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The occurrence of five major Newcastle disease virus genotypes (II, IV, V, VI and VIIb) in Bulgaria between 1959 and 1996

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SUMMARY

Partial sequence and restriction enzyme cleavage site analyses of the fusion protein gene were used to genotype 47 Newcastle disease virus strains isolated between 1959 and 1996 in Bulgaria. Viruses belonged to five major genotypes that appeared to be associated with epizootics characterized by temporal and/or geographical restrictions. Genotype IV viruses (responsible for the European branch of the first panzootic) dominated the scene up to the early 1980s, interspersed with sporadic outbreaks caused by genotype II (US strains causing pneumoencephalitis) viruses. Genotype V viruses (transmitted by psittacines from South America) were first shown in 1973 and persisted until the late 1980s. Genotype VI (earliest members from the Middle-East 1968/70 outbreaks) was represented by scattered isolations between 1974 and 1996. A genotype VIIb (recent Middle East epizootic) virus was isolated as early as in 1984. Newcastle disease epizootics in Bulgaria were highlighted by multiple infection with more than one genotype at any one time.

INTRODUCTION

Newcastle disease (ND) is one of the most devastating diseases of poultry. It is caused by Newcastle disease virus (NDV), the only member of *Avian paramyxovirus-1* of the genus *Rubulavirus* in the family *Paramyxoviridae*. The genome of NDV is a contiguous, single-stranded, negative-sense RNA consisting of approximately 15 kb and 6 genes (3′-NP-P-M-F-HN-L-5′) that encode 6 major polypeptides [1].

The epidemiology of ND is characterized by enzootic infections of many developing countries throughout the world (mainly in Asia, Africa and South America) and epizootic-free periods of various length in countries where the disease has been brought under control (e.g. in Europe and the United States). These are interrupted by epidemics of varying severity, caused by viruses introduced from mostly unidentified sources [2]. The first major wave of ND in Europe coincided with the beginning of the Second World War [3] and was part of the worldwide dissemination of the disease also referred to as the first panzootic [4]. Genetic analysis of a limited number of NDV strains derived from early worldwide epidemics (into the 1960s), however, showed that these viruses fell into at least three distinct genetic lineages (genotypes II, III and IV) that also reflected geographical restriction [5–7]. A longitudinal analysis of NDV strains from Italy has shown that only genotype IV viruses occurred in the early period [7]. The next major

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European epizootic started in Western Europe in 1970 and in the succeeding years ND swept through other countries [4, 7, 8]. During this period viruses belonging to a novel genetic lineage (genotype V) could be isolated in England, Hungary [5] and Italy [7]. Another severe wave of outbreaks was recorded in a number of countries in Western Europe commencing in 1992 [9] caused by a genetic lineage (VIIa) that is most likely prevalent in the Far East [6].

In an effort to reconstruct the possible movements of viruses and the epidemiological links of past epizootics in Europe, 47 NDV strains derived from Bulgaria in the past 4 decades were genotyped. We have addressed questions concerning the number and duration of genotypes present in the country and their relationships to contemporaneous epizootic viruses isolated in Europe and elsewhere.

MATERIAL AND METHODS

Viruses

The viruses were isolated from chicken flocks with ND except for BG-109/84 that derived from a pigeon. Strains were grown in 9- to 10-day-old embryonated eggs and allantoic fluids were stored at $-70\ ^{\circ}C$ until used. All strains proved to be velogenic in animal tests [10 and references therein]. In addition to the Bulgarian strains, viruses isolated in Hungary and the United States were also included into the study. The designations and origin of NDV strains, and accession number of sequences are listed in Table 1. For labelling, two letter country abbreviation and a serial number in the order of arrival were used. Designations were kept for already published reference strains [5–7, 11].

