

Virulence and VP2 sequences of Infectious Bursal Disease Virus isolates from recent outbreaks in The Netherlands and one Hungarian isolate.

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Abstract

In The Netherlands severe outbreaks of infectious bursal disease (IBD) occurred from 1988 till 1990. During several years in the 90's the IBD situation in The Netherlands was quiet. However, starting from half 1996 till 1998 severe outbreaks occurred again in several parts in The Netherlands.

We wanted to know whether these outbreaks could be a result of the occurrence of variation in antigenicity or variation in virulence (even more virulent strains) of Infectious Bursal Disease Virus (IBDV) strains or whether the stringent vaccination management procedures were loosened.

Both virulence and sequence data of the HVR of VP2 of recent IBDV isolates were compared with the very virulent IBDV (vv) isolate D6948, originally isolated in 1989 in The Netherlands. The virulence was tested in SPF chickens. The clinical signs, mortality and histopathology were scored and compared. The sequence of a Hungarian isolate was also determined and compared. There is no indication from our data that recent isolates are different from D6948 either in virulence or in amino acid sequence of the variable region of VP2. The sequence of the Hungarian isolate is identical to D6948. From several farms with a case history including lower mortality (1996), only a vaccine strain was isolated or a combination of vaccine strain with a vvIBDV isolate.

Introduction

In the mid to late 1980's the poultry industry in Europe was affected by very virulent serotype I Infectious Bursal Disease Virus (vvIBDV) isolates. (Chettle *et al.*, 1989; van den Berg *et al.*, 1991). These very virulent field isolates were capable of establishing themselves in the face of high levels of maternal antibodies that normally were protective. They cause more severe clinical signs during an outbreak and are now found globally (e.g. Europe, Japan, Israel and Asia). In The Netherlands severe outbreaks occurred from 1988 till 1990. (Kouwenhoven and van den Bosch, 1995). Hot and intermediate plus vaccines were developed and used after the first outbreaks with vvIBDV. Also the timing of the active vaccination was better estimated by the breeder or chick titer and the half-life of antibodies of approximately 3.5 days. (De Wit and Van Loon, 1998; Kouwenhoven and Van den Bos, 1995).

During several years in the 90's the IBD situation in The Netherlands was quiet. However, starting from half 1996 till 1998 severe outbreaks with an incidence of 16% occurred again in several parts in The Netherlands, especially in the Southern part of the country (Heijmans and ter Huurne, unpublished data).

These outbreaks could be a result of the loosening of the stringent vaccination management procedures, variation in antigenicity or variation in virulence of IBD strains. The nucleotide sequence of the polyprotein encoding part of the A-segment of the Del, the GLS and the DS326 antigenic variant IBDV isolates has been determined (Vakharia *et al.*, 1994). Most of the amino acid changes were found in a specific region of the VP2 protein, the so-called hypervariable region (HVR). Furthermore it was found that the epitopes which are capable of inducing neutralising antibodies are conformation dependent and are clustered in the HVR. This region consists of a domain with a high hydrophobicity index (amino acid 224 to 314 of pVP2, corresponding with amino acid 224 to 314 of the polyprotein) which is flanked by two small hydrophilic regions, each spanning about 12 amino acids (Vakharia *et al.*, 1994; Heine *et al.*, 1991). Amino acid substitution both within the hydrophobic region and within the hydrophilic regions might be involved in the antigenic variant character of these isolates.

The molecular determinants that distinguish vvIBDV from classical IBDV isolates are not exactly known. No specific antibodies, which exclusively recognise the vvIBDV isolates have been described yet (Eterradossi *et al.*, 1997). The lack of discriminating antibodies makes direct diagnosis difficult. Most attention has been given to sequence comparison between the hypervariable region of VP2 of classical isolates and of very virulent isolates. Sequence analysis of the vvIBDV isolate UK661 showed that only three unique (i.e. not found in non-vvIBDV isolates) amino acid substitution are present within the hypervariable region of the VP2 protein. (Brown and Skinner, 1996).

In this paper we describe the results of virulence tests of several isolates from broiler farms during these outbreaks. The mortality and bursa/body weight ratios are given. The results of histopathology and immunohistochemistry are not reported. We show the sequence data of the HVR of VP2. Both virulence and sequence data of new IBDV isolates were compared with the vvIBDV isolate D6948.

