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ISOLATION OF AVIAN PARAMYXOVIRUS TYPE 1 (APMV-1) FROM FREE-LIVING MALLARDS (*ANAS PLATYRHYNCHOS*) (PRELIMINARY COMMUNICATION)

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Summary

Zarkov, I., I. Bochev, N. Oreshkova & R. Manvell, 2005. Isolation of avian paramyxovirus type 1 (APMV-1) from free-living mallards (*Anas platyrhynchos*) (Preliminary comminication). *Bulg. J. Vet. Med.*, **8**, No 3, 173–181.

In our attempts of virus isolation we examined 236 samples (faecal, cloacal, intestinal content, visceral organs). The samples were obtained from 16 species of wild, free-living birds from 06.12.2003 to 25.03.2004. They were collected from 5 different areas, situated in Southeastern Bulgaria.

Viruses were isolated from the 3 consecutive passages, carried out on chicken embryos, in 5 samples (2.12% of the total number). All of them were from a single avian species (*Anas platyrhynchos*) and from one region.

The isolates were defined by the haemagglutination test, followed by the haemagglutination inhibition test, as avian paramyxovirus type 1. In a consecutive study through RT-PCR carried out in the UK (Veterinary Laboratories Agency Weybridge, UK) they were related to the lentogenic representatives.

Key words: APMV-1, *Anas platyrhynchos*, ducks, mallards, virus isolation

INTRODUCTION

Avian paramyxoviruses belong to the Avulavirus genus of the Paramyxovirine subfamily, Paramyxoviridae family (Mayo, 2002). There are 9 serotypes (APMV 1–9). A prototype of the most important one (APMV-1) is the Newcastle disease virus (NDV). Also, viruses with various virulence are included (lentogenic – avirulent and mildly virulent, mesogenic – moderately virulent, velogenic – markedly virulent). In pathogenic APMV-1, the Fo glycoprotein (fusion glycoprotein) could be cleaved by cellular proteases to functionally active segments F1 and F2. Lentogenic viruses are dependent on trypsin-like enzymes. The latter are found in respiratory and gastrointestinal tracts and this results in viral localization. The determination of amino acid sequence of the Fo glycoprotein (at the site of cleavage) between positions 112 and 117 is an important genetic test included in the prerequisites to define isolate's pathogenicity (Nagai *et al.*, 1976; Anonymous, 2002; Zorman *et al.*, 2002).

The hosts of APMV-1 are among about 250 avian species from the Gallinaceae family (Alexander, 1997) – domestic and wild (Mackenzie *et al.*, 1984; Lipkind

et al., 1987; Alexander, 1991; Graham *et al.*, 1996; Shihmanter *et al.*, 1997; Pfitzer *et al.*, 2000; Clavijo *et al.*, 2001; Zohari *et al.*, 2001; Ursula *et al.*, 2002; Jorgensen *et al.*, 2004). The presence or absence of disease and its severity in affected birds depends on viral properties as well as on the avian species and the rearing conditions. Chickens are more sensitive whereas goose and ducks – more resistant (Alexander, 1995; 1997).

The role of free-living waterfowl in APMV-1 distribution is important (Muller *et al.*, 1999). They are less sensitive to the development of clinical disease despite the presence of infection (Alexander, 1995). Migrating waterfowl that are carrying or releasing the virus are moving in large flocks and contribute to virus dissemination (elements, present in our country along the migration pathway Via Pontica).

The isolation of the agent is the most positive and preferred diagnostic approach that directly confirms or rejects the presence of APMV-1 in good-quality clinical material. For primary isolation, the most suitable and most commonly used are chick embryos (CE). In successful isolation, there is an opportunity for more extensive studies of identification and differentiation of isolates that are carried out by various serological, antigenic, molecular-genetic and immunological tests.

Various factors are influencing the results obtained by the isolation method such as the use of suitable specimens, way of storage, proper system of isolation etc.

The aim of this study was to determined the potential prevalence of avian paramyxoviruses in mallards in Bulgaria and to characterize the respective isolates.

MATERIALS AND METHODS

Birds

A total of 236 wild free-living birds, belonging to 5 orders, 5 families, 9 genera and 16 species were studied (Table 1).

