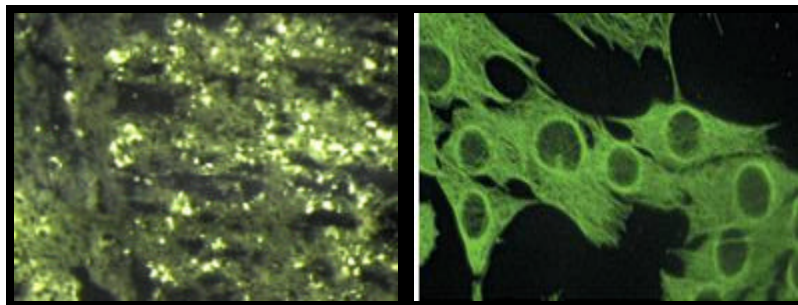


Fig. 3: Bright intracytoplasmic fluorescence was seen when reovirus infected CER cells. Cells were examined 24 hours postinfection by the indirect fluorescent antibody method. X100

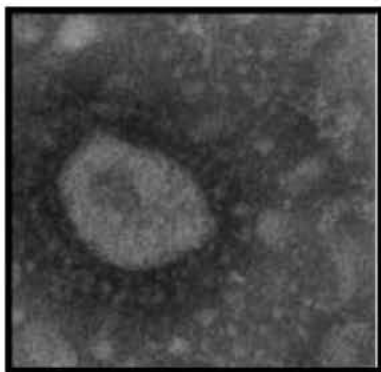


Fig. 4: The isolated reovirus electronmicrography X150,000

cultures at this time, the indirect fluorescent antibody method using positive serum revealed bright granular and diffuse intracytoplasmic fluorescence (Fig. 3) which progressively decreased in intensity and incidence (number of cells showing fluorescence) and eventually disappeared as the serum was diluted. No fluorescence was seen in negative controls. The mean of the end point of serum Antibodies of each governorate was shown in Table 2.

Electron Microscopy: Reoviruses (Fig. 4) averaged 75-80 nm in diameter. The virus contains two capsids outer and inner. The Reovirus is featureless.

DISCUSSION

Avian reoviruses have been associated with viral arthritis/tenosynovitis, malabsorption syndrome, stunting/runting syndromes, enteric disease, immunosuppression and respiratory disease. Reoviruses have also been isolated from birds with mal-absorption/mal-digestion/pale bird syndrome. Affected birds are stunted, unthrifty, have poor feed conversions and generally look sick. Orange tinged diarrhea may be present as can be various degrees of diarrhea and mal-digestion. Some birds may lose color in the legs and beak while others may have various feather problems. Mortality is usually low although numerous birds can be affected.

In the present study SNT, FAT and EM were used for virological investigations and their efficiency was compared for routine examination of specimens, because of their relative simplicity speed and sensitivity.

Virus propagation showed typical CPE of reovirus, especially syncytia formation. The present study showed

clearly that nuclei in the ARV-induced syncytia had a proliferating ability before the nuclear number had reached a maximum which comes in agreement with Biswas *et al.* [10]. SNT is a very sensitive technique, especially for enteric viruses. In the current study it was used to identify the virus and the virus infectivity decreased in concentration increased manner [15], where the infectivity titer of prepared virus was decreased 2.98 (log₁₀) when treated with specific anti ARV hyperimmune serums. Moreover, FAT was used to compare the incidence of the virus in different governorates, whereas, incidence of the virus was the highest in Qalubia followed by El-Sharkia then El-Gharbia Governorates. Bright granular and diffuse intracytoplasmic fluorescence indicates presence of the virus and its component inside the cytoplasm where it replicate which is very pathognomonic to ARV this agrees with Ide [16]. While EM examination showed reovirus outer and inner membrane, which comes in agreement with Woolcocka and Shivaprasad [18]. Pu *et al.* [6] and Biswas *et al.* [10] used the same tests and they were very reliable to diagnose ARV.

In conclusion, this study showed that the causative agent of the reported symptoms and deaths was ARV. The study also proved that SNT, FAT and EM are very accurate and reliable tests to detect avian reovirus outbreak.

