

## **SIGNIFICANCE OF VIRAL TITRE IN REISOLATION OF *AVIAN PARAMYXOVIRUS 1* IN CHICKEN EMBRYOS**

**Kiril Dimitrov**

*National diagnostic and research veterinary medical institute  
Bulgaria, Aksakovo, 9154, 2 "Batova" str.  
dimitrov\_kiril@yahoo.com*

### **ABSTRACT**

*Avian paramyxovirus 1* is a pathogen of huge social and economic importance, and the disease it provokes is often devastating, especially in domestic fowl. The isolation and reisolation of the virus in chicken embryos followed by typing in HA and HIA tests is the "gold standard" in disease diagnostics. The present report describes a study on the amount of Newcastle disease virus in the inoculum for its reisolation in chick embryos. It was found out that when the initial haemagglutination titre was high, the dilution of allantoic fluid in *APMV 1* serial passage in chick embryos resulted in higher virus yield.

*Key words: AMPV 1, NDV, reisolation, HI titre, dilution.*

### **INTRODUCTION**

*Avian paramyxovirus 1* (*APMV 1* = Newcastle disease virus, NDV) causes the Newcastle disease (ND) in birds. NDV is a pathogen of huge social and economic importance, and the disease it provokes is often devastating, especially in domestic fowl (Alexander, 1988; Pfitzer et al., 2000). A number of tests for ND diagnostics are available. Conventional diagnostic approaches provide reliable information. The most commonly used method for NDV isolation is the infection of chicken embryos (VI). They are the most appropriate biological medium for virus replication. Chick embryos are inoculated with the supernatant from suspensions obtained from organs and tissues of dead or sick birds. The medium from processing of cloacal or tracheal swabs could be also used as inoculum. Inoculation is done aseptically in the allantoic cavity with an amount of 0.1 to 0.3 ml. Infected embryos are incubated at 37°C for 96 to 120 hours and candled once or twice daily depending on the experimental design. VI is used also both for reisolation and replication of NDV. The presence of *APMV 1* in the allantoic fluid of infected embryos is confirmed by means of the haemagglutination (HA) and haemagglutination inhibition (HIA) assays with known monospecific serum (Senne, 1998). HA is used for detection of haemagglutinating agents and their titration. It is performed as drop agglutination as well as in microvariant – in microtitre plates. Suspensions of washed red blood cells (most commonly chicken, but also from turkeys) are used. This reaction could not be used as a stand-alone proof for the presence of NDV, as other avian viral pathogens (orthomyxoviruses, coronaviruses, adenoviruses) also exhibit haemagglutination activity (Gulka et al., 1984; Jonassen et al., 2005; Hafez, 2011).

HIA is described for the first time by Hirst (1941) in a study on influenza viruses. The method is widely used for detection of isolated haemagglutinating agent such as the ND virus. It is based on the ability of antibodies to inhibit haemagglutination activity of the HN-antigen of NDV. A serious drawback of the HIA test is that it is very laborious. Due to cross-reactivity between some different *APMV* types, for instance 1 and 3 (Stanislawek et al, 2001; Nayak et al., 2012), the interpretation of results could be impeded. In Bulgaria, the method is successfully used by Surtmadzhiev et al. (1980) for evaluation of the immune status of flocks in industrial poultry farms with respect to Newcastle disease.

The purpose of the present study was to establish the significance of *Newcastle disease virus* titre in reisolation of the pathogen in chick embryos.

**MATERIALS AND METHODS**

Viruses

In this study, two NDV strains were used – NDV/chicken/Kardam/2008 and NDV/pigeon/NovoYankovo/2009, whose features are presented in Table 1.

Table 1. Strains of Newcastle disease viruses used in the present study – species and origin of birds, type of samples.

Isolate	Bird species	Settlement	Region	Sampling date	Sample type
NDV/chicken/Kardam/2008	chickens	Kardam	Dobrich	15.02.2008	carcasses
NDV/pigeon/NovoYankovo/2009	pigeons	Novo Yankovo	Shoumen	05.03.2009	organs

Monospecific serum for HIA.

NDV Ulster 2C serum (manufactured by Istituto Zooprofilattico Sperimentale delle Venezie, Italy) was used.

Biological systems.

