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Heterogenicity of Bovine Viral Diarrhea Virus in Egypt

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Abstract: Bovine viral diarrhea virus (BVDV) of genotype-1 was identified in Egypt, BVDV-1 is detected in two Egyptian governorates (Fayoum and Alexandria). The percentage of BVDV infected cases was 6.7 and 8.4 in Fayoum and Alexandria respectively. The isolated viruses were discriminated on the basis of the sequence of the 5' non-coding region (5'-UTR) using reverse-transcription polymerase chain reaction (RT-PCR). Eleven BVDV strains obtained from cattle during this study belong to BVDV of genotype 1. The Egyptian isolates formed at least three branches originated from the existing branches of 1a, 1b and 1f genotypes. Two viruses from 1a which contain also the vaccine strain used in Egypt indicating their possible origin, while 7 viruses from 1b indicating spared of this genotype in the two regions. While 2 viruses were belong to 1f genotype. So far this is the first report describing multiple genotypes of BVD in Egypt; however it is spread in different areas in the Middle East. These findings suggest that extensive genetic diversity was found within BVDV type 1 isolates from two governorates in Egypt. The isolates were grouped with the BVDV type 1 strains, particularly of the Middle East origin, indicating genetic diversity of circulating Egyptian viruses that possibly originated from a common ancestor of old Egyptian viruses. Further studies in different localities are needed to investigate the situation all over the country.

Key words: Bovine viral diarrhea virus (BVDV) · Egypt · Genotyping · Phylogenetic analysis · Cattle

INTRODUCTION

Bovine viral diarrhea virus (BVDV) is an economically important viral pathogen of cattle worldwide, causing a wide range of clinical syndromes and together with hog cholera virus (HCV) and border disease virus (BDV), are members of the genus Pestivirus in the family Flaviviridae [1-6]. BVDV was also previously detected in African buffaloes [7] and in water buffaloes in Egypt and in India [8, 9]. The genome of BVDV is a single-stranded, positive sense, non-polyadenilated RNA of approximately 12.5 Kb in length. It has two non-coding regions at the 5'end (5'UTR) and at the 3'end (3'UTR) of the genome [10-11]. Translation occurs in a cap-independent manner from a single large open reading frame (ORF) that encodes a polyprotein of about 4000 amino acids.

BVDV viruses were usually typed based on comparison of genomic sequences from the 5-untranslated region (5-UTR), Npro and E2 region. Vilcek *et al*. [12-13] identified 11 genetic groups (sub-genotypes) of BVDV-1 and confirmed a new sub-genotype, BVDV-1k, isolated from cattle in Switzerland. BVDV isolates from India were typed as BVDV-1b [14] whereas BVDV-1c is a predominant sub-genotype in Australia [15].

Two genotypes of BVDV have been described on the basis of the 5'UTR analysis, namely BVDV type 1, which is subdivided into subgroup 1a and represented by the reference strain NADL and subgroup 1b, represented by the reference strain Osloss. BVDV type 2 has strain 890 as reference and comprises especially isolates associated with a new form of acute infection in cattle, the

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haemorrhagic syndrome, originally described in North America [16]. BVDV type 2 has also been isolated from sheep [17].

In Egypt, BVDV was previously isolated as the Egyptian strains (Iman and Kenna) in 1975 and 1982, respectively and were typed as CP-BVDV 1 and the strain Behera-CP 58/99 that isolated from the milk and identified as CP-BVDV 2 [18, 19]. The first report of a BVDV-2 infection in dromedary camels in Egypt was reported by Yousif *et al*. [20].

We have reported the epidemiological surveillance of BVDV infection of cattle and buffaloes in two governorates of Egypt (Fayoum and Alexandria) using various diagnostic methods [21].

The aim of the present study was to continue our investigations to identify the genotypes of BVDV currently isolated from cattle in two Egyptian governorates (Fayoum and Alexandria).