Preparation of viral RNA, reverse transcription (RT), polymerase chain reaction (PCR) and restriction enzyme cleavage site analysis

These procedures were performed without modification [5–7]. Briefly, a 1349 bp (approximately 75%) portion of the fusion (F) protein gene (between nucleotides 334 and 1682) was amplified by RT–PCR and products were digested with each of the following restriction enzymes (RE) *Hin*fI, *Bst*OI and *Rsa*I. DNA fragments were separated by agarose gel electrophoresis and physical maps of fragments and restriction sites were constructed. Group-specific cleavage sites or combinations of sites have been described in detail in previous papers [5–7, 11].

Sequence analysis

A portion of the F gene between nucleotides 47 and 420 was sequenced as described [6, 7, 11] and phylogenetic analysis was performed by the programme TREECON for Windows 1.3b that created a distance tree by the neighbour-joining algorithm [12]. Distance values (Table 2) were taken from a distance matrix (not shown) prepared by CLUSTAL W of the MEGALIGN programme.

RESULTS AND DISCUSSION

The first cases of ND in Bulgaria were diagnosed in 1943 around a German-occupied airport from where the disease spread rapidly and it was only in 1951 that the disease was brought under control by vaccination with the mesogenic vaccine strain Hertfordshire [13]. By the end of the decade the epizootic flared up again and the incidence diminished to below 100 cases per year only by the end of the 1960s [8]. NDV strains have been available for study only since that time.

NDV genotypes and subtypes

Phylogenetic analysis of NDV strains from the Bulgarian collections allowed the recognition of five major genotypes described previously [6, 7, 11] and certain genetic sublineages (or subtypes) as demonstrated by the dendrogram (Fig. 1). Strains were also screened by restriction site analysis of the F gene [5] to confirm groupings and to reveal possible mixed isolates or vaccine contaminants. Unique cleavage sites were utilized as shared derived characters in confirming the monophyletic nature of certain subtypes, and provided additional information to distance data. Here we show only representative physical maps of fragment and cleavage site patterns characteristic for the major genotypes and subtypes of reference strains and local isolates (Fig. 2). Results of the grouping based on both techniques are also shown in Table 1. The appearance of genotypes in the country followed the chronological order described below.

Before the 1970s, viruses belonging to two genetic groups (II and IV) could be identified in Bulgaria. It appears that genotype IV viruses dominated and persisted until the early 1980s. The group is composed of several subtypes as demonstrated by the extent of mutational distance (up to 10·9%) between isolates (Fig. 1, Table 2). The branching structure of the dendrogram shows that the majority of Bulgarian isolates group into subtypes (arbitrarily termed a_1, a_2

