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Original Contribution

MOLECULAR APPROACH IN INVESTIGATION OF NEWCASTLE DISEASE STRAINS, ISOLATED IN BULGARIA DURING 2005-2007.

N. Oreshkova, G. Goujhgoulova, G. Georgiev, K. Dimitrov*

National Diagnostic and Research Veterinary Medicine Institute "Prof. Dr. G. Pavlov", Bulgaria, Sofia

ABSTRACT
Newcastle disease virus is a pathogen of major social and economic importance. The disease it provokes is often devastating, particularly in domestic poultry. The infection in wild birds, especially aquatic species, is often asymptomatic and they are therefore considered to be a reservoir of the virus in the wild. Several teams of scientists have proposed different grouping of the isolates from all over the world, according to genetic similarity. The molecular analysis of the Bulgarian strains isolated from poultry outbreaks during 2005-2007 and also from wild birds in the same period, allows determination of the presumable origin of the viruses.
KEYWORDS
Newcastle disease, Bulgaria, molecular, epizootology

INTRODUCTION

Taxonomically, the Newcastle disease virus belongs to Order Mononegavirales, family Paramyxoviridae, subfamily Paramyxovirinae, genus Avulavirus. The Avulavirus genus includes 9 serologically distinct avian paramyxoviruses (APMV-1 – APMV-9) (1, 2). APMV-1 is synonymous to the Newcastle disease virus (NDV), and the term pigeon paramyxovirus type 1 is used to distinguish the antigenic variant of APMV-1 (3). The natural hosts of APMV-1 are all domestic and many wild avian species (1, 4, 5, 6).

The penetration of the virus in the host cell occurs by fusion and is mediated by a protein of the external viral envelope, called fusion-protein (F-protein). It is synthesized as a precursor (F0) and needs to be cleaved into two smaller fragments in order to be active (F1 and F2). Studies evidence, that the pathogenicity of the ND strains is determined by the amino acid sequence at the site of F-protein cleavage (7, 8). The proteins with single basic amino acid sequences at the cleavage-site could be hydrolyzed only by tripsin and similar enzymes, present in a restricted number of tissues and organs in the organism of susceptible animals. Thus, viruses with such F-protein possess a limited tropism and cause subclinical or unapparent infections. Unlike them, proteins with several basic amino acids (arginine - "R" and lysine - "K") at the site of F-protein cleavage as well as phenylalanine at position 117 (9) could be cleaved by proteases, present in cells everywhere in the macroorganism. This determines the generalized infection with severe course, caused by viruses with such F-protein.

In Bulgaria, the Newcastle disease was first detected in 1943 by Semerdzhiev (10) and the author assumed that the infection was probably introduced from Romania. Later on, Hrischev (11) demonstrated that the origin of the first isolated virus is Germany and it was imported with food. During the following years, the infection spread throughout the whole country and until 1982 multiple outbreaks were registered each year.

Several investigations for genetic typization of Newcastle disease isolates have been carried out. The first one was that of Ballagi-Pordany et al. (12) and it was performed with more than 200 isolates by means of restriction site analysis. On this background, six major groups of isolates have been established (I – VI). Using the same method, Lomniczi et al. (13) studied isolates involved in the epizootics in West Europe in 1992-1996 and concluded that they belonged to 2 various

*** Correspondence to**: *Kiril Dimitrov, Bulgaria, 9154 Aksakovo, 2 "Batova" str., phone/fax: +359 52764427, e-mail: dimitrov_kiril@yahoo.com*

genotypes. Some of the viruses belonged to group VI. Unlike them, the viruses that caused epizootics in Germany, Belgium, Spain and Italy, could be classified in a new group – VII, undiscovered in Europe so far. The information was further completed by Herzeg (14), who added a new group – VIIb, that unlike VIIa (the previous VII) comprises isolates from North Africa and Mozambik obtained during 1990 to 1995 and isolates from Bulgaria and Greece from the next two years. At the same time, the author recognizes also group VIII from so far unknown European isolates, originating from South Africa. E. Aldous et al. (15) performed a new research on 174 isolates of avian paramyxoviruses type 1 on the basis of sequencing of the viral F-protein genome. They divided the isolates into 6 groups and several subgroups, summarizing the available information **(Table 1)**.