MATERIALS AND METHODS

A total number of 1698 and 1602 animals (cattle and buffaloes) was screened for detection of BVDV infection from the Fayoum and Alexandria governorates respectively. Blood samples were collected on EDTA as anticoagulant for separation of buffy coat to be used for BVDV RNA detection by real- time PCR. Blood samples were pooled as 5 samples per one pool for screening using RT-PCR test and positive pools were investigated individually. A total of 249 BVDV positive samples (114 from Fayoum and 135 from Alexandria) were used for virus isolation from our previous work [21]. RNAs extraction and genotyping of eleven noncytopathic isolates from cattle of Fayoum and Alexandria were used for sub-genotyping and phylogenetic analysis.

RNAs were extracted from buffy coats of tested pooled blood samples or from the supernatant of cell culture after virus isolation [21] using QiAmp Viral RNA Mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. A set of primers were used for the RT-PCR reaction and for the subsequent sequence analysis using forward and reverse PCR primers of BVD targeting the 5- untranslated region UTR using primers as follows (corresponding to positions 105–125 and 399–378, respectively, in the sequence of BVDV strain NADL):

Forward – 5'-AGCCATGCCCTTAGTAGGACT-3'
Reverse –5'-ACTCCATGTGCCATGTACA-3'[22]

The one-tube RT-PCR was carried out according to the manufacturer instruction (OneStep RT-PCR Kit, Qiagen, Germany). The 50 µl reaction mixture contained 10 µl of extracted RNA, 10 µl of 5x RT-PCR buffer, 2 µl primer F, 2 µl primer R, 2 µl dNTP mix containing 400 µM each dATP, dGTP, dCTP, dTTP and 2 µl of QiagenOneStep Enzyme Mix. A fragment of 300bp of the 5'-noncoding region was amplified by PCR thermo cycling using (T3 Biometra- Germany) as follows: 50 °C for 30 min, 94 °C for 15 min, followed by 40 cycles of 95 °C for 15 s, 56 °C for 30 s and 72 °C for 2 min. Final extension was completed at 72 °C for 10 min. After amplification, PCR products of 5µl were analyzed by electrophoresis on a 1.5% agarose gel containing ethidium bromide with final concentration of 0.5µg/ml at 95 V for 30 min in 1x TBE buffer, against GeneRuler™100 bp Plus DNA Ladder (Fermentas). Images of the gels were taken on BioDoc Analyze Digital Systems (Biometra, Germany). Gel containing DNA band of the expected size (300 bp) was excised and purified with the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer instruction. The purified PCR products were sequenced directly using the ABI PRISM®BigDyeTM Terminators v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and the ABI PRISM®3130 genetic analyzer (Applied Biosystems) with 80 cm capillaries.

The assembly of the consensus sequences and alignment trimming was performed with the Lasergene DNASTAR group of programs (DNASTAR Inc., Madison,WI), Using Clustal V method and the identity percent and divergence between all viruses was also carried out. BLAST similarity analysis for partial nucleotide sequences of 5-untranslated region UTR for Egyptian viruses was carried out using BLAST tool of NCBI. The phylogram was drawn using MEGA6 software with other representative Egyptian viruses from the Genebank and in comparison with other international strains. Reference strains of BVDV type 1 and 2 and viruses from a previous investigation on BVDV in Africa, Middle East and worldwide were included for comparative purposes.

RESULTS

The results of positive BVDV antigen in blood using RT-PCR are presented in Tables (1). The percentage of BVDV infected cases was 6.7 and 8.4 in Fayoum and Alexandria respectively. The results of genotyping for the Egyptian viruses indicated successful identification for the presence of BVDV genotype-1 in 40 samples from Fayoum and 39 samples from Alexandria with total of 79 positive samples in both governorates (Table 1).

Table 1: BVDV detection by RT-PCR test:

		RT-PCR positive BVDV Antigen		
Governorates	# of examined animals	Number	Percentage	BVD virus isolates
Fayoum	1698	114	6.7%	40
Alexandria	1602	135	8.4%	39
Total	3300	249	7.5%	79