Table 1. *Origin and grouping of NDV strains and nucleotide sequences*

Designation	Place of isolation (province)	Genetic grouping		Accession number
		CSP*	Sequence	
BG-1/59	Plovdiv	IV	IVa_1	AF402103
BG-5/67	Vratza	IVa	IVa_2	AF402104
BG-110/68	Sofia	II	II	AF402105
BG-6/68	Pasardjik	IVa		
BG-7/68	Plovdiv	IVa		
BG-8/68	Haskovo	IVa	IVa_1	AF402106
BG-10/68	Haskovo	IVa		
BG-83/68	Kardjali	IVa		
BG-13/69	Sofia	II	II	AF402107
BG-85/69	Haskovo	IVa	IVa_1	AF402108
BG-11/69	Haskovo	IVa	IVa_1	AF402109
BG-15/70	Haskovo	IVa	IVa_1	AF402110
BG-14/70	Haskovo	IVb	IVb_1	AF402111
BG-16/73	Haskovo	II	II	AF402112
BG-88/73	Haskovo	Va	Va	AF402113
BG-18/74	Haskovo	IVb	IVb	AF402114
BG-17/74	Kardjali	Va	Va	AF402115
BG-72/74	Shumen	VI	VId	AF402116
BG-19/74	Haskovo	VI	VIc	AF402117
BG-58/75	V. Tarnovo	IVa		
BG-55/75	Iambol	Va	Va	AF402118
BG-24/75	Vidin	II	II	AF402119
BG-21/75	Haskovo	IVa		
BG-20/75	Haskovo	IVb	IVb	AF402120
BG-92/77	Haskovo	IVa	IVa_2	AF402121
BG-90/77	Haskovo	Vb	Vb	AF402122
BG-25/78	Montana	Vb_1	Vb_1	AF402123
BG-47/79	Vidin	VI	VIc	AF402124
BG-26/79	Vidin	Vb	Vb	AF402125
BG-43/80	Vratza	Vb_1	Vb_1	AF402126
BG-45/80	Sofia	IVa		
BG-50/80	Montana	Vb_1	Vb_1	AF402127
BG-27/80	Montana	Vb_1	Vb_1	AF402128
BG-95/80	Sofia	Vb_1		
BG-60/81	Pernik		IVea	AF402129
BG-101/82	Kustendil	Vb_1		
BG-100/82	Montana	Vb_1	Vb_1	AF402130
BG-99/82	Shumen	VI	VId	AF402131
BG-44/82	Sofia	IVa	IVa_1	AF402132
BG-109/84	Pernik		VIIb	AF402133
BG-102/86	Vratza	Vb_1	Vb_1	AF402134
BG-29/86	Vratza	VI	VIc	AF402135
BG-104/88	Vratza	Vb_1	Vb_1	AF402136
BG-105/92	Montana	VI	VIc	AF402137
BG-48/95	Vidin	VI	VIc	AF402138
BG-30/95	Montana		VIIb	AF136781
BG-31/96	Silistra		VIIb	AF136782
HU-35/79	Hungary	II	II	AF402102
US(Ca)-11914/43	California	II	II	[30]
US(Purdue)/49	Indiana	II	II	AF401646

* Cleavage site pattern.

Table 2. *Relationships of time of isolations and mutational distances of NDV strains*

Genotype	BG viruses	Subtype*	Examples	Time range (years)	Distance‡ (%)
II				34	7·4
	BG		BG-110/68, BG-24/75†	7	1·4
			BG-13/69, HU-35/79	10	0
IV				49	10·9
	BG		BG-20/75, BG-92/77†	2	10·9
		Intra-subtype			
		ea	Herts 33, BG-60/81	48	3·0
		a_1	BG-1/59, BG-11/69	10	0·8
			BG-85/69, BG-44/82	13	0
		a_2	BG5/67, BG-92/77	10	0·8
		b	BG-14/70, BG-20/75	5	0·3
		Inter-subtype			
		a_2/a_1	BG-5/67, BG-11/69	2	6·8
		a_1/b	BG-11/69, BG-14/70	1	9·0
		b/a_2	BG-20/75, BG92/77	2	10·9
V				18	3·3
	BG		BG-88/73, BG-104/88†	15	2·7
		b_1	BG-25/78, BG-104/88	10	0
VI				27	9·9
	BG		BG-72/74, BG-48/95†	21	7·4
		c	BG-29/86, BG-48/95	9	2·2
			BG-19/74, BG48/95	21	0
		d	BG-72/74, BG-99/82	8	0·3
		d/c	BG-99/82, BG-29/86	4	7·1
VIIb				13	5·3
	BG		BG-109/84, BG-31/96†	12	0·3

* See Figure 1.
† Examples illustrate the largest genetic distance between BG strains.
‡ Data were taken from a distance matrix (not shown).

