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Molecular investigation of Bovine Ephemeral Fever in Iraq

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Abstract

Bovine ephemeral fever (BEF) is an important viral disease of cattle and water buffaloes that causes severe economic losses. This study sought to detect BEF virus in Babylon Province by using reverse transcriptase PCR (RT-PCR) during the period from May-September 2012. During the BEF outbreak, 150 blood samples were collected from cross breed cattle of different ages, from 4 different locations in Babylon Province. Only 37 cases (24.44%) showed positive result for BEF in RT-PCR, with significant result (P< 0.05) between positive and negative cases. It was proven that 6 blood samples were positive by using RT-PCR at age of less than 1 year, while the higher percentage 37.83% of positive cases was reported at the age of >1 year - 3 years. The results showed significant differences of (P<0.05) between all positive cases in different age groups. According to the sex, the result showed that the highest percentage of BEF virus infection was reported in females (14%) and the lower percentage (10.66%) was in males. Statistically, there was no significant differences between female and male of all positive cases. The results of this study showed also variation in the distribution of the positive cases between the 4 different locations in Babylon Governorate. The percentage of positive cases was 30.10% and 16.66% in Al-Qasim Municipality and A-Madhatia respectively while the percentage of Al-Taleaha and Al-Hashemia Municipalities was 14.28%.

Keywords: BEF, Al-Qasim, RT-PCR, Babylon Province, cross breed cattle.

Introduction

Bovine Ephemeral fever(BEF) also known as Three Day Sickness is an arthropod vector-borne disease of cattle and occasionally buffaloes which is caused by the genus *Ephemerovirus* of the *Rhabdoviridae* family (Uren *et al.*, 1992; Wunner *et al*., 1995; Nandi and Negi,1999; Walker, 2005).The causative virus of the ephemeral fever is bullet shaped consists of a negative –sense, single- stranded RNA genome and 5 structural

proteins, including a Nucleoprotein (N), Polymerase – associated protein (P), Matrix protein (M), Large RNA-dependent RNA polymerase (L) and a surface glycoprotein (G), which induces the production of protective neutralizing antibody(Uren *et al*., 1994; Zaghloul *et al*., 2012). The disease is characterized clinically by sudden onset of fever, stiffness, lameness, nasal and ocular discharges, depression, cessation of rumination and constipation (St. George *et al.,* 1984). These clinical signs can be exacerbated by severe environmental stress or forced exercise (St. George, 1985).

Bovine ephemeral fever is common in tropical and subtropical regions of Africa, Asia, Australia and Middle East (St. George, 1997; Wang *et al.,* 2001; Venter *et al.,* 2003; Yeruham *et al.,* 2003) and spread of the disease depends on the season and weather condition (Kirland, 1995; Walker, 2005). Mosquitoes and Culicoides midges involved in transmitting the virus (Kirland, 1995).

The Disease was described in Iraq in 1991 when Al–Bana, (1991) studied the influence of theileria vaccine and the infection of Three-day sickness virus on the efficacy of rinderpest vaccine, whereas Poushijian, (1997) isolated the virus of BEF in 1996 in Ninavah .In Iraq there is an essential need for introduction of molecular techniques such as PCR which provide a rapid, sensitive and specific diagnosis of the disease, that will help us in the control of the disease and consequently reduce the economic loss, therefore, the aims of this study were to diagnose BEFV infection using the reverse transcriptase RT-PCR molecular technique and to study some of the epidemiological aspects of the disease.

Materials and Methods

Animals investigated

The present study was carried out in Babylon Governorate during the period from May to September 2012 on 150 clinically infected cattle of both sexes with ages ranged between 8 months to more than 4 years. Some of these animals showed clinical signs suggesting infection with BEF.

Blood samples collection

Blood samples were collected from 150 cows and bulls during a natural outbreak in Babylon Province, showing clinical signs similar to those caused by BEFV infection mostly fever, anorexia, depression and stiffness. The blood samples (10-15ml) were collected from the jugular vein of infected cattle in EDTA anticoagulant evacuated tubes and then transferred in cool bag (ice bag) to Al-Hashemia hospital.