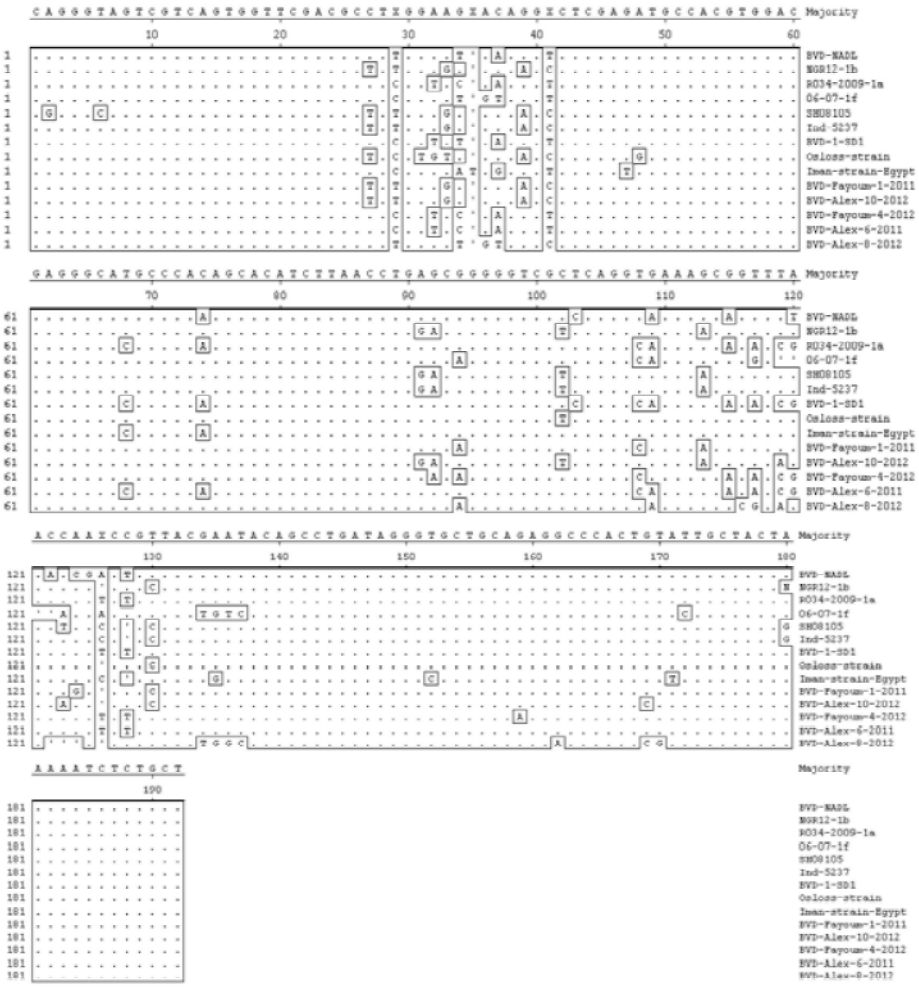

Fig. 1: Results of partial nucleotide sequence alignment for 5'UTR of BVDV

BLAST similarity analysis for partial nucleotide sequences of 5-untranslated region UTR for eleven Egyptian viruses of this study showed the different range of genetic relatedness to other international BVDV strains, the difference among the blast results for the viruses under study depend on the sub-grouping of BVD viruses (Table 2).

The results of genetic analysis of 5'UTR of Egyptian viruses of BVD indicate their grouping in different groups at least 3 genotypes. The similarity percent of strain BVD-Fayoum-1-2011 and BVD-Alex-10-2012 was 96% and 97.9% with NGR12 of 1b genotype respectively. While strains BVD-Fayoum-4-2012 and BVD-Alex-6-2011 were very close to strain R034-2009 of 1a genotype with 96.9% to 100% respectively. The strain BVD-Alex-8-2012 was very close to 06/07 strain of 1f genotype with 92.2% (Fig. 1, 2).