and b) that are separated both from the bulk of the Italian (IT) strains and the cluster that contains the earliest (ea) NDV isolates available. The presence of area-specific restriction sites has previously confirmed that viruses in subtype IT have a monophyletic relationship [7]. Based on cleavage site analysis, a large portion of the Bulgarian isolates displayed either pattern IVa or b, congruent with sublineages a_1/a_2 and b, respectively (Fig. 2), and this confirms their monophyletic descent. The majority of strains had restriction site patterns designated IVa that differed by several sites from those of the early isolates represented here by strain Italien/45. Pattern IVa had an additional *Hin*fI site at position 736 and a *Bst*OI site at 1260, and had lost *Rsa*I 1593. IVb exhibited further variation with two extra sites at *Hin*fi 1603 and *Rsa*I 1055 and two missing at *Bst*OI 752 and 1601. Mutational distances within and between subtype sequences are shown in Table 2. Intra-subtype distances indicate that the rate of change of field isolates can be estimated at about 1% (or less) per decade. This agrees well with values obtained in a separate study in which samples were taken yearly over a decade during an epizootic [14]. Similar values were also found in other studies [6, 7, 11]. Moreover, several examples of no change in nucleotide sequence for periods of 1–2 decades were also encountered (Table 2). In contrast, large genetic distances (6·8–10·9%) were seen between near contemporaneous members of distinct subtypes (inter-subtype comparisons in Table 2), and this points to a much longer evolutionary process than can be accounted for by the earliest date of the beginning of the European branch of the first panzootic [2–4].

The occurrence of strains belonging to genotype II was an unexpected finding because these viruses have previously been thought to occur only in the United States mainly before the 1960s. Uniquely among all other groups, it has evolved into distinct pathogenicity categories and comprises velogenic (e.g. US(Ca)-11914/43 and Texas GB/48), mesogenic (Beaudette C/45 and Roakin/46) and lentogenic (LaSota(V1)/46