The collected blood samples centrifuged at 3000 rpm for 15 minutes in order to separate the Buffy coat. The plasma was removed and discarded from the tube leaving a small amount just above the Buffy coat. The remaining plasma, Buffy coat, and the RBCs were transferred to clean Eppendorf tubes and stored at -20 ℃ until PCR was done.

Viral RNA extraction

Viral RNA was extracted from the Buffy coat of blood samples by using AccuZol^{TM} Total RNA extraction kit (Bioneer, Korea) and done according to the company instructions.

Reverse Transcription Real-Time PCR (RT-PCR)

The primers and probe that used in this study designed by using the complete sequence of glycoprotein gene of BEFV isolate LS11 (Gene Bank No JX564637.1). The NCBI Gene Bank, online primer 3, used and supplied by Bioneer Company (Korea). Reverse transcription of RNA was carried out with primer FG 5′ACTTAGCTCCCACAAGACCAG3′as a forward primer and primer RG 5′TCCCCCTCTTGTTGATGTTCTC3′asa reverse primer. The technique was carried out according to method described by Stram *et al*., (2005).

Real-Time PCR master mix preparation

Real-Time PCR master mix was prepared by using one step Reverse Transcription and Real-Time PCR detection kit (AccuPower® RocketScriptTM RT-qPCR PreMix, PreMix, Bioneer. Korea), and done according to the company instructions.

The RT-PCR master mix reaction components were added into RT-PCR tube containing (8 wells strips tubes containing Rocket Script reverse transcriptase and TaqMan probe premix). Then all strips tubes components were mixed by vortex and centrifuged for 3000 rpm for 3 minutes in ExiSpin™ centrifuge. Later on all samples were transferred into Exicycler Real-Time PCR thermocycler.

Real-Time PCR Thermocycler conditions

Real-Time PCR Thermo-Cycler conditions were set according to primer annealing temperature and RT-PCR TaqMan kit instructions. The reaction conditions were: One cycle of reverse transcriptase at 50 ℃ for 15 minutes, one cycle of pre-denaturation at 95 ℃ for 5 minutes, 45 cycles of denaturation at 95 ℃ for 20 seconds, 45 cycle of annealing (extension) at 60 ℃ for 30 seconds and 45 cycles for scanning the virus at 60 ℃ for 30 seconds.

Real-Time PCR Data analysis

RT-PCR data analysis was performed by calculation the threshold cycle number (CT value) that represented the positive amplification of alleles gene in Real-time cycle number.

Statistical analysis

Data were analyzed statistically by Chi-Square test (X²) and ANOVA on the basis of SPSS program (Snedecor and Cochran, 1980).

Results and Discussion

Detection of BEFV by RT-PCR Technique

Out of 150 blood samples collected during an outbreak of bovine ephemeral fever disease from different areas of Babylon Province during summer season, 37 cases were positive for the BEFV infection at percentage of 24.66% by using RT-PCR. There were significant differences (P< 0.05) between positive and negative cases in RT-PCR technique, as shown in Table (1), Figure (1).

Table (1) Percentage of positive BEFV in blood samples by RT-PCR.

Result	No. of tested samples	%*
Positive	37	24.66%
Negative	113	75.33%
Total	150	100%

*P< 0.05 (X² test)

Our study was compatible with previous study of Momtaz *et al*., (2012) who found that out of 600 samples, 150 (25%) exhibited the 1870 bp segmented of bovine ephemeral fever G gene in RT-PCR. While the percentage of positive cases was lower in comparison with the result of Degheidy *et al*., (2011), who proved that by using PCR, 10 samples out of 22 Buffy coat samples were positive with percentage of 45.4%.

The present investigation found to be higher than the finding recorded by Nawal *et al*. (2001) during isolation of BEFV in outbreak 2000 in Egypt as 9%-16% with average of 13.4% from the collected samples. The variation in the percentage of positive cases may be related to the location (Degheidy *et al*., 2011; Zheng and Qiu, 2012) and density of insect vectors, breed and immunity of the animal, severity of the outbreaks and animals vaccination programs (Radostits *et al*., 2007; Yeruham *et al*., 2010).

In conclusion, this study showed that the Reverse Transcriptase RT-PCR was designed and applied to diagnose BEF virus in cattle for the first time in Iraq and BEFV infection occurred in a relatively high percentage which considered as an outbreak.

