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Research Article

Presence of a Very Virulent Genotype of Infectious Bursal Disease Virus in Vaccinated Layer Hens in Turkey

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Abstract: Despite vaccinations against very virulent (vv) strains of infectious bursal disease virus (IBDV) or Gumboro, Gumboro outbreaks in vaccinated hens in Turkey are still reported. These outbreaks suggested that new vv strains might have emerged and the present vaccines might not be able to provide protection against infections with these strains. The objective of this study was to characterize the viruses isolated from vaccinated layer hens. For this purpose, the hypervariable region of the VP2 gene of the viruses was amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) and the amplification products were sequenced. These results showed that the field isolates of IBDV were more closely related to the European and Asian vv strain rather than the classical virulent strain, and the Gumboro outbreaks in Turkey were not derived from a new vv strain.

Key Words: IBDV, VP2 gene, sequence analysis

Türkiye'de Aşılanmış Yumurtacı Tavuklarda Enfeksiyöz Bursal Hastalığı Virüsünün Oldukça Virülent Genotipinin Varlığı

Özet: Türkiye'de enfeksiyöz bursal hastalık virusu (IBDV) yada Gumboro'nun oldukça virulent (vv) suşlarına karşı aşılamalara rağmen, aşılanmış tavuklarda IBDV salgınları hala rapor edilmektedir. Bu salgınlar, mevcut aşıların korumadığı, yeni vv türlerin ortaya çıkmış olabileceğini akla getirmektedir. Bu çalışmada, aşılı yumurtacı tavuklardan izole edilen virusların karakterizasyonu amaçlanmıştır. Bu amaçla, VP2 gen bölgesinin oldukça değişken bölgesi tersine transkripsiyon-polimeraz zincir tepkimesi (RT-PCR) ile çoğaltıldı ve çoğaltılan ürünler sekanslandı. Bu sonuçlara göre, IBDV'nin saha izolatlarının klasik virulent suşdan ziyade Avrupa ve Asya vv suşlarına daha yakın ilişkili olduğu ve salgınların yeni bir vv suşundan köken almadığı görülmüştür.

Anahtar Sözcükler: IBDV, VP2 geni, sekans analizi

Introduction

Infectious bursal disease virus (IBDV) is a member of the family *Birnaviridae*. It is a nonenveloped icosahedral virus with a diameter of about 60 nm and contains a double-strand RNA genome with 2 segments (1). The virus has 2 serotypes, named serotype I and serotype II. Serotype I viruses are pathogenic for chickens while serotype II viruses are avirulent. The classical form of the disease, known as Gumboro disease, affects young broiler chickens. The disease was characterized by destruction of the follicles of the bursa of Fabricius (BF) and resulted in high morbidity and mortalities in 1960s to 1980s throughout the world.

Very virulent (vv) strains, which were detected in Asia and Europe in the late 1980s, caused high mortality in not only young chickens, but also in older animals. In addition, it was shown that vv strains cause more severe disease, even in vaccinated flocks (2-4).

A vaccine must accurately reflect the antigenic types present in a country. The antigenic diversity of IBDV needs to be considered when designing and constructing effective vaccination strategies for this virus. Different antigenic and pathogenic types emerge as a result of mutations formed in the genome of IBDV. The most crucial mutations have been observed after nucleotide changes occurred in the hypervariable region of the VP2

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gene of the virus (5-8). Furthermore, nucleotide sequence comparisons of the VP2 gene are crucial for detection of genetic diversity of the field isolates.

Very virulent strains in Turkey were partly characterized by serological and restriction fragment length polymorphism analysis (9,10). Vaccinations against IBD were performed during the years following the outbreaks with vv strains. Despite vaccinations against vv strains of IBDV, outbreaks in vaccinated hens in Turkey are still reported. These outbreaks suggested that new vv strains might have emerged and the present vaccines may not be able to provide protection against infections with these strains. The objective of this study was to characterize the VP2 gene region of the viruses isolated from vaccinated layer hens.

Materials and Methods

Samples

Gumboro outbreaks were reported in Elazığ, Turkey, between 2002 and 2004. Samples were collected at the beginning of the outbreak from a 20,000 commercial layer flock of White Leghorns at 16 weeks of age. The animals were vaccinated with intermediate-hot vaccine strains (Bursine-2; Fort Dodge Animal Health, Iowa, USA, and Tad-gumboro-vac-forte; Lohmann Animal Health, Germany) by a private company at 14, 21, and 28 days of age. The mortality rate was 60%-70%, whereas the morbidity rate was approximately 90%. Twenty animals that died at the beginning of the outbreak were necropsied and examined macroscopically. Tissue samples including the bursa of Fabricius, liver, lungs, intestines, spleen, cerebrum, kidneys and cerebellum were collected and fixed in 10% formalin, and the samples were stained with hematoxylin-eosin (H&E). A portion of the bursa samples was stored at -80 °C and reserved for PCR analysis and virus isolation.