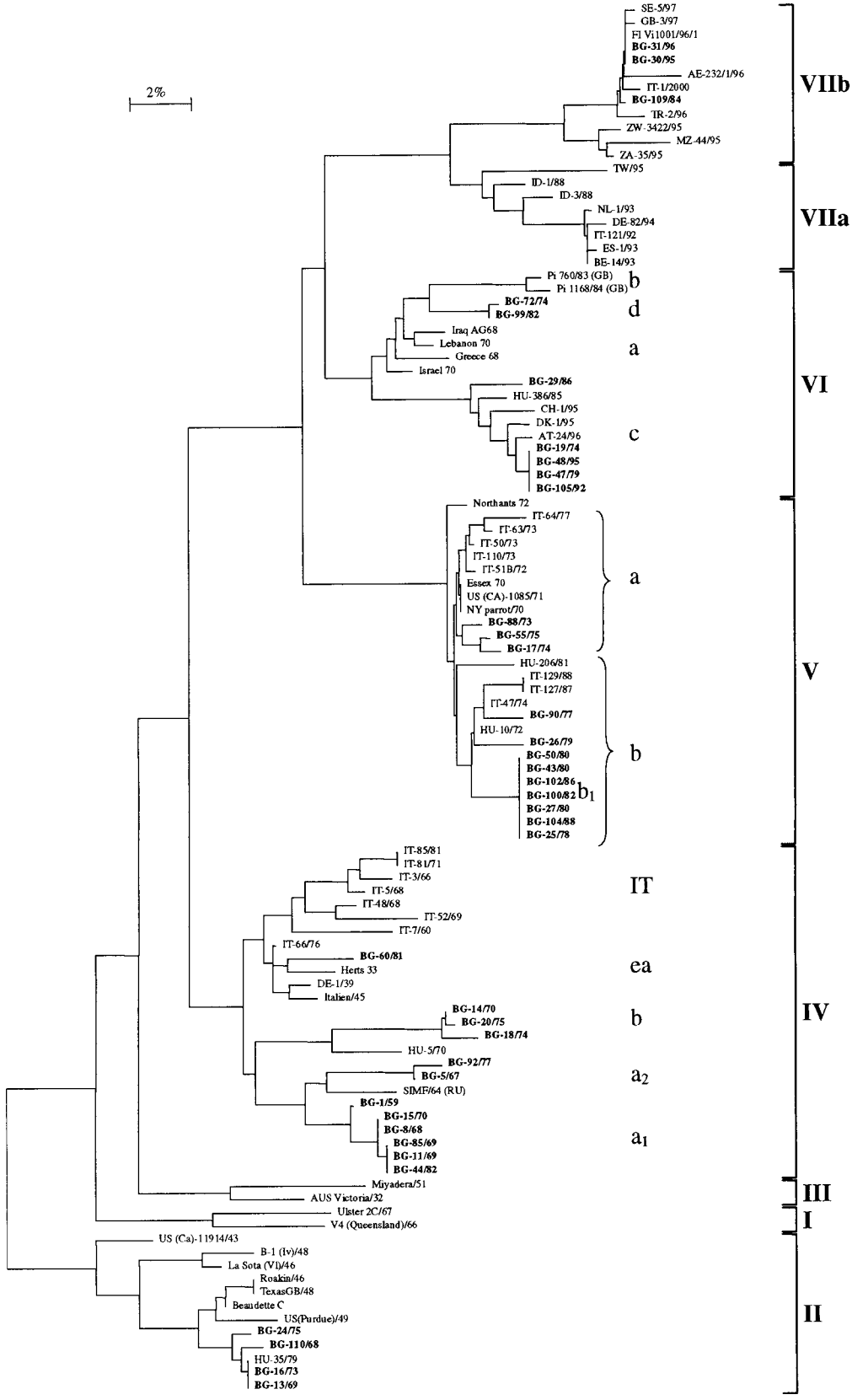

Fig. 1. Phylogenetic tree based on partial sequences (nt 47–420) of the F gene of NDV strains. Accession numbers of viruses in bold type are shown in Table 1. Sequences with normal letters were taken from GenBank [6, 7, 11]. Genotypes are shown on the right, and certain sublineages are also indicated.

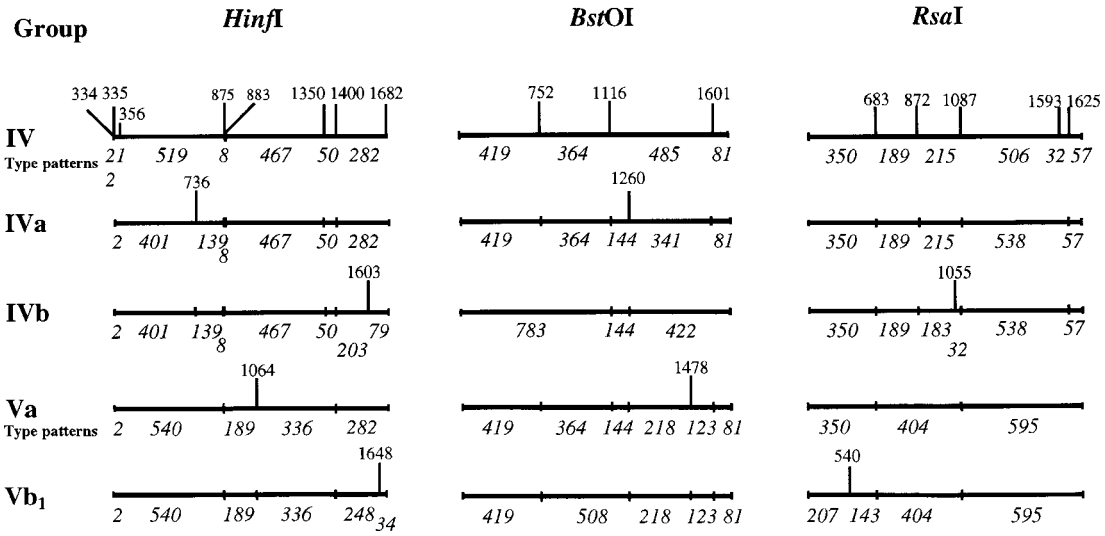

Fig. 2. Physical maps of restriction fragments and cleavage sites of the F genes of NDV strains. Cleavage sites are designated by the last nucleotide of the fragment (except position 334 that is the first nt of the amplified region). Fragment lengths (in italics) are in bp.

and B-1(IV)/48) strains [5, 15, 16]. American velogenic viruses are also distinct in causing pneumoencephalitis rather than being viscerotropic velogenic [17]. The Bulgarian genotype II viruses and a single isolate from Hungary (HU-35/79) group into a cluster with restricted divergence (<1·4%). The shortest distance between BG and US strains is not more than 1·9% while divergence of US isolates presented here reaches 7·4% (Fig. 1). All non-US strains possess group II specific cleavage sites such as *Bst*OI 979 and *Rsa*I 1160 [5] but they also display variations (presence of *Bst*OI 752 and lack of *Rsa*I 1625) that appear to be characteristic for these local isolates (not shown).