Table1. *Genetic groups of Newcastle disease isolate (Aldous et al. (15)*

Group	Sub group	Old genetic group	Divergence (%)	Year	Pathotype	Geographic origin	Hosts
1		I	14.3	1967-2000	Asymptomatic intestinal	Worldwide	ducks, chickens
2		II	8	1945-2000	Mixed	Worldwide	chickens, ostriches
3			25				
	3a	III		1932-1999	Velogenic	Asia, Australia, Europe	chickens
	3b	IV		1933-1989	Velogenic	Asia, Africa, Europe	parrots, chickens
	3c	V		1970-1997	Velogenic	Africa, America, Europe	cormorants, chickens peasants, turkeys
	3d	VIII		1965-1994	Velogenic	Asia, Africa, Europe	chickens, turkeys
4			29.3				
	4a	VIa/VIe		1968-1996	Velogenic	Africa, Europe, Near East	falcons, chickens
	4b	VIb		1984-2000	Velogenic	Europe, Near East	pigeons
	4c	VIc		1989-1999	Velogenic	Europe, Near East	falcons, chickens
	4d	VId		1989-1999	Velogenic	Europe	chickens, pigeons, ostriches
5			15.9				
	5a	VIIa		1988-1996	Velogenic	Europe	chickens, pheasants
	5b	VIIb		1982-2000	Velogenic	Asia, Africa, Europe	chickens, pheasants, falcons, turkeys
	5c	VIIc		1984-1999	Velogenic	Asia, Europe	chickens, pigeons
	5d	VIId		1997-2000	Velogenic	Asia, Near East	chickens, ostriches
	5e			1994-1997	Velogenic	Taiwan	chickens, finches
6			25.9	1977-1998	Lentogenic	Worldwide	ducks, chickens

Bulgarian isolates included in the study of Aldous et al. (15) are 5 and they belong to various genetic lineages, shown in **Table 2**.

Table 2. *Bulgarian NDV isolates included in the investigation of Aldous et al. (15).*

Name of the isolate	Year of isolation	Genetic lineage acc. to Aldous et al. (15)
Chicken/BG/Haskovo/91	1991	3b
Chicken/BG/Stremtsi/93	1993	5b
Pigeon/BG/Novo selo/95	1995	4d
Chicken/BG/Montana/95	1995	5b
Chicken/BG/Silistra/96	1996	5b

The aim of the current paper is to perform an epizootological analysis of the Newcastle disease outbreaks in Bulgaria during 2005-2007 using data from molecular researches.

MATERIALS AND METHODS

During 2005-2007 4527 samples from both domestic and wild birds were collected (Table 4). The samples were treated with antibiotic medium (homogenization was firstly performed for organ samples). After mixing and centrifugation at 1000xg for 10 min at 4 °C, the supernatant fluids were obtained. Nine-to-eleven-day-old embryonated chicken eggs were inoculated with 0.2 ml of these supernatant fluids into the allantoic sac and incubated for 94 hours at 37 °C and 65% relative humidity. Eggs were candled twice daily to assess embryo viability. For eggs, which have died in 24 hours post inoculation, the death was considered as nonspecific and they were discarded. Allantoic fluids were collected from all dead later than 24 hours post inoculation embryos and from all still live once at the end of the incubation time as well. The live embryos were first chilled at 4 °C for at least 2 hours before the allantoic fluids were collected. The fluids were then tested in HA reaction, following the standard procedure (16). The samples that showed hemagglutination activity were further processed using real- time reverse- transcriptase polymerase chain reaction (one- and two- step) **(Table 3)** with primers and probes previously described by Wise et al. (17). Confirmation of the obtained results and nucleotide sequencing of F-protein cleavage site were made at Avian Virology Department, Veterinary Laboratories Agency, Weybridge, Addlestone, Surrey, United Kingdom, EU, OIE and WHO reference laboratory for AI and ND.