Table 1. Avian species and number of obtained samples included into analyses

Order	Family	Genus	Species	Samples
Gaviformes	Gavidae	Gavis forst	*Gavia arctica*	1
Anseriformes	Anatidae	Anser	*Anser albifrons*	59
			Anser anser	28
			Anser erythropus	5
		Branta scop.	*Branta ruficollis*	4
		Anas	*Anas clypeata*	1
			Anas crecca	1
			Anas platyrhynchos	54
			Anas Penelope	2
		Todorna flem.	*Todorna todorna*	1
		Aythia boie	*Aythia ferina*	1
Charadriiformes	Laridae	Larus	*Larus melanocephalus*	4
			Larus cachinans	35
Pelecaniformes	Phalacro-coracidae	Phalacro-corax briss	*Phalacrocorax pygmaeus*	2
			Phalacrocorax carbo	17
Gruiformes	Rallidae	Fulica	*Fulica atra*	21
			Total number	236

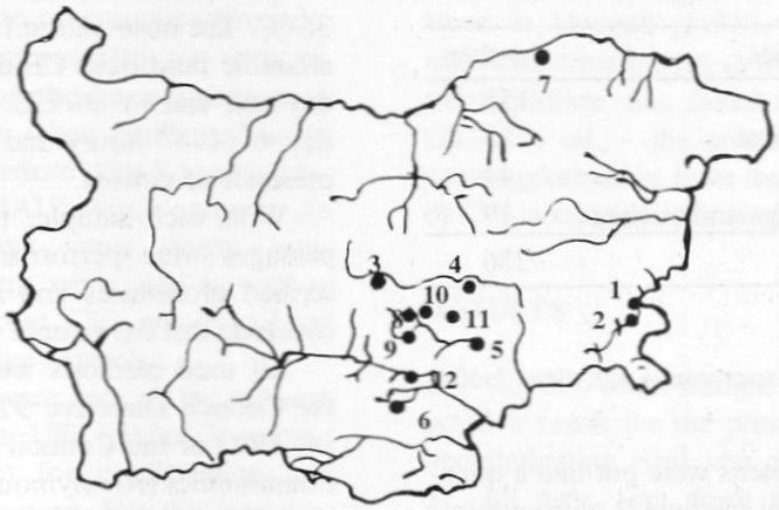

Fig. 1. Map of Bulgaria showing the places from which the samples were obtained: 1 – Burgas, Vaya lake; 2– Burgas, Mandra dam, the Poda zone; 3 – Koprinka dam; 4 – Zhrebchevo dam; 5 – Ovcharitsa dam; 6 – Trakiets dam; 7 – Silistra, Srebarna; 8 – Stara Zagora, Zeleni Balkani; 9 – Madzherito; 10–Badeshte; 11 – Sabrano; 12 – Dimitrovgrad.

Geographical regions

The overall number of studied areas was 12. The inhabitat of studied birds was Southeastern Bulgaria with the exception of 2 specimens obtained from the region of Silistra (Fig. 1). The inhabitat included water basins populated by waterfowl. The larger number of birds originated from 5 water basins: 4 dams (Koprinka, Ovcharitsa, Trakiets, Mandra-Poda zone) and 1 lake (Vaya). Two of them were in the region of the Burgas moist zones – Poda and Vaya. In the region of the dam Ovcharitsa, the specimens were obtained from its both ends – in the vicinity of Radnevo town and Skalitsa village. From an ornithological point of view, important inhabitats were Poda zone (protected area since 20.04. 1989, *Official Gazette (Bulgaria)* 37/1989), Vaya lake – (protected area since 30.11.1973, *Official Gazette (Bulgaria)* 101/1973) and Ovcharitsa dam.

Specimens

For virus isolation, specimens from the gastrointestinal tract (faecal, intestinal content, cloacal samples) and the respiratory system (lung, trachea) were used. The total number of gastrointestinal samples was 217, with prevailing of faecal ones– 155 (Table 2). Respiratory samples were 19. Fresh faecal samples were collected from the shores of water basins after chasing away the birds from places where they gathered together. Cloacal samples were obtained from birds captured by ornithologists or by us. Intestinal content and parts of pulmonary organs were collected from dead birds (killed in a chase etc.) The specimens were obtained within the period 06.12.2003 – 25.03.2004, when the active migration of wild free-living birds occurred.