Genotype V strains were first seen in Bulgaria in 1973. These are represented by isolate BG-88/73 that showed 99·2% similarity with Essex 70 and US(CA)-1083/71, the reference strains of the ND epizootics that commenced in England [2, 4, 8] and California [18–20] in 1970 and 1971, respectively. Genotype V viruses display a lesser degree of diversity (maximum 3·3%) than members of other genotypes. When compared to genotype IV, group V restriction patterns are specified by the appearance of novel cleavage sites for *Hin*fI at position 1064 and *Bst*OI at 1478 coupled with the lack of *Hin*fI site 883, *Rsa*I 872 and 1625 (Fig. 2) [5]. Strains can be classified into Va and b according to the absence or presence, respectively, of an *Rsa*I site at 540. Diversity of isolates (Va) even from different countries is within 2% up to the second half of the 1970s, whereas it increases in the succeeding years. Subtypes separated by relatively longer branches are also distinguishable by further cleavage site differences. For example, members in subgroup Vb_1, have an additional *Hin*fI site at position 1648 and lack *Bst*OI site at 1116 (Fig. 2). On the other hand, their genetic stability is remarkable: most late isolates have identical sequences, showing no evolution at all in a space of 10 years (Table 2). We have found that late Italian isolates (e.g. IT-127/87) also have an area-specific marker (*Hin*fI 1515) [7].

Genotype VI viruses were first found in Bulgaria in the mid-1970s. The earliest ND epizootic that could be clearly associated with genotype VI viruses was the one that caused severe losses in the Middle East in the late 1960s. Isolates derived from these cases are designated VIa [5, 6]. Pigeon paramyxovirus-1 strains, the causative agents of an ND-like disease that emerged among show pigeons in the early 1980s, form another sublineage termed VIb [2, 4–6]. The Bulgarian isolates of the current study fell into two further subtypes, designated VIc and VId. In addition to local isolates, VIc also comprises strains that derived from sporadic ND cases in Western Europe (AT-24/96, DK-1/95 and CH-1/95) in the mid-1990s (Fig. 1) [6]. In spite of the fairly limited representation of genotype VI viruses in the collection, their presence could be demonstrated in Bulgaria with an interval of two decades.

Genotype VIIb is composed of viruses collected during epizootics that spread from the early 1990s onwards in the southern part of Africa and in the Middle East [11, 21]. The Middle East sublineage has slightly diverged from the South African branch and

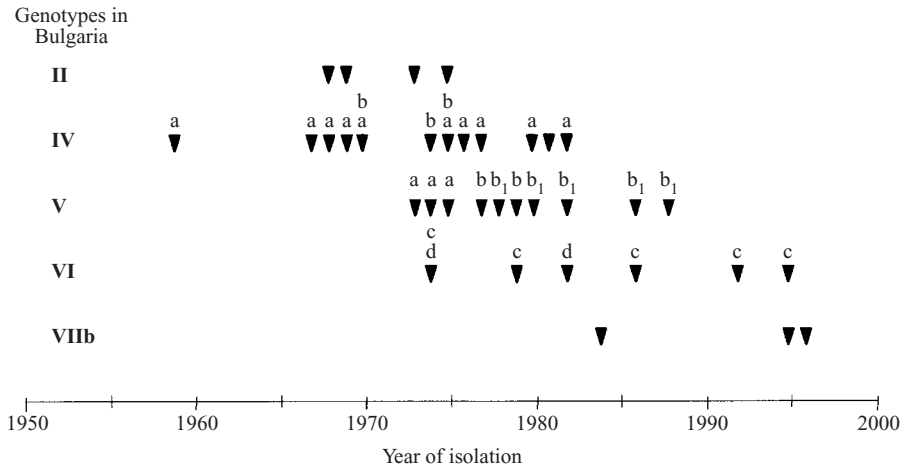

Fig. 3. Temporal distribution of NDV genotypes and subtypes in Bulgaria. Each arrowhead represents one or more isolates.