Table 3. *Phases of the performed rRT-PCR.*

Step	One- step rRT-PCR	Reagents and kits used	Two- step rRT-PCR	Reagents and kits used
RNA extraction	Performed in BSL II+ and kit's manual procedure was followed	Roboscreen® mini kit QIAamp® Viral RNA Mini Kit	Performed in BSL II+ and kit's manual procedure was followed	Roboscreen® mini kit QIAamp® Viral RNA Mini Kit
Reverse transcription	N/A		10 min at 25°C 45 min at 37°C	High-capacity cDNA Archive kit®
PCR	30 min at 50°C 15 min at 95°C	Qiagen® One-Step RT-PCR Kit, primers and probes (17)	2 min at 50°C 10 min at 95°C	TaqMan® Universal PCR Master Mix, No AmpErase® UNG, primers and probes (17)
	40 cycles 10 sec at 95°C 40 sec at 52°C		40 cycles 10 sec at 72°C 10 sec at 94°C 30 sec at 52°C	
Machine used: Applied Biosystems 7300 machine (Applied Biosystems, Foster City, CA).				

RESULTS

Out of the 4527 submitted samples during 2005-2007, Newcastle disease virus has been detected in 160 of them and 22 strains were isolated (20 from domestic and 2 from wild birds) **(Table 4)**. The locations where from are the samples that gave positive results are presented on **Figure 1.**
The performed nucleotide sequencing analysis showed that all but two of the isolates from poultry were velogenic. The two lentogenic strains from domestic birds were from mule ducks and chickens and were from Simeonovgrad (Haskovo region) and Hubavene (Vratsa region) respectively **(Table 5)**.
Two strains of the Newcastle disease virus were isolated from wild birds (Table 5). The isolates were determined as lentogenic due to absence of multiple basic amino acids residues at their F-protein cleavage site. They were obtained from swan (Cygnus olor) and pigeons (Columbidae) and originated from regions (Burgas and Dobrich) that are on main flyway of migrating birds – Via Pontica.

Table 4*. Number of collected and positive for NDV samples from domestic and wild birds during 2005-2007.*

Type of samples	Number of samples from poultry	Number of positive samples	Number of samples from wild birds	Number of positive samples
Faeces	535	0	2	0
Organs	129	30	83	1
Carcasses	221	78	76	1
Cloacal swabs	3050	48	181	0
Tracheal swabs	194	2	56	0
TOTAL	4129	158	398	2

Figure 1. Schematic representation of Newcastle disease outbreaks and isolates from wild birds in Bulgaria during 2005 - 2007

Table 5. *Strains of NDV isolated from poultry and wild birds in Bulgaria during 2005-2007.*

Name of the isolate	Date of isolation	Sequence of F-protein cleavage- site	Pathogenicity	Genetic lineage acc. to Aldous et al. (15)
Chicken/BG/Borovan/07	28.09.2007	RRQKR↓F	Velogenic	5d
Chicken/BG/Ohoden/07	28.09.2007	RRQKR↓F	Velogenic	5d
Chicken/BG/Hubavene/07	28.09.2007	GRQGR↓L	Lentogenic	2
Chicken/BG/Vidno/07	25.09.2007	RRQKR↓F	Velogenic	5d
Chicken/BG/Galatin/07	24.09.2007	RRQKR↓F	Velogenic	5d
Chicken/BG/Moravica/07	24.09.2007	RRQKR↓F	Velogenic	5d
Chicken/BG/Smolianovtsi/07	17.09.2007	RRQKR↓F	Velogenic	5d
Chicken/BG/Krapets/07	17.09.2007	RRQKR↓F	Velogenic	5d
Chicken/BG/Ruska Bela/07	13.09.2007	RRQKR↓F	Velogenic	5d
Chicken/BG/Radovene/07	12.09.2007	RRQKR↓F	Velogenic	5d
Chicken/BG/Pudria/07	04.09.2007	RRQKR↓F	Velogenic	5d
Chicken/BG/Krvoder/07	30.08.2007	RRQKR↓F	Velogenic	5d
Chicken/BG/Medeni poliani/07	25.07.2007	RRQKR↓F	Velogenic	5d
Mule Duck/BG/Simeonovgrad/07	03.04.2007	GRQGR↓L	Lentogenic	2
Chicken/BG/Kostalevo/07	05.02.2007	RRQKR↓F	Velogenic	5d
Chicken/BG/Gigen/07	07.01.2007	RRQKR↓F	Velogenic	5d
Chicken/BG/Iuper/06	01.12.2006	RRQKR↓F	Velogenic	5d
Chicken/BG/Vodniantsi/06	27.11.2006	RRQKR↓F	Velogenic	5d
Chicken/BG/Slanchogled/06	24.07.2006	RRQKR↓F	Velogenic	5d
Chicken/BG/Zaichar/06	17.04.2006	RRQKR↓F	Velogenic	5d
Chicken/BG/Skrat/05	25.01.2005	RRQKR↓F	Velogenic	5d
Chicken/BG/Gabrene/05	23.01.2005	RRQKR↓F	Velogenic	5d
Swan/BG/Ravna gora/2006	07.02.2006	GRQGR↓L	Lentogenic	2
Pigeon/BG/Shabla/2006	09.01.2007	EKQER↓L	Lentogenic	1