Propagation of IBDV

Field isolates were propagated in primary chicken embryo fibroblast (CEF) cells. Monolayer CEF cells were infected with the bursal samples with suspected IBDV and incubated at 37 °C for 4-5 days. After 4 days, the infected cells were frozen and thawed. After 2 blind passages, the cell culture supernatant was collected at 1500 xg for 15 min. Presence of IBDV in the cell culture supernatant was confirmed by PCR analysis.

RNA extraction and RT- PCR

RNA samples were extracted from the bursal homogenates and IBDV infected CEF cells using an EZ-RNA total RNA isolation kit by following the manufacturer's recommendations (Biological Industries Co., Israel). The RNA pellets were resuspended in 30 µl of RNase-free H_2O. Approximately 70 µl of DMSO was added. After 5 min incubation at 100 °C, RNA was stored at –80 ºC until used for RT-PCR analysis.

The RT-PCR procedure was performed as previously described (11). A total of 25 µl of mixture containing 10 µl of sample RNA, 5 µl of 5x M-MLV buffer (250 mM Tris-HCl, pH 8.3, 375 mM potassium chloride, 15 mM magnesium chloride, 50 mM DTT), 2 mM of each deoxynucleotide, 20 U M-MLV reverse transcriptase (Promega), and 1.25 µM of each primer (IBDV B3; 5´ CCC AGA GTC TAC ACC ATA 3´ and IBDV B4; 5´ TCC TGT TGC CAC TCT TTC 3´) were used in the RT reaction. The reaction was performed at 37 °C over 1 h. The PCR reaction was performed with a total of 50 µl of mixture containing 10 µl of the sample cDNA, 5 µl of 10x PCR buffer (100 mM Tris-HCl, pH 8.0, 500 mM potassium chloride, 15 mM magnesium chloride), 2 mM of each deoxynucleotide, 1 U Taq DNA polymerase (Bioron), and 1.25 µM of each primer. For amplifications, 34 cycles were used consisting of 94 °C for 1 min, 42 °C for 1 min, and 72 °C for 2 min. A final extension step was performed at 72 °C for 10 min. The amplified PCR products were stained with ethidium bromide and visualized under ultraviolet light on 2% agarose gel.

Sequencing

RT-PCR products belonging to 3 isolates chosen randomly were purified using a DNA purification system (Promega). Then, purified DNAs were sequenced by using the ABI 310 Genetic Analysis System (Iontec Co., İstanbul, Turkey). The position of isolates in the phylogenetic tree was checked by a neighbor-joining method from the PHYLIP inference software package.

Results

All bursa samples had massive necrosis, a swollen appearance and a hemorrhagic mucosal surface. There were mild to moderate foci of hemorrhages on the skeletal muscles of the legs, and the mucosal surface between the muscular and glandular stomach. The kidneys were pale and swollen.

The most notable changes occurred in the bursa of Fabricius including lymphoid follicular necrosis, hemorrhage, edema, and mononuclear cell infiltration, predominantly heterophiles. Most of the lymphocytes throughout the follicles were pyknotic and karyorectic.

In the RT-PCR, a 470 base pair (bp) long amplification product was detected from the bursal samples of the animals with suspected IBDV (data not shown).

In the genetic analysis, sequences of 3 isolates selected randomly were shown to be the same. Gene sequences belonging to the hypervariable region of the VP2 gene of the Turkish isolate (EL2004-1/TR) amplified by the PCR are shown in Figure 1. The nucleotide sequence of the isolate was deposited in GenBank under accession number EF043078. These gene sequences were compared to the sequences obtained from the EMBL database (Figure 1). In the genetic analysis, 26 nucleotide changes were determined between the same gene region of F 52/70/UK classical virulent isolate (D12610) (7) and sequence of the VP2 gene region of the Turkish isolate (Figure 1).

Amino acid sequences of the hypervariable region of the VP2 gene of the Turkish isolate (EL2004-1/TR) and amino acid sequences of the same region of viruses isolated in different countries are shown in Figure 2.

Figure 1. Alignment of nucleotide sequences of the hypervariable region of the VP2 gene of the Turkish isolate (T 2004/23/TR) with the classical virulent strains (F 52/70/UK) and 4 very virulent strains (UK 661/UK, 96108/France, OKYM/Japan, SDH1/Iran).