comprised viruses from Bulgaria, Great Britain, the Scandinavian countries and most recently, Italy [7]. The occurrence of these viruses has already been reported in Bulgaria [11] but the present study has revealed that very closely related members, represented by BG-109/84, must have been present a decade earlier. The mutational distance between the latter and recent members is only 0·3%.

Evolutionary and epidemiological aspects

Using phylogenetic information it is possible to define epizootics aetiologically, that is to delimit, in space and time, a stream of infection that is initiated by a particular founder virus and maintained by a population of closely related descendants belonging to the same monophyletic lineage. Information on the rate of sequence divergence during the natural course of epizootics has enabled us to ascertain or exclude epidemiological links between outbreaks. Based on the results of previous studies [6, 7, 11, 14] and the current work, a maximum of 1% sequence change per decade has been adopted when classifying strains into an epidemiologically connected group (see intra-subtype comparisons in Table 2).

Our analysis of the Bulgarian collection indicates that at least five but probably more introduction events occurred during the past five decades. As a consequence, several streams of infection affected the country, resulting in up to four distinct genotypes or subtypes circulating simultaneously in the region (Fig. 3). For example, in the middle of the 1970s, in a southern province (Haskovo) alone, viruses belonging to seven epidemiologically unrelated genotypes and subtypes (II, IVa_1, IVa_2, IVb, Va, Vb and VIc) were isolated (Table 1).

The different degree of intragroup diversity reflects differences not only in the number of introductions but also in the history of the genotypes. In this context, in the pre-1970 era genotype IV dominated the scene with several distinct subtypes (a_1, a_2 and b). The possibility that these evolved recently (i.e. they are epidemiologically linked) is questionable because the 2–3 decades that have elapsed since the first apparent epidemic of the disease either in Europe or in Bulgaria (in the early 1940s) must have been insufficient for varieties with 6·8–10·9% mutational distance to emerge (see inter-subtype comparisons in Table 2). It is more plausible that separate introductions of different sublineages occurred that evolved independently elsewhere. For example, it is likely that the ancestor of a more recent isolate, BG-60/81, was really introduced around the early 1940s from a virus pool in Western Europe, as indicated by the close relationship between this strain and the earliest isolate in Europe, Herts 33 (only 3% divergence in 48 years). Another possibility is that ND arrived in the region much earlier but remained unrecognized for some time, thus allowing variations to develop locally. Indeed, there is diagnostic evidence that ND was already present in Europe in the first decade of the century [22]. The situation in Bulgaria shows some parallels with the history of ND in Italy where genotype IV viruses were also found to be prevalent for several decades after the Second World War [7]. However, the majority of the Italian viruses appeared to be organized into a distinct monophyletic group with 5·9–10·6% genetic distance from contemporaneous Bulgarian isolates suggesting again

local evolution over a considerable time. While no evidence of interchange of strains with other localities was found in Italy, the Bulgarian sublineages comprise strains from other East European countries (from Hungary, HU-5/71 and the former Soviet Union, SIMF/64 (RU)) as well (Fig. 1). There are data that genotype IV viruses also occurred prior to 1970 in Australia and Germany [5]. An important conclusion is that for the genetic diversity of these viruses to develop, much longer field evolution is required than can be accounted for by dates of the beginning of the European branch of the first panzootic [3, 7].