Table 2. Type of analysed materials

Type of samples	Number
Faecal	155
Intestinal content	18
Cloacal	44
Viscera – Lungs and trachea	19
Total	236

Preparation of specimens for virus isolation

Obtained specimens were put into a transportation nutrient medium B with antibiotics – penicillin G 2×10^6 IU/L (Antibiotic Co, Bulgaria), streptomycin 200 mg/L (Antibiotic Co, Bulgaria), polymyxin B 2×10^6 IU/L (Fluka Biochemika, Switzerland), gentamicin sulfate 250 mg/L (Veterin S.A.), nystatin dihydrate 0.5×10^6 IU/L (Fluka Biochemika, Switzerland), sulfamethoxazole 0.2 g/L (Fluka Biochemika, Switzerland). It was also supplemented with sterile 0.5% foetal calf serum and the pH was corrected to 7.2–7.4. The medium was poured into 2 mL flasks prior to the transportation. The ratio sample:medium was about 10–20 % (w/v).

The obtained specimens were processed by manual shaking, centrifugation at 1000 g for 20 min, filtration with filters (Syringe filter Cameo 17GP – Sigma, cat No S 6062) with pore size of 0.22 μm and volume of 12 mL, then they were transferred into new sterile flasks and stored at temperatures not higher than–30 °C until analysis.

Virus isolation

In our experiments, 9–10-days old CE were used, inoculated with 0.3 mL of the prepared suspension into the allantoic cavity. Three embryos were used for each sample. Inoculated CE were incubated at 36 °C. The observation lasted 6 days. The allantoic fluid from CE died before the 6th day and that of all CE was collected by day 6 (144th hour) and was studied for presence of viruses.

With each sample, three consecutive passages were performed using the described procedures and methods prior to conclude that the sample was virus-free.

All used methods were complying to the Council Directive 92/66/EEC of July 14, 1992 of the Council of the European Communities (Anonymous, 1992).

Isolate identification

The presence of haemagglutinating viruses was proved by the haemagglutination (HA) test (with 1% chick erythrocytes) in its micro variant (MHA test) with component volumes of 0.05 mL by the routine technique (Hirst, 1942) in polypropylene plates with 96 wells (Corning Inc. costar, № J14831, USA).

The identification of haemagglutinating isolates as APMV serotype 1 was done by the standard β procedure haemagglutination inhibition test (Salk, 1944) in its micro variant (MHI test). The samples with haemagglutination titres of 1:8 were accepted as positive. A polyclonal hyperimmune serum (HIS) obtained against APMV-1 (La Sota strain) in twofold increasing dilutions from 1:2 to 1:1024 was used.

The HIS was obtained from 5 chickens at the age of 4 months, sensitive and non-vaccinated against NDV. They were treated with undiluted allantoic fluid of vaccinal NDV La Sota strain (with MHA test titre 1:256), cultivated in CE. The chickens were inoculated three times (for a 75-day period at 21-day intervals) with increasing doses alantoic fluid of 1 mL, 2 mL and 3 mL. For the 2nd and 3rd inocula-

tion, the allantoic fluid was supplemented with equal volume of complete Freund's adjuvant. The obtained HIS was with antibodies, inhibiting the haemagglutination in titres of 1:512, when performed using the standard procedure with 8 haemagglutination units (HAU) viral antigen. As controls, negative sera from non-inoculated chickens were used as well as allantoic fluid from CE, non-inoculated with virus (negative controls).

The isolates were sent to the referent laboratory in the UK (Avian Virology, VLA Weybridge) for confirmation and pathological typization. For this purpose, they were studied in a reverse-transcriptase polymerase chain reaction system (RT-PCR).

Isolate titration

The titration of viruses was done in CE (6 for each dilution) and serial 10-fold dilutions of the virus from 10^{-1} до 10^{-12}, at a volume of 0.1 mL/CE. The 50% embryo lethal dose (ELD_{50}/0.1 mL) and the 50% embryo infective dose (EID_{50}/0.1 mL) were determined by the method of Reed & Muench (1938). For ELD_{50}/0.1 mL determination, the death rate of CE in viral dilution was detected whereas for EID_{50}/0.1 mL – the presence of positive haemagglutination from the allantoic fluid of CE, inoculated with viral dilutions.

RESULTS

In 5 (2.12%) of all studied 236 samples, a positive result for the presence of a haemagglutinating virus was obtained (Table 3). All they were from mallards (*Anas platyrhynchos)* originating from the Burgas region and both water basins– 2 avian specimens (7.14% positive) collected in the Poda zone inhabitat and 3 (16.67% positive) – in Vaya lake inhabitat.

The haemagglutination after passages has become stable (Table 4). After the third passage, the titres increased to 1:16 – 1:256. The maximum titres in 4 isolates (No. No. 15, 17, 23, 35) were 1:16, whereas in 1 (No. 5) – 1:256.