Percentage of positive BEFV samples according to animal age

Out of 37 positive cases to BEFV infection, 6 animals blood samples were positive by using RT-PCR at age of less than 1 year, while the higher percentage of positive cases were at the age of >1 -2years and >2-3 years at a percentage of 37.83% for both age groups (Table 2).

The results showed significant differences of (P<0.05) between all positive cases in different age groups .

The present results were in agreement with the results of Momtaz *et al.,* (2012), who proved that the infection rate had gradually and significantly increased in older animals, but the more susceptible age was in cattle of more than 5 years old, while in our results the highly susceptible age was in cattle of more than 1-3 years .

Generally, most researchers revealed that the disease is milder in young animals than mature, in lean animals than fat animals, in light steers, and cows than heavy bulls, in dry cows than those in heavy lactation (St.George, 1988; Wenbin *et al.,* 1991; Yeruham *et al.,* 2003).

On the contrary, in the study of Zaher and Ahmed, (2011) which pointed that the severity of the disease was more obvious in younger than older animals.
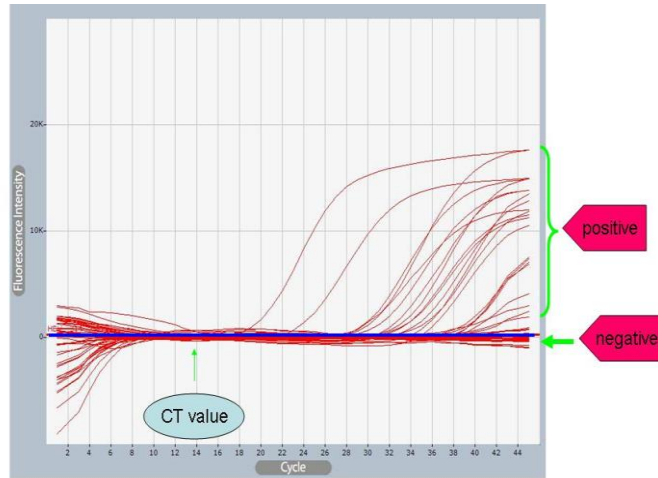

Figure (1): Reverse Transcription Real-Time PCR amplification plot shown the positive and negative BEFV samples

Table (2) Percentage of positive cases in BEFV infection according to animal age.

Total samples	Age of animals	Positive cases	% cases
37	< 1 year	6	16.21%
	> 1 – 2 years	14	37.83%
	> 2 – 3 years	14	37.83%
	> 3-4 years	3	8.10 %

Percentage of positive BEFV samples according to sex of animals

According to the sex, the result showed that the highest percentage of BEF virus infection has been found in females14%   and the lower percentage 10.66% was in males. Statistically, there was no significant differences between female and male of all positive cases (Table 3).

Table (3) Percentage of positive BEFV samples according to sex.

Total samples	Animal sex	Positive cases	Percentage *
37	Females	21	14%
	Males	16	10.66%

$*P < 0.05$

Our results found to be in agreement with the finding recorded by Momtaz *et al*.(2012) who proved that viral infection was significantly more frequently observed in females than in males and particularly in cattle , While in buffaloes , no significant difference was evidenced between males and females. The researchers explained the higher susceptibility of females to the viral infection might be related to the inclination of carrier insects to sting females more than males. But in our opinion, it may be due to exposure of females to biological and physiological stress factors such as pregnancy and lactation.

Percentage of positive BEFV samples according to location

Hundred and fifty blood samples were collected from 4 different locations in Babylon Province. Positive blood samples were distributed as follow: 28 out of 93 (30.10%) from Al-Qasim Municipality, 6 out of 36 (16.66%) from Al-Madhatia Municipality,2 out of 14 (14.28%) from Al-Taleaha and 1 out of 7 (14.28%) from Al-Hashemia Municipalities. The result in table (4) showed that there were no significant differences to all positive cases in different locations. But the high occurrence of the positive cases of BEFV in Al-Qasim area might be due considering the area as a strong commercial exchange for cattle via Iraqi farmers and merchants.

Table (4) Percentage of positive cases in BEF according to location.

No of Sample	Positive	Location	%
93	28	Al- Qasim	30.10
36	6	Al- Madhatia	16.66
14	2	Al- Taleah	14.28
7	1	Al-Hashemia	14.28

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