DISCUSSION

The historical review showed that the isolates from Bulgaria during 1991-1996 belong to 3 different genetic lineages. Comparing the readings from Table 1 (15) and Table 2, it is visible that these strains belong to different epizootological waves passing through Europe at that time (19, 20) which affected Bulgaria as well. Unlike them, almost all Bulgarian isolates from domestic fowl during 2005- 2007 belong to lineage 5d (Table 5). The phylogenetic analysis determined that the divergence among the velogenic strains during this period was not more than 0.3÷0.5% (21, unpublished data). A very close relation between them and isolates from Greece and Turkey in 2005 was found out (divergence 0.8%) (22) as well as with isolates from Hungary and Romania in 2006 (divergence 0.5 ÷ 1.3%) (21,23, unpublished data).

These data showed that during the studied period, an epizootic of Newcastle disease was observed on the Balkans, including our country and it affected our neighbors Greece, Romania and Turkey as well. The picture of the epizootic was masked by the widespread application of vaccine that is readily available and builds a stable immunity against all APMV-1 strains. On the other hand, because the immunity of the population was not uniform and dense, the virus succeeded to produce outbreaks of infection against non-immune birds. These data suggest that the virus was incessantly circulating over our territory. The genetic analysis showed that it was the same virus for the past three years (with insignificantly mutations between strains). Its

introduction was probably spread by most common routes: vehicles, illegal import of birds, poultry products or fodder from our neighbor countries, as well as by dissemination by wild birds (5). The last statement however was not supported by any direct evidence.

Unlike most of the isolates from domestic fowl, strains obtained from wild birds and two of the isolates from poultry during the same period belong to other groups according to the classification of Aldous et al. (15) The pigeon isolate belongs to lineage 1 and is 98.9% homologous with isolates from wild birds in Ireland during 2005 and Italy during 2006 and 98.4% homologous with isolate in UK during 2007 (22, 23, unpublished data). The swan isolate and the two lentogenic strains from poultry belong to lineage 2. The swan strain is 100% homologous with viruses isolated from poultry in Israel in 2006 and in Bulgaria in 2004 (unpublished data). Zarkov et al. (17) isolated NDV from mallards in 2004. Their strains (Mallard/BG/Vaya lake/2004 and Mallard/BG/Poda/2004) also belong to lineage 2 and have the same sequence motif at the site of F-protein cleavage as the swan strain and the two lentogenic strains from domestic birds (Table 5). It should be noted that the isolates belonging to lineage 2 are phylogeneticaly close to the vaccinal strain La Sota.

The similarity of some of the Bulgarian wild bird isolates with the La Sota vaccinal strain raise the question about the source of the infection in these cases – whether it was due to transfer of a vaccinal virus from domestic fowl immunized with live lentogenic vaccine or to circulation of this virus among wild birds. This topic is open to further research.

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ABBREVIATIONS

AI = Avian influenza
APMV 1÷9 = Avian paramyxoviruses 1÷9
F-protein = Fusion-protein
HA = Hemagglutination test
ND = Newcastle disease
NDV = Newcastle disease virus
PPMV 1 = Pigeon paramyxovirus type 1
rRT-PCR = Real- time reverse- transcriptase polymerase chain reaction

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