Phylogenetic Analysis: On the basis of partial nucleotide sequence alignment for 5'UTR the Egyptian viruses of BVDV showed the grouping of the Egyptian viruses in

Table 2: BLAST similarity analysis for partial nucleotide sequences of 5-untranslated region UTR for Egyptian viruses in comparison to international strains

Virus isolate	Related Strain	Country	Year	Genotype	BLAST similarity
BVD-Alex-8-2012,	CRO/10-15-30	CROutia	2010	1f	95%
BVD-Alex-9-2012,	strain TR-2007-B-AG5	Turkey	2007	1b	95%
BVD-Fayoum-1-2011	Bovine viral diarrhea virus 1 isolate O-1897/00-175	Slovenia	2000	1b	95%
BVD-Fayoum-3-2011	pestivirus type 1 strain M65CK/96	Sweden	1996	1a	94%
BVD-Fayoum-5-2012	IT99-6869	Italy	2003	1a	93%
BVD-Alex-10-2012	Bovine viral diarrhea virus 1 isolate Ind 5237	India	2007	1b	98%
BVD-Alex-11-2012	Bovine viral diarrhea virus 1 strain MS1	USA	2006	1b	98%
BVD-Fayoum-2-2011	Bovine viral diarrhea virus 1 isolate KA07	Korea	2008	1b	97%
BVD-Fayoum-4-2012	Bovine viral diarrhea virus 1 isolate SH08105	China	2008	1b	97%
	Bovine viral diarrhea virus 1 strain MS14	USA	2007	1b	97%
	Bovine viral diarrhea virus 1 strain MS7	USA	2007	1b	97%
	Bovine viral diarrhea virus 1 strain Yili0698	China	2006	1b	97%
BVD-Alex-6-2011	Bovine viral diarrhea virus 1-SD1	USA	1992	1a	99%
BVD-Alex-7-2011	AQHY95BI12	USA	2007	1a	99%
	isolate H1-181/AU	Australia	2011	1a	98%
	7153506 (B)	USA	2006	1a	97%
	Bovine viral diarrhea virus 1-NADL	USA	1988	1a	94%

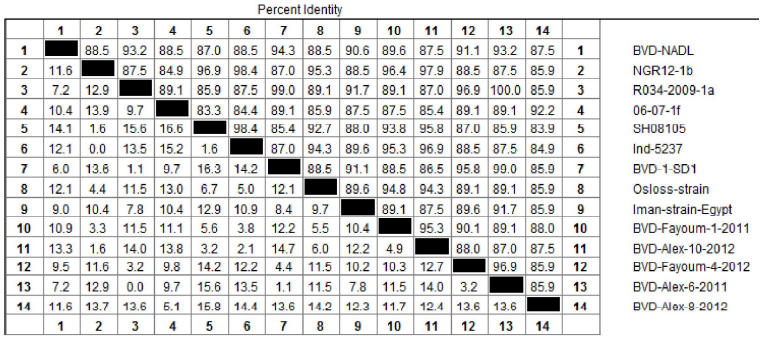

Fig. 2: Identity percent of nucleotide sequence for 5'UTR of BVDV

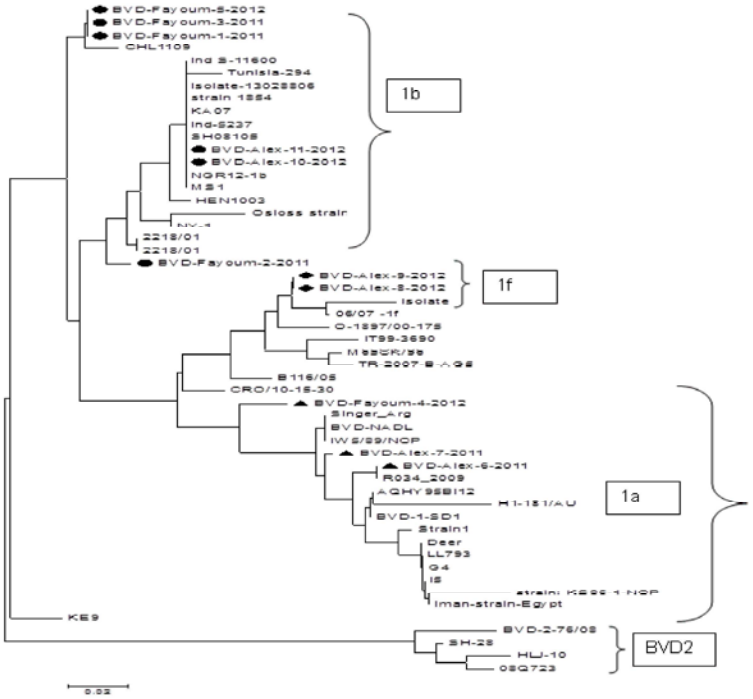

Fig 3: Phylogentic analysis of BVD viruses shows 2 main groups for world BVDV (BVD-1 & BVD-2) and 3 subgroups for BVD-1 (BVD-1a & BVD-1b).

comparison other parts of the world and showed at least 3 subgroups for BVDV Egyptian viruses related to BVD type 1 (Fig. 3).