Material and methods.

Isolates

D6948 was originally isolated in 1989 by the Poultry Health Service (Doorn, The Netherlands). It was purified by 5 passages in embryonated eggs and two subsequent passages in SPF leghorn chickens. All recent IBDV strains tested were isolated from bursa material of affected chickens by Animal Health Service (AHS) Deventer by 3 to 5 passages in embryonated eggs. IBD was first diagnosed at the AHS by positive Immunofluorescence Assay (IFA) of bursal sections with polyclonal anti-IBDV antiserum. The chickens derived from several farms with different case histories during outbreaks of IBDV during 1996 and 1997.

Virulence test

The virulence was tested in two separate experiments (A and B) (Table 2 and 3). In both experiments SPF chickens were inoculated with 200 ELD50 (Egg Lethal Doses) per chicken, nasally and by eye-drop. D6948 was used in the positive control group and PBS was used in the negative control group. In experiment A 6 field isolates from 1996 were tested in 3-weeks-old chickens. The chickens were followed on clinical signs

and mortality during 10 days. In experiment B 4 field isolates from 1997 were tested in 4-weeks-old chickens. These were followed during 21 days (to be able to check on antibodies against CAV). One field isolate (97-5123) was tested in a separate similar experiment. At the end of the experiments, the surviving chickens were necropsied, bursa and body weights were determined and bursa samples were taken for histopathology.

Determination and sequence analysis of the hypervariable region of the VP2-gene of IBD-virus.

In each group 5 extra chickens were inoculated to be sacrificed three days post infection to collect bursa of Fabricius. Two volumes of tryptose phosphate buffer (TPB) was added. Identical mixtures of bursa samples containing either a Dutch isolate (97XX, not tested experimentally) and a Hungarian isolate obtained from Dr Nick Dren were treated in the same way.

Genomic dsRNA was isolated from infected bursa homogenates resolved in TPB with the QIAamp Tissue Kit (Qiagen GmbH) and precipitation with ethanol. Viral dsRNA was mixed with two primers and a reaction was carried out with one primer. Primers AC0 and ANC5 or ANC2 (see table I) were used respectively. The dsRNA mixture was prepared for Reverse Transcription by heating the sample for 3 min to 100°C and immediately afterwards the denatured RNA was stored on ice until the reaction was started. Reverse Transcription was carried out by adding to the RNA-mixture First strand buffer (Gibco Brl), 200 mM Dithiothreitol (Gibco Brl), 40 U/ul Rnasin (Promega), 10 mM dNTP's, and 200 U of superscript II reverse transcriptase (Gibco Brl) in a total volume of 20 ul. The total volume of the reaction mixture was kept at room temperature for 10 min and then incubated at 42°C for 1 h.

The HVR of the VP2-gene was amplified using AmpliTaq® DNA Polymerase (Perkin Elmer) under standard PCR conditions in a Biometra thermal cycler (2' 94°C; 35*(1' 94°C, 1' 55°C, 3' 72°C); 5' 72°C) using the first strand cDNA as template. Two oligonucleotide primers designated as AC3 and ANC5 (see table I) were used to obtain the HVR of the VP2-gene.

The amplification products were analysed on a 1% agarose gel and extracted with the Qiaex II Agarose Gel Extraction kit (Qiagen GmbH). The amplification products were then ligated into the pGem-T Easy vector using pGem-T Easy Vector system I (Promega Corp.). *Escherichia coli* DH5 α -cells were made competent and transfected with plasmid DNA. Plasmid DNA was purified for cycle sequencing using Flexiprep (Pharmacia Biotech). Primers used AC3; AC4; AC5; AC6 and ANC5 (see table I). For each IBDV-strain 2 independent clones were sequenced. One primer of each of the five primers listed above was added to the sequencing reaction which was carried out according to the supplier's instructions (ABI PRISM dRhodamine cycle sequencing ready reaction dye terminator kit on the ABI 310 Genetic Analyser). The sequence data were aligned by using the computer programs AutoAssembler and GeneWorks® 2.3 copy.