736

F 52/70/UK
CCCAGAGTCTACACCATAACTGCAGCCGATGATTACCAATTCTCATCACAGTACCAACCA
EL2004-1/TR
CCCAGAGTCTACACCATAACTGCAGCCGATGATTACCAATTCTCATCACAGTACCAA**G**CA
UK 661/UK
CCCAGAGTCTACACCATAACTGCAGCCGA**C**GATTACCAATTCTCATCACAGTACCAA**G**CA
96108/France
CCCAGAGTCTACACCATAACTGCAGCCGA**CA**ATTACCAATTCTCATCACAGTACCAA**G**CA
OKYM/Japan
CCCAGAGTCTACACCATAACTGCAGCCGATGATTACCAATTCTCATCACAGTACCAA**G**CA
SDH1/IRAN
CCCAGAGTCTACACCATAACTGCAGCCGATGATTACCAATTCTCATCACAGTACCAA**G**CA

796

F 52/70/UK
GGTGGGGTAACAATCACACTGTTCTCAGCCAACATTGATGCTATCACAAGCCTCAGCATT
EL2004-1/TR
GGTGGGGTAACAATCACACTGTTCTCAGC**T**AA**T**AT**C**GATGC**C**ATCAC**G**AGCCTCAGCAT**C**
UK 661/UK
GGTGGGGTAACAATCACACTGTTCTCAGC**T**AA**T**AT**C**GATGC**C**ATCAC**A**AGCCTCAGCAT**C**
96108/ France
GGTGGGGTAACAATCACACTGTTCTCAGC**T**AA**T**AT**C**GATGC**C**ATCAC**A**AGCCTCAGCAT**C**
OKYM/Japan
GGTGGGGTAACAATCACACTGTTCTCAGC**T**AA**T**AT**C**GATGC**C**ATCAC**A**AGCCTCAGCAT**C**
SDH1/Iran
GGTGGGGTAACAATCACACTGTTCTCAGC**T**AA**T**AT**C**GATGC**C**ATCAC**A**AGCCTCAGCAT**C**

Figure 1. Contunued.

856

F 52/70/UK
GGGGGAGAGCTCGTGTTTCAAACAAGCGTCCAAGGCCTTGTACTGGGCGCCACCATCTAC
EL2004-1/TR
GGGGGAGA**A**CT**T**GTGTTTCAAACAAGCGTCCAAGGCCTT**A**TACTGGG**T**GC**T**ACCATCTAC
UK 661/UK
GGGGGAGA**A**CTCGTGTTTCAAACAAGCGTCCAAGGCCTT**A**TACTGGG**T**GC**T**ACCATCTAC
96108/France
GGGGGAGA**A**CT**T**GTGTTTCAAACAAGCGTCCAAGGCCTT**A**TACTGGG**T**GC**T**ACCATCTAC
OKYM/Japan
GGGGGAGA**A**CTCGTGTTTCAAACAAGCGTCCAAGGCCT**CA**TACTGGG**T**GC**T**ACCATCTAC
SDH1/Iran
GGGGGAGA**A**CTCGTGTTTCAAACAAGCGTCCAAGGCCTT**A**TACTGGG**T**GC**T**ACCATCTAC

916

F 52/70/UK
CTTATAGGCTTTGATGGGACTGCGGTAATCACCAGAGCTGTGGCCGCAGATAATGGGCTG
EL2004-1/TR
CTTATAGGCTTTGATGGGACTGCGGTAATCAC**T**AGAGCTGTGGCCGCAGA**C**AATGGGCT**A**
UK 661/UK
CTTATAGGCTTTGATGGGACTGCGGTAATCACCAGAGCTGTGGCCGCAGA**C**AATGGGCTG
96108/ France
CTTATAGGCTTTGATGGGACTGCGGTAATCACCAGAGCTGTGGCCGCAGA**C**AATGGGCTG
OKYM
CTTATAGGCTTTGATGGGACTGCGGTAATCACCAGAGCTGTGGCCGCAGA**C**AATGGGCT**A**
SDH1/Iran
CTTATAGGCTTTGATGGGACTGCGGTAATCACCAGAGCTGTGGCCGCAGA**C**AATGGGCT**A**