There are case reports from Great Britain [23], Germany [24], Hungary [25] and Japan [26] on the occurrence of American type ND (pneumoencephalitis) in the early 1950s which was attributed to transmission in connection with the movements of American troops and purchase of breeding stocks [2, 23]. This is, however, the first work that provides aetiological evidence that pneumoencephalitis cases outside North America were indeed caused by US-type (genotype II) viruses. The more restricted genetic diversity of the Bulgarian cluster suggests a single introduction followed by spread in the region (e.g. to Hungary). The large mutational distance (13–20%) of genotype II from all the others is consistent with the notion that is separation happened long ago [15, 16] and suggests that these viruses could be indigenous in North America.

The present study lends further support to the notion that the first panzootic which commenced in the 1920s, can be broken down to geographically separated epizootics [5], with no sign that a single lineage conquered the globe at that time [27]. On the contrary, evidence is mounting that before 1970 at least three distinct genotypes (II, III and IV) participated in maintaining ND outbreaks throughout the world and they were probably specific to different geographic areas [5–7, 11]. Thus it seems that genotype III was responsible for at least a portion of early Far East epizootics with over 60 years between the first (AUS-Victoria/32) and latest isolate (TW/95-3 from Taiwan) available [28]. As indicated by the current work, the European branch was most likely maintained exclusively by genotype IV viruses that have also persisted for at least four decades [5–7, 11]. Now it has been shown that genotype II viruses also achieved transcontinental movement, albeit due to special circumstances of transmission. It is interesting that infections due to genotypes IV and II had a tendency to disappear from Bulgaria by the early 1980s. It is difficult to explain why they were replaced when the efficacy of control might not have improved significantly as demonstrated by the introduction and spread of two novel genotypes, V and VI.

Investigations established an epidemiological link between infected parrots imported from South America and outbreaks in California [18–20], whereas the transmission to Europe was confirmed by retrospective analysis of strains derived from imported birds (NY parrot/70) and from chickens in California (US(Ca)-1085/71) and England (Essex 70) [5, 6, 29]. Since no strains are available from 1971 and 1972 in the Bulgarian collection, the earliest outbreak elicited by a group V virus cannot be determined. Epizootic data, however, appear to support the delayed introduction of this virus to Bulgaria [8]. The more restricted intra-group diversity ($<2\%$) of the early isolates of genotype V and the apparent random distribution of sequences on the tree from different countries argue for a rapid dissemination and probable cross-transmission of viruses between countries. This is also consistent with the extensive outbreaks reported in the continent in the early 1970s [8]. It remains to be seen if the slightly higher divergence (up to 3·3%) found in the 1980s is the result of separate introductions or rapid local evolution.

We have preliminary evidence that genotype VI is a highly divergent group of Asian and African lineages (E. Wehmann et al., unpublished observations) whose representatives VIc and VId, respectively, were found in Bulgaria. With 4–8% mutational distance between contemporaneous clusters such as VIc and VId, or VIa, and VId the outbreaks caused by these viruses cannot be regarded as epidemiologically connected. Only VIc appears to have survived in Bulgaria and might have spread into other European countries, perhaps on more than one occasion (Fig. 1).

In contrast to the above, the northern clade (occurring in the Middle East and Europe) of group VIIb, in spite of the distant geographical locations, displays unexpectedly narrow sequence variations ($<0{\cdot}5\%$ between members in Bulgaria and Western Europe) indicating either direct epidemiological link between them or possibly introductions from a common source. As these viruses were present in Bulgaria as early as in 1984, the latter proposition is less probable. It is more likely that sporadic outbreaks in Western and Northern Europe in 1997–8 [21], the more severe infections in Italy in 2000 [7] and former cases in Bulgaria have epidemiological links based on the virtual identity of NDV strains involved (e.g. FI Vi

1001/96/1, GB-3/97, IT-1/2000, BG-30/95 and BG-31/96). It is to be noted, that these latest outbreaks in Western Europe are epidemiologically unrelated to those that commenced in 1992 and were caused by VIIa type viruses of Far East origin (Fig. 1) [6].

In summary, the longitudinal analysis of NDV strains from Bulgaria has contributed to our understanding of the history of ND epizootics both locally and in a wider perspective. Analyses such as these will be of use in assessing control measures and retrospectively determining the nature of past epizootics.

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