Results

Virulence tests

Results of the virulence tests are given in Tables 2 and 3. As mentioned before only mortality, appearance of gross lesions at post mortem, and bursa/body weight ratios are given in this report. Generally several isolates from 1996 appeared to be a-virulent as far as mortality and gross lesions were concerned. The bursa/body weight ratios were however not different from the ratio induced by the vvIBDV D6948. Isolate

906-B6 did not induce any pathologic changes. This must have been due to a too low virus titre in the suspension used to inoculate the chickens with, since no viral RNA could be isolated from the bursa samples of this group either. All Dutch isolates from 1997 appeared to be virulent with mortality rates that were even higher than that of D6948. The results of the virulence tests were in accordance with the history in the flocks, except for isolate 97-B3 that originated from a flock with high mortality, but did not induce mortality in experimental chickens.

Sequence analysis.

Results of the sequence analysis are given in Figure 1. The deduced amino acid sequences of the VP2 HVR of the Dutch and one Hungarian IBDV isolates are compared with known sequences of vvIBDV isolates, several variant strains and vaccine strains. The conclusion drawn from the sequence analysis is given in Table 2. Three out of 6 isolates from 1996 seemed to be vaccine strains based on sequence analysis. One isolate (96-B5) contained both vaccine and vv virus. The isolates from 1997 and the Hungarian isolate have amino acid sequences of the VP2 HVR identical to known vvIBDV isolates and D6948. One exception is 97-B3, which has a substitution located in the hydrophobic part of the HVR (D279N).

Discussion

A selection of IBDV isolates obtained from farms in different regions in The Netherlands during outbreaks of IBD in 1996 and 1997 were tested for virulence and compared with a Dutch vvIBDV strain isolated in 1989. The amino acid sequence of the VP2 HVR was determined. Also, the amino acid sequence of one Hungarian isolate was determined. The aim of this study was to verify whether outbreaks of IBD in The Netherlands during 1996 and 1997 could be a result of the occurrence of variation in antigenicity or variation in virulence (even more virulent strains) of IBDV strains or whether the stringent vaccination management procedures were loosened.

Based on virulence tests in 3- or 4-weeks-old SPF chickens, the isolates either were a- or low virulent (96-B4, 96-B6, 96-C4, 96-C5, 97-B3), virulent (96-B5) or very virulent (D6948, 96-C6, 97-B4, 97-B5, 97-B6, 97-5123, Hungarian isolate). However, the discrimination between virulent and very virulent is non-conclusive based on challenge in SPF chickens. The mortality in SPF chickens caused by D6948 may vary from 40 to 100% depending on strain of chickens, passage in chickens or embryonated eggs. (ter Huurne *et al.*, unpublished results). The best way to discriminate between classical virulent and vvIBDV isolates in a challenge model is to use maternal immune chicks. The vvIBDV isolates are able to break through levels of antibodies that are protective against classical IBDV isolates. (Chettle *et al.*, 1989; van den Berg *et al.*, 1991). In our own challenge experiments we found that D6948 could break through antibody titres that were 4 to 8 times higher than could the virulent strain Faragher 52/70, depending on the vaccine used in breeders (Maas *et al.*, manuscript in preparation).

A reason for outbreaks of IBD could be the occurrence of antigenic variant strains of IBDV. The nucleotide sequence of the polyprotein encoding part of the A-segment of the Del, the GLS and the DS326 antigenic variant IBDV isolates have been determined (Vakharia *et al.*, 1994). Most of the amino acid changes were

found in HVR of the VP2 protein. Based on our sequence data we have no indication for the occurrence of antigenic variants in The Netherlands.

The molecular determinants, which distinguish vvIBDV from classical IBDV isolates, are not exactly known. No specific antibodies that exclusively recognise the vvIBDV isolates have been described yet (Etteradossi *et al.*, 1997)). The lack of discriminating antibodies makes direct diagnosis difficult. Most attention has been given to sequence comparison between the HVR of VP2 of classical isolates and of very virulent isolates. Sequence analysis of the vvIBDV isolate UK661 showed that only three unique (i.e. not found in non-vvIBDV isolates) amino acid substitutions are present within the HVR of the VP2 protein. Other amino acid substitutions are present in the remaining part of the pVP2 protein and in the genes encoding the other viral proteins (Brown and Skinner, 1996). We have decided to determine and compare the amino acid sequence of the HVR of the VP2 protein. All isolates that were very virulent based on the challenge in SPF chicks had a deduced amino acid sequence of the HVR of VP2 identical to D6948 and the other already published vvIBDV isolates (Figure 1). The before-mentioned virulent isolate 96-B5 contained a combination of amino acid sequences of very virulent isolates and of an attenuated (vaccine) strain. Several vaccine strains and other attenuated strains have been described. Known sequence data are included in Figure 1 for comparison.