The Egyptian viruses were clustered into 3 distinct genotypes of BVD-1 that were related to 1b, 1a and 1f genotypes. However, the three isolates (Fayoum-1, 3/2011 and Fayoum-5/2012) showed a higher genetic divergence among other viruses of 1b genotype.

DISCUSSION

In the early genetic typing of BVDV-1 viruses, only two sub-genotypes were recognized—BVDV-1a and BVDV-1b [10, 16]. Later analysis revealed an additional two to three sub-genotypes [23, 24]. More recent analysis has revealed at least 11 BVDV-1 genetic groups [12] indicating considerable genetic diversity within this pestivirus genotype.

In this study, reverse-transcription polymerase chain reaction (RT-PCR) was used to amplify specific sequences from the 5'NTR of the genome. The oligonucleotide primers corresponding to positions 105–125 and 399–378, respectively, in the sequence of BVDV strain NADL, were used to generate the PCR products. BVDV of genotype 1 was identified in the present study in both Egyptian governorates cattle (Alexandria and Fayoum) and they were discriminated on the basis of the sequence of the 5' non-coding region (5' UTR) using real-time PCR. The results of genotyping for the Egyptian viruses indicated successful identification for the presence of BVDV genotype-1 in the 2 Egyptian governorates (40 samples from Fayoum and 39 samples from Alexandria) with total of 79 positive samples in both governorates (Table 1).

A number of 11 of the Egyptian viruses from cattle were sequenced and genotyped as genotype 1 (BVD-1) in 3 groups related to 1a 1b and 1f genotypes (Figure 1). Comparing the sequencing data, genotypes BVD-1 of 1b and 1f genotypes from the field viruses did not cluster with vaccine strain currently used in field in Egypt of genotype 1a (Iman strain) indicating progressive genetic evolution of BVDV in Egypt.

In previous work, BVDV-1 isolates were clustered into potentially 16 genetic subtypes, 1a through 1p [25]. Two isolates from Finland and one from Egypt formed a group, which was tentatively labelled as BVDV-1j [11]. In Egypt, BVD strains, Iman and Kenna were typed as CP-BVDV-1 and the strain Behera-CP 58/99 was identified as CP-BVDV-2 [18, 19].

The present work revealed the presence of BVDV genotype 1 in cattle in Egypt based on the high sequence similarity between genotype 1 field strains and other strains from Middle East and Africa. The presence of genotypes -1b and 1f that phylogenetically belong to different clusters and coexist in Egypt is consistent with the possibility of vaccination failure due to low similarity with the vaccine. The clustering of the Egyptian viruses is not related to geographical location as the 3 groups were found in the two examined governorates. Vilcek *et al*. [13] also indicated that distribution of subgenotypes has no relationship to the geographic origin of viral isolates. There was no relationship between geographic origin, the nature of the clinical signs and typing of BVDV. This was also reported in BVD isolates from South Africa [26].

This is the first report of the presence of multiple genotypes like 1b and 1f in cattle in Egypt; however they were frequently reported from different areas in Europe and in the Middle East. This situation is indicating genetic diversity of circulating Egyptian BVD viruses that possibly originated from a common ancestor of old Egyptian viruses and linked to heterogenic BVD strains reported all over the world.

The results from this study will allow better understanding of the genetic variability of BVD1 field isolates in Egypt and will contribute towards development of more effective vaccines that may provide adequate protection against BVD infection in cattle and improve the currently existing programs to Nationally eradicate BVDV.

CONCLUSION

The results from this work represent the presence of 3 different clusters of BVDV genotype 1 (1a, 1b and 1f) in Egyptian cattle and the importance to continue surveillance all over the country to investigate the spread of these viruses in Egypt that will help to adopt measures to control the disease.

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