Table 3. Results from the attempts of virus isolation

Region	Number of studied samples	Samples with RHA – number (%)	Positive sample No
Burgas – Vaya lake	18	3 (16.67%)	5, 15, 17
Burgas – Poda zone	28	2 (7.14%)	23, 35
Koprinka dam	63	0	
Zhrebchevo dam	1	0	
Ovcharitsa dam	58	0	
Trakiets dam	43	0	
Silistra – Srebarna	2	0	
Stara Zagora – Zeleni Balkani	8	0	
Madzherito	4	0	
Badeshte	7	0	
Sabrano	3	0	
Dimitrovgrad	1	0	
Total	236	5 (2.12%)	

Table 4. Results from the haemagglutination reaction (RHA) of isolates from passages I, II and III

Region	Sample No.	Passages with haemagglutination and titres		
		I	II	III
Burgas – Vaya lake	5	1:8*	1:32*	1:256*
Burgas – Vaya lake	15	1:2	1:8	1:16*
Burgas – Vaya lake	17	0–1:2	1:8	1:16*
Burgas – Poda zone	23	1:2	1:8	1:16*
Burgas – Poda zone	35	0	1:8	1:16*

* the embryos died

In one sample, infected CE died in the first passage (No. 5) and for the other specimens – only after the 3rd passage. The lethality in all occurred after the 5th day (hour 120th) of the inoculation. In infected and dead CE there were haemorrhages on the embryo (Fig. 2) and on the chorioallantoic membrane (Fig. 3).

After titration of the isolate from sample No. 5, originating from Burgas – Vaya lake (Table 4) at the 5th passage level there was a difference in ELD_{50}/0.1 mL and EID_{50}/0.1 mL. The average embryo lethal dose was $10^{-6.5}$ ELD_{50}/0.1 mL, whereas the average embryo infective dose – $10^{-10.17}$ EID_{50}/0.1 mL (Fig. 4).

There was a difference in the values of haemagglutinins from the allantoic fluid for the various dilutions. The highest values were observed in CE inoculated with virus in dilution of 10^{-2}. In dilutions 10^{-2},10^{-3}, 10^{-4} and 10^{-5}, the values were higher than the observed average HA concentrations obtained in all used dilutions.

A 100% death rate of CE occurred in dilutions of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}.

The data from the RT–PCR study of the pathotype (Table 5) showed that the sector of the genome between the 112th and the 117th amino acids of studied field APMV-1 isolates from ducks was with equal sequence to those of reference lentogenic APMV-1 La Sota and Hitchner B1 strains (Table 5).

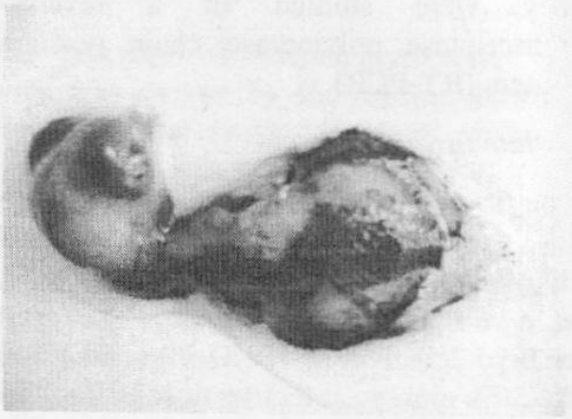

Fig. 2. Haemorrhages on the embryo (isolate of sample No. 5, 3rd passage), 2048×1536 ×24B.

Fig. 3. Haemorrhages on the chorioallantoic membrane (isolate of sample No 5, 3rd passage) 2048×1536× 24B.