976

F 52/70/UK
ACGGCCGGCACCGACAATCTTATGCCATTCAATATTGTCATTCCAACCAATGAGATAACC
EL2004-1/TR
ACGGCCGGCAC**T**GACAA**C**CTTATGCCATTCAATATTGT**G**ATTCCAACCA**GC**GAGATAACC
UK 661/UK
ACGGCCGGCAC**T**GACAA**C**CTTATGCCATTCAATATTGT**G**ATTCCAACCA**GC**GAGATAACC
96108/France
ACGGCCGGCAC**T**GACAA**C**CTTATGCCATTCAATATTGT**G**ATTCCAACCA**GC**GAGATAACC
OKYM/JAPAN
ACGGCCGGCAC**T**GACAA**C**CTTATGCCATTCAATATTGT**G**ATTCCAACCA**GC**GAGATAACC
SDH1/Iran
ACGGCCGGCAC**T**GACAA**C**CTTATGCCATTCAATATTGT**G**ATTCCAACCA**G**TGAGATAACC

1036

F 52/70/UK
CAGCCAATCACATCCATCAAACTGGAGATAGTGACCTCCAAAAGTGGTGGTCAGGCAGGG
EL2004-1/TR
CAGCCAATCACATCCATCAAACTGGAGATAGT**T**ACCTCCAAAAGTGGTGGTCA**A**GC**G**GGG
UK 661/UK
CAGCCAATCACATCCATCAAACTGGAGATAGT**A**ACCTCCAAAAGTGGTGGTCAGGC**G**GGG
96108/ France
CAGCCAATCACATCCATCAAACTGGAGATAGT**A**ACCTCCAAAAGTGGTGGTCAGGC**G**GGG
OKYM/Japan
CAGCCAATCACATCCATCAAACTGGAGATAGTGACCTCCAAAAGTGGTGGTCAGGC**G**GGG
SDH1/Iran
CAGCCAATCACATCCATCAAACTGGAGATAGTGACCTCCAAAAGTGGTGGTCAGGC**G**GGG

Figure 1. Contunued.

1096

F 52/70/UK
GATCAGATGTCATGGTCGGCAAGTGGGAGCCTAGCAGTGACGATCCATGGTGGCAACTAT
EL2004-1/TR
GATCAGATGTCATGGTC**A**GCAAGTGGGAGCCTAGCAGTGACGATCCA**C**GGTGGCAACTAT
UK 661/UK
GATCAGATGTCATGGTC**A**GCAAGTGGGAGCCTAGCAGTGACGATCCA**C**GGTGGCAACTAT
96108/ France
GATCAGATGTCATGGTC**A**GCAAGTGGGAGCCTAGCAGTGACGATCCA**C**GGTGGCAACTAT
OKYM/Japan
GATCAGATGTCATGGTC**A**GCAAGTGGGAGCCTAGCAGTGACGATCCA**C**GGTGGCAACTA**C**
SDH1/Iran
GATCAGATGTCATGGTC**A**GCAAGTGGGAGCCTAGCAGTGACGATCCA**C**GGTGGCAACTAT

1156

F 52/70/UK
CCAGGGGCCCTCCGTCCCGTCACACTA
EL2004-1/TR
CCAGGGGCCCTCCGTCCCGTCACACTA
UK 661/UK
CCAGGGGCCCTCCGTCCCGTCACACTA
96108/ France
CCAGGGGCCCTCCGTCCCGTCACACTA
OKYM/Japan
CCAGGGGCCCTCCGTCCCGTCACACTA
SDH1/Iran
CCAGGGGCCCTCCGTCCCGTCACACTA

Figure 2. Alignment of the amino acid sequences of the hypervariable region of the VP2 gene of the Turkish isolate (T 2004/23/TR) with the classical virulent strains (F 52/70/UK) and 4 very virulent strains (UK 661/UK, 96108/France, OKYM/Japan, SDH1/Iran).

203

F 52/70/UK
PRVYTITAADDYQFSSQYQPGGVTITLFSANIDAITSLSIGGELVFQTSVQGLVLGATIY
EL2004-1/TR
PRVYTITAADDYQFSSQYQ**A**GGVTITLFSANIDAITSLSIGGELVFQTSVQGL**I**LGATIY
UK 661/UK
PRVYTITAADDYQFSSQYQ**A**GGVTITLFSANIDAITSLSIGGELVFQTSVQGL**I**LGATIY
96108/ France
PRVYTITAAD**N**YQFSSQYQ**A**GGVTITLFSANIDAITSLSIGGELVFQTSVQGL**I**LGATIY
OKYM/ Japan
PRVYTITAADDYQFSSQYQ**A**GGVTITLFSANIDAITSLSIGGELVFQTSVQGL**I**LGATIY
SDH1/IRAN
PRVYTITAADDYQFSSQYQ**A**GGVTITLFSANIDAITSLSIGGELVFQTSVQGL**I**LGATIY

Figure 2. Contunued.