REFERENCES

1. Clarke P. and K.L. Tyler, 2007 Down-regulation of cFLIP following reovirus infection sensitizes human ovarian cancer cells to TRAIL-induced apoptosis. *Apoptosis*, 12: 211-223.
2. Glass, S.E., S.A. Naqi, C.F. Hall and K.M. Kerr, 1973. Isolation and characterization of a virus associated with arthritis of chickens. *Avian Disease*, 17: 415-424.
3. Joklik, W.K., 1983. The members of the family reoviridae. In: Joklik WK, Editor, *The Reoviridae*. New York/London: Plenum, pp: 11-30.
4. Kobayashi, T., L.S. Ooms, J.D. Chappell and T.S. Dermody, 2009 Identification of Functional Domains in Reovirus Replication Proteins {micro} NS and {micro} 2. *J. Virolol.* Jan 28. [Epub ahead of print]
5. Demordy, T.S., M.L. Nibert, R. Bassel-Duby and B.N. Fields, 1990. A s1 region important for hemagglutination by serotype 3 reovirus strains. *J. Virol.*, 64:5173-5176.
6. Pu J, X. Liu, Y. Guo, Y. Cao, J. Zhao and G. Zhang, 2008. Seroprevalence of avian reovirus in egg-laying chicken flocks in China. *Avian Disease*, 52: 675-679.

7. Weiner, H.L., K.A. Ault and B.N. Fields, 1980. Interaction of reovirus with cell surface receptors. I. Murine and human lymphocytes have a receptor for the hemagglutinin of reovirus type 3. *J. Immunol.*, 124: 2143-2148.
8. Ni, Y. and R.F. Ramig., 1993. Characterization of avian reovirus-induced cell fusion: the role of viral structural proteins. *Virology*, 194: 705-714.
9. Schiff, L.A. and B.N. Fields, 1990. Reoviruses and their replication. In B.N. Fields, D.M. Knipe, *et al.* editor, *Virology*. Raven Press, New York, N.Y., pp: 1275-1306.
10. Biswas, P.K., H. Barua, G.M. Uddin, D. Biswas, A. Ahad and N.C. Debnath, 2009. Serosurvey of five viruses in chickens on smallholdings in Bangladesh. *Preventive Veterinary Medicine* Jan 1, 88 (1): 67-71.
11. McNulty, M.S., 1993. Reovirus. In: *Virus infection of birds*. J.B. McFerran and M.S. McNulty (Eds.). Oxford: Elsevier Science Publishers, pp: 81-193.
12. Robertson, M.D. and G.E. Wilcox, 1986. Avian reovirus. *Veterinary Bulletin*, 56: 154-174.
13. Rosenberger, J.K. and N.O. Olson, 1991. Reovirus infection. In: *Disease of Poultry*, Calnek, B.W., H.J. Barnes, C.W. Beard, W.M. Reid and H.W. Yoder editors. 9th Edn. Ames: Iowa State University Press, pp: 639-647.
14. Duncan, R., Z. Chen, S. Welsh and S. Wu, 1996. Avian reovirus-induced syncytium formation is independent of infectious progeny virus production and enhances the rate, but is not essential, for virus-induced cytopathology and virus egress. *Virology*, 224: 453-464.
15. Madbouly, H.M., M.A.B. Sagheer, N.S.S. Ata, M.A. Kutkat and K.S. Zaher, 2005. Identification and purification of a local isolate of ILTV. *Egyptian J. Vet. Sci.*, 39: 11-20.
16. Ide, P.R., 1982. Avian Reovirus Antibody Assay by Indirect Immunofluorescence Using Plastic Microculture Plates. *Canadian J. Comparative Med.*, 46: 39-42.
17. Snedecar, G.W. and W.G. Cochran, 1976. *Statistical methods*. The sixth edition, Iowa State University Press, Ames, Iowa, United States.
18. Woolcocka, P.R. and H.L. Shivaprasad, 2008. Electron microscopic identification of viruses associated with poultry enteritis in turkeys grown in California 1993-2003. *Avian Disease*, 52: 209-213.