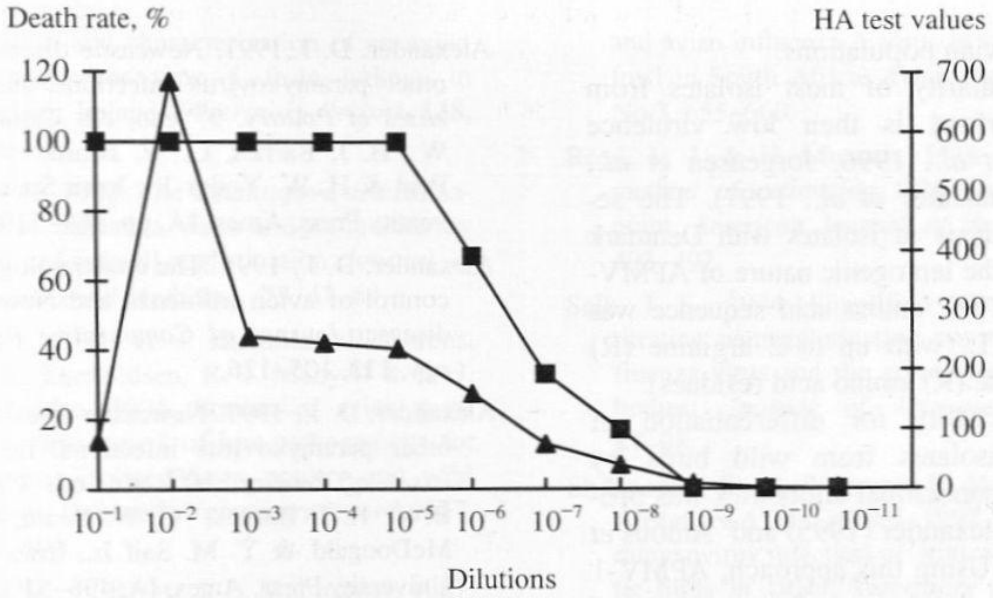

Fig. 4. Summarized data of titrages of APMV-1 isolate. Death rate percentage for the different dilutions (–■–) (the average death rate for all dilutions in the range from 10^{-1} to 10^{-11} was 75%); HA test values for the different dilutions (–♦–) (the mean HA test value for all dilutions in the range from 10^{-1} to 10^{-11} was 1:178).

Table 5. Amino acid sequence in the fusion glycoprotein (F0 glycoprotein) between functionally active segments F_1 and F_2

Strain	Amino acid sequence between F1 and F2	Group
Velogenic strain Herts 33 (referent)	.....112**RRQRR**↓F118.....	
Lentogenic APMV-1La Sota, Hitchner B1 strains (referent)	.....112G**R**QG**R**↓L118.....	Group E
Isolate (tested)	.....112G**R**QG**R**↓L118.....	

DISCUSSION

In Bulgaria, this is the first case of APMV-1 isolated from mallards. From the literature data it is seen that the reports for APMV-1 isolates from this avian species are relatively few (Alexander & Manvell, 2001a; Alexander & Manvell, 2001b). Such data were reported for Denmark (Jorgensen *et al.*, 2004), Israel (Shihmanter *et al.*, 1997), North Ireland (Graham *et al.*, 1996), but only in Denmark and Israel the isolates were from mallards.

The isolation of APMV-1 strains from a single region suggests the following possible sources with regard to their origin:

- transfer of a vaccinal strain from domestic birds, in which regular vaccinations with lentogenic NDV strain have been performed;
- persistence and circulation of the virus in wild birds at inhabitats with a large population and big diversity of species.

The hypotheses of APMV-1 isolates' origin from mallards in Denmark, where domestic birds are not vaccinated assumed a natural circulation of lentogenic NDV strains and an endemic distribution among

domestic avian populations.

A peculiarity of most isolates from wild waterfowl is their low virulence (Graham *et al.*, 1996, Jorgensen *et al.*, 2004, Shihmanter *et al.*, 1997). The sequence analysis of isolates with Denmark confirmed the lentogenic nature of APMV-1 strains. Their amino acid sequence was GGKQGR↓L (with up to 2 arginine (R) and/or lysine (K) amino acid residues).

A possibility for differentiation of APMV-1 isolates from wild birds by means of monoclonal antibodies was proposed by Alexander (1995) and Aldous *et al.* (2003). Using this approach, APMV-1 strains were divided into 3 groups, one of them being group E, where a part of APMV-1 strains from Denmark, our isolates and vaccinal NDV strains (B1, La Sota) do belong. Those data allow to work on the hypotheses upon the origin of APMV-1 isolates from wild birds, i.e. whether they are vaccinal or other, specific for wild birds circulating strains.

The data obtained in Israel (Alexander, 1991) showed a mixed infection in mallards with APMV-1 and APMV-4. In our samples, this was not observed.

In conclusion, for the first time in our country, AMPV-1 were isolated from freely living mallards *(Anas platyrhynchos).* The isolates originated from one region in Southeastern Bulgaria and were from a single avian species. The isolated strains were lentogenic and possessed the characteristics of vaccinal strains, used in Bulgaria.

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