262

F 52/70/UK
LIGFDGTAVITRAVAADNGLTAGTDNLMPFNLVIPTNEITQPITSIKLEIVTSKSGGQAG
EL2004-1/TR
LIGFDGTAVITRAVAADNGLTAGTDNLMPFN**I**VIPT**S**EITQPITSIKLEIVTSKSGGQAG
UK 661/UK
LIGFDGTAVITRAVAADNGLTAGTDNLMPFN**I**VIPT**S**EITQPITSIKLEIVTSKSGGQAG
96108/ France
LIGFDGTAVITRAVAADNGLTAGTDNLMPFN**I**VIPT**S**EITQPITSIKLEIVTSKSGGQAG
OKYM/ Japan
LIGFDGTAVITRAVAADNGLTAGTDNLMPFN**I**VIPT**S**EITQPITSIKLEIVTSKSGGQAG
SDH1/IRAN
LIGFDGTAVITRAVAADNGLTAGTDNLMPFN**I**VIPT**S**EITQPITSIKLEIVTSKSGGQAG

332

F 52/70/UK
DQMSWSASGSLAVTIHGGNYPGALRPVTL
EL2004-1/TR
DQMSWSASGSLAVTIHGGNYPGALRPVTL
UK 661/UK
DQMSWSASGSLAVTIHGGNYPGALRPVTL
96108/ France
DQMSWSASGSLAVTIHGGNYPGALRPVTL
OKYM/JAPAN
DQMSWSASGSLAVTIHGGNYPGALRPVTL
SDH1/IRAN
DQMSWSASGSLAVTIHGGNYPGALRPVTL

Discussion

In this study, in order to characterize the viruses isolated from vaccinated hens during an IBDV outbreak in Turkey, the isolates in the VP2 gene region were sequenced. Many nucleotide changes determined in comparison with the classical virulent virus of the Turkish isolate are also present in the vv isolates (Figure 1). In the comparison with vv strains of EL2004-1/TR, 6 nucleotide changes with UK 661 (X92761) (12), SDHI/Iran (AY323952) (13), and 96108/France (AJ001948) (7) and 5 nucleotide changes with OKYM/Japan (D49706) (14) were determined. Four of the 26 nucleotide changes were defined in only Turkish isolates. The phylogenetic tree showed that the Turkish isolates had nucleotide sequences typical of vvIBDV (data not shown).

Although 26 nucleotide substitutions were found in Turkish isolates in comparison with the VP2 gene region of the classical virulent isolate, merely 5 of them resulted in amino acid differences. Thus, 21 nucleotide differences were evaluated as a silent mutation. The mutations elicited of amino acid substitutions were determined in nucleotide positions 794, 896, 1010, 1026 and 1027 in the VP2 gene. As shown in Figure 2, as the result of these nucleotide changes, 4 amino acid substitutions emerged at positions 222 (Pro-Ala), 256 (Val-Ile), 294 (Leu-Ile), and 299 (Asn-Ser) of the VP2 protein. The amino acids substitutions described in the Turkish isolates were also present in vv strains UK 661, SDHI/Iran, and OKYM/Japan.

These findings showed that the genome structure of the VP2 hypervariable region of the Turkish isolate was similar to the vv isolate genome structure rather than the classical virulent virus. Moreover, when the genomic identity of Turkish IBDV isolates and Iranian IBDV isolates and also the nearness of these 2 countries are taken into consideration, there may be an epidemiologic relation between these 2 isolates. Yet, there is no certain information about the origins of vvIBDV at present.

In the early 1990s in Turkey, epidemic diseases brought about by vv types were recorded and the viruses isolated during these epidemics were partly characterized by serological and restriction fragment length polymorphism analysis (9,10). Furthermore, following these epidemics, vvIBDV specific vaccines have been used. Despite vaccinations against vv strains, Gumboro outbreaks in vaccinated hens in Turkey are still reported. These outbreaks suggested that new vv strains might have emerged and the present vaccines may not be able to provide protection against infections with these strains. Therefore, the hypervariable region of the VP2 gene of the viruses isolated from vaccinated layer hens was amplified by RT-PCR and the amplification products were sequenced. Sequence or phylogenic analysis showed that sequences of the field isolates of IBDV were more closely related to European and Asian vv strain sequences rather than the classical virulent strain sequences.

According to these results, the outbreaks were not derived from a new vv strain. Yet, some studies showed that VP2 was not the sole determinant for virulence (15-17). Therefore, it is concluded that the VP2 protein alone may not be the antigenic determinant. As another probability, although vaccination was applied according to the vaccination programs recommended by the vaccination firms in these flocks, it is supposed that these programs may not protect mature animals connected with decreases in antibody titers under the protection level. Therefore, we recommend the determination of antibody titers or booster vaccination of adult hens.

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