The before-mentioned a-virulent isolates all had amino acid sequences comparable to known classical vaccine strains (D78, Lukert strain) or attenuated strains like CEF-94 (Boot *et al.*, 2000) and attenuated Cu-1. Several of these isolates had the two amino acid substitutions (D279N and A284T) that Lim *et al.* (1999) had introduced into the non-CEF-adapted vvIBDV isolate HK46 to turn it into a CEF-adapted isolate. These mutations were most probably based on data of Yamaguchi *et al.* (Yamaguchi *et al.*, 1996), which showed that these specific mutations were found in two independent experiments in which a vvIBDV isolate (OKYM) was adapted for growth on primary chicken embryo fibroblast cells (OKYMT). Sequences of OKYM and OKYMT are also given in Figure 1. Dutch field isolate 97-B3 is an exception in that it did not induce any mortality or lesions, but has an amino acid sequence of the HVR of VP2 that is almost identical to that of vvIBDV. The difference in this sequence is substitution D279N that was also named by Yamaguchi *et al.* (1996) to be important for adaptation and attenuation. The broilers on this farm had been vaccinated with the hot vaccine 228 E. We suppose that the isolate that we have obtained from this farm might be this vaccine strain. However we cannot be sure, since the sequence of vaccine strain 228 E is not published.

In conclusion, there is no indication from our data that recent isolates are different from D6948 either in virulence or in amino acid sequence of the HVR of VP2. The sequence of the Hungarian isolate is identical to D6948. From several farms with a case history including lower mortality (1996), only a vaccine strain was isolated or a combination of vaccine strain with a vvIBDV isolate. Moreover it has been confirmed that the diagnosis IBV cannot be based on current techniques like IFA on bursa since this test cannot discriminate between vaccine and field strains. This test is more valuable if the flock history is taken into consideration.

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Legends to Figure 1.

Deduced amino acid sequence of the VP2 hypervariable region from positions 207 to 350 (numbering according to Bayliss *et al.*, 1990). The sequence of the Dutch vIBDV isolate D6948 and of UK661 are used as reference. A dash indicates sequence not determined. A dot indicates where sequences are identical to UK661 and D6948. Dark shading indicates VP2 major hydrophilic peaks A and B (Azad *et al.*, 1987) pale shading indicates minor hydrophilic peaks 1 and 2 also reported to influence IBDV antigenicity (van den Berg *et al.*, 1996). The attenuated isolate CEF94 has been described by Boot *et al.* (2000).

Table 1: Primers used for sequencing the hypervariable region of the VP2-gene:

| Primers | Sequence | Orientation | Position |
|---------|------------------------------|-------------|--------------------------------|
| AC0 | 5'-ATACGATCGGTCTGACCCCGG-3' | + | 3/23 |
| AC3 | 5'-GGTAGCCACATGTGACAG-3' | + | 709/726 |
| AC4 | 5'-ACCCAGCCAATCACATCC-3' | + | 1034/1051 |
| AC5 | 5'-aaggcCTTCATGGAGGTGGCCG-3' | + | 1405/1421 (<i>Stul</i> -site) |
| AC6 | 5'-TTCACCTGGGGTACTCCG-3' | + | 1741/1758 |
| ANC2 | 5'-CTGCCTGTCCCTGGAGCC-3' | - | 326/342 |
| ANC5 | 5'-CCCATCTGGAGCATATCC-3' | - | 1287/1304 |

Table 2: Results of virulence test and sequence analysis of IBDV isolates from broiler farms during outbreaks in 1996.

| Isolate/group | History of mortality in flock | Mortality ¹ | Bursa/body weight ratio *1000 ² | Pathology ³ | Sequence analysis ⁴ |
|------------------|-------------------------------|------------------------|--|------------------------|--------------------------------|
| Negative control | NA | 0/15 | 7 | None | NA |
| D6948 | Yes, high | 8/15 | 0.8 | Severe | Vv |
| 96-B4 | No | 0/15 | 1 | None | Vaccine |
| 96-B5 | Yes, low | 3/15 | 2 | Severe | Vv + vaccine |
| 96-B6 | No | 0/15 | 7 | None | ND ⁵ |
| 96-C4 | No | 0/15 | 2 | None | Vaccine |
| 96-C5 | No | 0/15 | 2 | None | Vaccine |
| 96-C6 | Yes, high | 11/15 | 2 | Severe | Vv |

¹ Mortality is indicated in number of deaths per total number of chickens in group.