Biological systems used for reisolation of ND viruses were 9–11 days-old chick embryos, obtained from disease-free unvaccinated flocks.

Chicken red blood cells.

Chicken red blood cells collected from healthy birds, negative for antibodies against NDV, at 10% and 1% solutions were used. Erythrocytes were obtained after processing of collected blood in Alsever’s solution and triple washing with PBS (pH 7.2-7.4).

Reisolation of *Avian paramyxovirus 1*.

For reisolation of NDV, 0.2 ml of isolates were inoculated in the allantoic cavity of 9–11 days-old chick embryos (three for each strain). The procedure described in the OIE Manual OIE (2012) and Council Directive 92/66/EEC was followed. Infected chick embryos were incubated at 37°C and candled at a daily basis. Upon death occurrence, dead embryos were separated and placed at 4°C, and on the 120<sup>th</sup> hour, those remaining alive were cooled too. The allantoic fluid of each embryo was tested in HA for presence of haemagglutinating agent.

Detection of the affiliation of isolates to *APMV 1* in HIA.

The type of isolates was confirmed in HIA with use of monospecific serum.

The performance of HA and HIA also followed the procedure described in OIE Manual (2012) and Council Directive 92/66/EEC.

Passages of isolates

Isolates were tested in three consecutive passages. For infection of chick embryos in each passage, allantoic fluid from the previous one was used. When the haemagglutination titre was high, the subsequent passages were carried out in two variants:

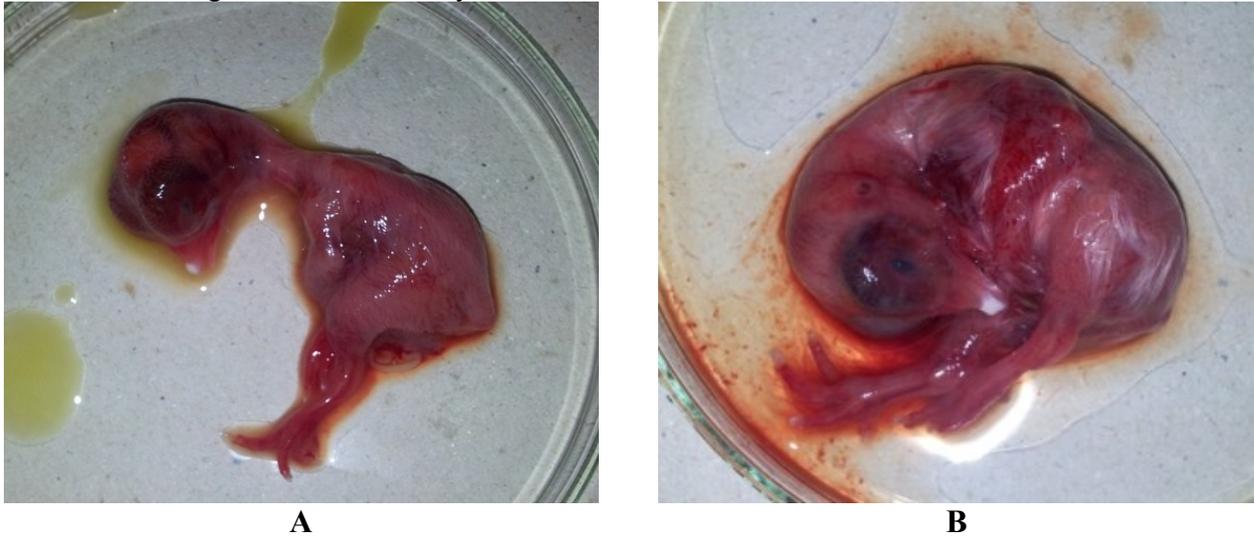
- with undiluted allantoic fluid from the previous passage;
- with inoculum diluted 1/50 when the titre from the previous passage was  $\leq 1/128$  and 1/100 when the titre from the previous passage was  $> 1/128$ .

**RESULTS**

Detection of isolates type in HIA.

Reisolated viruses were titred by means of HA-microvariant. On the basis of determined haemagglutination titres, dilution of isolates at 4 HAU was performed. With this dilution, HIA was carried out with monospecific serum against NDV. The presence of inhibited haemagglutinating activity allowed typing the pathogens as *APMV 1*. At the same time, stunted growth and petechiae on the trunk (Fig. 1A) and occipital area (Fig. 1B) were observed.