² bursa/body weight ratios are multiplied by 1000. These data are given for chickens necropsied at day 10.

³ Pathology indicates any gross lesions (like haemorrhages of muscles and bursa, oedema of bursa, degeneration of kidneys) observed during postmortems either after death or at day 10 PI.

⁴ Sequence analysis indicates conclusion drawn on sequence analysis given in Figure 1. Vv = very virulent sequence of HVR of VP2, vaccine = sequence identical to sequence of HVR of VP2 of vaccine strains.

⁵ No IBDV RNA could be obtained from the bursa samples.

NA = not applicable

ND = not determined

Table 3: Results of virulence test and sequence analysis of IBDV isolates from broiler farms during outbreaks in 1997.

| Isolate/group | History of mortality in flock | Mortality ¹ | Bursa/body weight ratio *1000 ² | Pathology ³ | Sequence analysis ⁴ |
|------------------|-------------------------------|------------------------|--|------------------------|--------------------------------|
| Negative control | NA | 0/14 | 7 | None | NA |
| D6948 | Yes, high | 9/13 | 0.6 | Severe | Vv |
| 97-B3 | Yes, high | 0/15 | 1 | Severe | Vv (hot vaccine) |
| 97-B4 | Yes | 14/14 | ND | Severe | Vv + vaccine |
| 97-B5 | Yes, high | 12/15 | 0.9 | Severe | Vv |
| 97-B6 | Yes, very high | 9/15 | 1.5 | Severe | Vv |
| 97-5123 | Yes, high | 9/10 ⁵ | 0.4 | Severe | Vv |
| 97-XX | Yes high | ND | ND | ND | Vv |
| Hungary | ⁶ | 80% | ND | Severe | Vv |

¹ Mortality in the experiment is indicated in number of deaths per total number of chickens in group, except for the Hungarian isolate.

² bursa/body weight ratios are multiplied by 1000. These data are given for chickens necropsied at day 21 PI.

³ Pathology indicates any gross lesions (like haemorrhages of muscles and bursa, oedema of bursa, degeneration of kidneys) observed during postmortems either after death or at day 21 PI.

⁴ Sequence analysis indicates conclusion drawn on sequence analysis given in Figure 1. Vv = very virulent sequence of HVR of VP2, vaccine = sequence identical to sequence of HVR of VP2 of vaccine strains.

⁵ The isolate 97-5123 was tested in a separate experiment.

⁶ The Hungarian IBDV isolate was isolated from the trachea of broilers with respiratory complaints.

NA = not applicable

ND = not determined

Figure 1. Deduced amino acid sequences of VP2 variable region

| | | major hydrophilic peak A | | | minor hydrophilic peak 1 | |
|-----------------|-------|-----------------------------|----------------|------------|-----------------------------|----------------|
| pHB22 (D6948) | YTIT | AADDYQ | FSSQYQAGGV | TITLFSANID | AITSLSIGGE | LVF-QTSVQG 255 |
| 5123 | | | | | | |
| UK661 | | | | | | |
| DV86 | | | | | | |
| 849VB | | | | | ...K..V... |R... |
| isolate Hungary | | | | | | |
| 97-B6 | | | | | | |
| 97-B5 | | | | | | |
| 96-C6 | ----- | | | | | |
| 96-B5 (2) | ----- | | | | | |
| 97-B4 | .L.. | | | | | |
| 97-xx | | | | | | |
| 97-B3 | | | | | | |
| OKYM | | | | | | |
| OKYM1 | | | | | | |
| 52-70 | | |P..... | | | |
| 002/73 | | |P..... | | ...N..V... | |
| GLS | | |T..... | |V... |K...HS |
| 96-C4 | | |L..... | | |N...HS |
| LukertBP | ----- | | | | |H...HS |
| D78 | | |P..... | |V... |H...HS |
| 96-B4 | ----- | |P..... | |V... |H...HS |
| 96-C5 | ----- | |P..... | |V... |H...HS |
| 96-B5 (1) | ----- | |P..... | |V... |H...HS |
| pHB23 (CEF-94) | | |P..... | |V... |H...HS |
| Cu-1 | | |P..... | |V... |H...HS |
| Edgar | | |P..... | | |H...HS |
| STC | | |P..... | |V... |H...HS |
| GBF-I | | |P..... | |V... |H...HS |
| Del E | |N..... |T..... | |V... |K...HS |
| Del A | | |Q..... | |V... |K...HS |
| 23/82 | |E..... |LIPS..... | KT..... | ...L..F.V... |S.VTIHS |
| OH | ..V. |E..... |LIPS..... | KT...T.... | ...L...V... |S.VTIHS |

Minor hydrophilic
peak 2

| | LILGATIYLI | GFDGTAVITR | AVAA | DNGLTA | GTDNLM | PFNI | VIPTSEITQP | 305 |
|-----------------|------------|------------|-------|--------|--------|------|------------|-----|
| pHB22 (D6948) | | | | | | | | |
| 5123 | | | | | | | | |
| UK661 | | | | | | | | |
| DV86 | | | | | | | | |
| 849VB | | | | | | | N | |
| isolate Hungary | | | | | | | | |
| 97-B6 | | | | | | | | |
| 97-B5 | | | | | | | | |
| 96-C6 | | | | | | | | |
| 96-B5 (2) | | | | | | | | |
| 97-B4 | | | | | | | | |
| 97-xx | | | | | | | T | |
| 97-B3 | | | | N | | | | |
| OKYM | | | | | | | | |
| OKYM1 | T | | | N | T | | | |
| 52-70 | V | | | | | | N | |
| 002/73 | V.N | V | T.T | G | | | | |
| GLS | V | S | | N | T | | N | |
| 96-C4 | A.N | T | S | T | I | | N | |
| LukertBP | A.N | T | S | T | I | | N | |
| D78 | V | T | | N | T | | N | |
| 96-B4 | V | T | | N | T | | N | |
| 96-C5 | V | | | Y | T | | N | |
| 96-B5 (1) | V | V | | XX | T | | N | |
| pHB23 (CEF-94) | V | | | N | T | | N | |
| Cu-1 | V | T | | N | T | | S.N | |
| Edgar | V | T | S | | | | N | |
| STC | V | F | T | | | | N | |
| GBF-I | V | ST | | X | | | N | |
| Del E | V | | | N | I | | N | |
| Del A | V | | | N | I | | N | |
| 23/82 | IEVDV | HF | D.AVK | T | F | T | N.V | V.N |
| OH | IEVDV | F | E.TVK | T | F | T | N.V | GG |

Major Hydrophilic
peak B

| | ITSIKLE | IVT SKSGGQAGDC | MSWSASGSLA | VTIHGGNYPG | ALRPV | 350 |
|-----------------|---------|----------------|------------|------------|-------|-----|
| pHB22 (D6948) | | | | | | |
| 5123 | | | | | | |
| UK661 | | | | | | |
| DV86 | | | | A..... | | |
| 849VB | | | | | | |
| isolate Hungary | | | | | | |
| 97-B6 | | | | | | |
| 97-B5 | | | | | | |
| 96-C6 | | | | | | |
| 96-B5 (2) | | | | | | |
| 97-B4 | | | | | | |
| 97-xx |V | | | | | |
| 97-B3 | | | | | | |
| OKYM | | | | | | |
| OKYM1 | | F | | | | |
| 52-70 | | | | | | |
| 002/73 | V..... | | L..N. | | | |
| GLS | | E | | | | |
| 96-C4 | | | | | | |
| LukertBP | | | | ---- | ---- | |
| D78 | | | R. | | | |
| 96-B4 | | | R. | | | |
| 96-C5 | | | R. | | | |
| 96-B5 (1) | | | R. | | | |
| pHB23 (CEF-94) | | | R. | | | |
| Cu-1 | | | K. | | | |
| Edgar | | | | | | |
| STC |V | | | | | |
| GBF-I | | | | | X. | |
| Del E | | D..E | | | | |
| Del A | | D | | | | |
| 23/82 | ...M... | V..Y.I..T..F | I..TV..T.. | ..V..... | | |
| OH | ...M... | V..Y.R..T..E | I..TV..T.. | ..V..... | | |