Figure 1. Chick embryos infected with the NDV/chicken/Kardam/08 strain.



Passages of isolates

The results from the titration in several consecutive passages in chick embryos of both isolates in the HA-microvariant test are shown in Table 2.

Table 2. Titration of NDV isolates by means of HA-microvariant without dilution of allantoic fluid from the previous passage.

Isolate	Haemagglutination titre of the virus and reciprocal value					
	Ist passage		II-nd passage		III-rd passage	
NDV/chicken/Kardam/2008	1/128	log <sub>2</sub> 7	1/256	log <sub>2</sub> 8	1/256	log <sub>2</sub> 8
NDV/pigeon/NovoYankovo/2009	1/16	log <sub>2</sub> 4	1/128	log <sub>2</sub> 7	1/128	log <sub>2</sub> 7

In the second passage, viral haemagglutination titre increased but in the third passage it was withheld for both isolates. When a preliminary dilution of the source material from previous passages was done, the results were different and haemagglutination titres were increased (Table 3).

Table 3. Titration of NDV isolates by means of HA-microvariant with dilution of allantoic fluid from the previous passage.

Isolate	Haemagglutination titre of the virus and reciprocal value					
	Ist passage		II-nd passage		III-rd passage	
Inoculum dilution			dilution 1/50 of passage I		dilution 1/100 of passage II	
NDV/chicken/Kardam/2008	1/128	log <sub>2</sub> 7	1/512	log <sub>2</sub> 9	1/1024	log <sub>2</sub> 10
Inoculum dilution					dilution 1/50 of passage I	
NDV/pigeon/NovoYankovo/2009	1/16	log <sub>2</sub> 4	1/128	log <sub>2</sub> 7	1/512	log <sub>2</sub> 9

## DISCUSSION

After three passages of isolates in chick embryos, the viral haemagglutination titres were determined in the HA test after each passage. The titre of the pigeon isolate was relatively low after the first passage – 1/16. After the second passage however, it increased, which was most probably due to its adaptation to the biological system. It is interesting to note that in both isolates, no increase in haemagglutination titres has occurred in the third passage. When the experiment was repeated, but with diluted allantoic fluid, haemagglutination titres increased after each passage. This was presumably due to a process, observed for the first time by von Magnus during his study on avian influenza virus (Stein et al., 1976) and termed “von Magnus phenomenon”. The infection with a high viral concentration results in production of virions with structural defects, which lose their antigenic properties. A possible reason for the observed event is also interferon-inducing function of NDV (Marcus et al., 1983). Nagai et al. (1981) demonstrated that L-cells infected with NDV, were not able to synthesize normally viral proteins. According to the authors, this was due to accumulation of interferon (IFN) in the biosystem. After addition of IFN-specific antiserum, the replication of the virus was already normal. At the same time, Huang et al. (2003) and Park et al. (2003) reported that when antigenic shift type mutation occurs, the so-called V-proteins and W-proteins with a strong interferon-antagonistic activity could be produced. Opposite to the present result, Zarkov et al. (2005) reported a stabilisation of haemagglutination activity of NDV isolates from wild birds after consecutive passages in chick embryos. For first-passage haemagglutination titres of 1/2 to 1/8, the authors observed increase up to 1/16, respectively 1/256 in the third passage. In this case however, the isolates were from wild ducks and increased haemagglutination titres could be attributed to the gradual adaptation of the virus to the biological system.

## REFERENCES

1. Alexander, D. J., 1988. Newcastle disease – An Avian Paramyxovirus. In: D.J. Alexander, (Ed). Newcastle disease, Development of veterinary virology, Kluwer Academic Publishers, Boston, p. 1-22.
2. Council of European Union, 1992. Council Directive 92/66/EEC of 14 July 1992 introducing Community measures for the control of Newcastle disease (цитат 03.06.2014 г.). Достъпен на: <http://eur-lex.europa.eu/legal-content/EN/ALL/?uri=CELEX:31992L0066>
3. Gulka, C. M., Piela, T. H., Yates, V. J., Bagshaw, C., 1984. Evidence of exposure of waterfowl and other aquatic birds to the hemagglutinating duck adenovirus identical to EDS-76 virus. *Journal of Wildlife Diseases*, 20(1):1-5.
4. Hafez, H. M., 2011. Avian Adenoviruses Infections with Special Attention to Inclusion Body Hepatitis/ Hydropericardium Syndrome and Egg Drop Syndrome. *Pakistan Veterinary Journal*, 31(2):85-92.
5. Hirst, G. K., 1941. The agglutination of red cells by allantoic fluid of chick embryos infected with influenza virus. *Science*, 94(2427):22-23.
6. Huang, Z., Krishnamurthy, S., Panda, A., Samal, S. K., 2003. Newcastle disease virus V protein is associated with viral pathogenesis and functions as an alpha interferon antagonist. *Journal of virology*, 77(16):8676-8685.
7. Jonassen, C. M., Kofstad, T., Larsen, I. L., Løvland, A., Handeland, K., Follestad, A., Lillehaug, A., 2005. Molecular identification and characterization of novel coronaviruses infecting graylag geese (*Anser anser*), feral pigeons (*Columbia livia*) and mallards (*Anas platyrhynchos*). *Journal of General Virology*, 86(6):1597-1607.
8. Marcus, P. I., Svitlik, C., Sekellick, M. J., 1983. Interferon induction by viruses. X. A model for interferon induction by Newcastle disease virus. *Journal of general virology*, 64(11):2419-2431.
9. Nagai, Y., Ito, Y., Hamaguchi, M., Yoshida, T., Matsumoto, T., 1981. Relation of interferon production to the limited replication of Newcastle disease virus in L cells. *Journal of general virology*, 55( 1):109.

10. Nayak, B., Dias, F. M., Kumar, S., Paldurai, A., Collins, P. L., Samal, S. K., 2012. Avian paramyxovirus serotypes 2-9 (APMV-2-9) vary in the ability to induce protective immunity in chickens against challenge with virulent *Newcastle disease virus* (APMV-1). *Vaccine*, 30(12):2220-2227.
11. OIE. Office International des Epizooties. World Organization for Animals Health. Manual of diagnostic tests and vaccines for terrestrial animals 2012, chapter 2.3.14. (цитат 03.06.2014 г.). Достъпен на: <http://www.oie.int/international-standard-setting/terrestrial-manual/access-online/>.
12. Park, M. S., Shaw, M. L., Muñoz-Jordan, J., Cros, J. F., Nakaya, T., Bouvier, N., Palese, P., Garcia-Sastre, A., Basler, C. F., 2003. Newcastle disease virus (NDV)-based assay demonstrates interferon-antagonist activity for the NDV V protein and the Nipah virus V, W, and C proteins. *Journal of virology*, 77(2):1501-1511.
13. Pfitzer, S., Verwoerd, D. J., Gerdes, G. H., Labuschagne, A. E., Erasmus, A., Manvell, R. J., Grund, Ch., 2000. Newcastle Disease and Avian Influenza A Virus in Wild Waterfowl in South Africa, *Avian Diseases*, 44, p. 655-660.
14. Senne, D. A., 1998: Virus propagation in embryonating eggs. In: Swayne, D. E., J. R. Glisson, M. W. Jackwood, J. E. Pearson, W. M. Reed (Eds.), *A Laboratory Manual for the Isolation and Identification of Avian Pathogens*, 4th edn, American Association of Avian Pathologists, Kennett Square, 235–240.
15. Stanislawek, W. L., Meers, J., Wilks, C., Horner, G. W., Morgan, C., Alexander, D. J., 2001. A survey for paramyxoviruses in caged birds, wild birds, and poultry in New Zealand. *New Zealand Veterinary Journal*, 49(1):18–23.
16. Stein, P., Gann, M. K., Baxter, W. L., 1976. Ultrastructural studies of influenza C. II. von Magnus phenomenon. *Microbios*, 15(60):135-43.
17. Surtmadzhiev, K., Gerganov, G., Kolarova, B., 1980. Establishing the immune status of poultry flocks against Newcastle disease in commercial poultry raising, *Veterinarnomedicinski nauki*, 17(5):25-31.
18. Zarkov, I., Bochev, I., Oreshkova, N., Manvell, R., 2005. Isolation of avian paramyxovirus – 1 (APMV-1) from wild feral birds, *Bulgarian Journal of veterinary Medicine*, 8(3):173-181.