JOURNAL OF VETERINARY MEDICAL RESEARCH 2016, 23 (2): 174- 183

Journal homepage:
http://www.bsu.edu.eg/bsujournals/JVMR.aspx

Online ISSN: 2357-0520 Print ISSN: 2357-0512

Original Research Article

Further evaluation of locally prepared live attenuated bovine ephemeral fever vaccine in cattle

Ibrahim[a,*] M.M., Amany El Zieny[b], Christine A. Mikhael[b], Soliman[a] Y.A.

[a] Central Laboratory for Evaluation of Veterinary Biologics, Abbasia, Cairo, Egypt. (CLEVB)
[b] Veterinary Serum and Vaccines Research Institute, Abbasia, Cairo, Egypt. (VSVRI)

ABSTRACT

Bovine ephemeral fever (BEF) is an acute, arthropod-borne disease of cattle. The disease is characterized by sudden onset of fever, high morbidity and very low mortality. Recovery occurs within three days of the onset of clinical signs. BEF is an important viral disease of cattle in Egypt so the live attenuated BEFV vaccine which is inactivated just before inoculation by reconstitute in PBS containing saponin. is extensive used for the prevention and control of the disease. Different assays were applied in the current study to quality control evaluate of that produce vaccine by detection of viral identity and viability before and after reconstitution by using real time quantitative reserve transcriptase polymerase chain reaction (qRT-PCR) and clinical findings (Body temperature and clinical signs) and potency by measuring the humoral immune response by serum neutralization test (SNT) and ELISA and cellular immune response by interferon gamma (IFN-γ) using ELISA kit and Quantitative real time polymerase chain reaction (qRT-PCR) and also by lymphocyte cell proliferation assay using tetrazolium salt(XTT).

ARTICLE INFO

Article history:

Received: 11 2016
Accepted: 12 2016
Available Online: 12 2016

Keywords:

Bovine Ephemeral Fever Virus, Vaccines, Evaluation, qRT-PCR, Saponins.

1. Introduction

Bovine Ephemeral Fever (BEF) is an acute arthropod born infectious viral disease caused by an enveloped bullet shaped virus belonging to the genus Ephemero virus Family Rhabdoviridae, (Dhillon, et al., 2000). Helical nucleocapsids comprise the negative-sense, single-stranded RNA genome tightly associated with the 52 kDa nucleoprotein (N) which, together with the 43 kDa phosphoprotein (P) and the large multi-functional enzyme (L) form aribonucleoprotein complex (Walker et al., 1991).

Bovine Ephemeral Fever Virus (BEFV) causes an acute febrile illness of cattle and water buffalo known as bovine ephemeral fever (BEF) or three-day sickness. It is characterized by biphasic fever, anorexia, stiffness in gait; lameness; high morbidity and low mortality and recumbency. Affected animals exhibit a rapid spontaneous recovery as clinical course lasts only 2 – 3 days in the majority of the affected animals (Kirkland, 2002 and Burgess and Spradbrow 1977).

The BEF disease presents a significant risk to dairy herds as the disease spreads rapidly through the herd, animals lose immunity during times of low

* Corresponding author: Ibrahim M.M., Central Laboratory for Evaluation of Veterinary Biologics, Abbasia, Cairo, Egypt.
Email: clevb@tedata.net.eg

exposure and become susceptible,usually more severe in bulls, fat, well-conditioned cows and pregnant and lactating cows and outbreaks depend on rainfall and warm temperatures, which increase insect populations. (Nandi and Negi, 1999 and Walker 2005).

In Egypt, an outbreak of BEF of limited severity was recorded by Hessian et al (1991) in the Lower Egypt. A sever outbreak was occurred in Shakia Governorate in Bahr El-Baker (Hassan, 2000) and was isolated and identified from an outbreak in Menufia Governorate in the farms of Tough Tumbashr (Soad et al.,2001), and was recorded in cattle and buffaloes in Dakahlia and Damiata Governorates during the summer of 2004 (Daoud et al., 2005 and Younis et.al, 2005).

The earliest BEF vaccines were based on field isolates of BEFV which were attenuated by repeated passages in suckling mice and/or cell cultures (Van der Westhuizen 1967). These vaccines were prepared with various adjuvants such as Freund's complete or incomplete adjuvant and aluminum hydroxide, (Tziporiand and Spradbrow 1973and Vanselow et al, 1995). Inactivation of BEFV has been achieved using a variety of agents such as formalin (Inaba et al., 1973), β-propiolactone (Della-Porta and Snowdon 1979), and binary ethyleneimine (Hsieh et al., 2006). Several adjuvants have been used for inactivated BEFV vaccines. These include aluminum phosphate gel, Freund's incomplete adjuvant and water-in-oil-in-water (w/o/w) emulsions. these vaccines provide variable protection against challenge. The use of inactivated vaccines is considered a safer approach. In the process of inactivation, the pathogen's ability to propagate in the vaccinated host is destroyed but the viral capsid remains intact, such that it is still recognized by the immune system. Also, live BEF vaccines were prepared successfully and showed good protection rates (Daoud et al, 2001a&b).

The live vaccines produced a long-lasting neutralizing antibody (NA) response which lasted more than 12 months after two vaccinations. (Tzipori and Spradbrow 1973and Tzipori and Spradbrow 1978) But due to some weaknesses of live vaccines include their potential for causing adverse clinical reactions (Della-Porte and Snowdon 1977) and their potential sensitivity to impairment by heat or light. A new live BEFV vaccine was modified to be inactivated on time of vaccination by addition of saponins to vaccine diluent to combine the advantage of both live attenuated and inactivated vaccines (Vanselow et al., 1995, Daoud et al., 2001and El-Behwar et al., 2010).

The present work was designed for further quality control of already locally produced live attenuated BEFV vaccine inactivated just before use by reconstitution in PBS solvent containing saponin for cattle by qRT-PCR, lymphocyte cell proliferation assay, serum neutralization test (SNT) and enzyme linked immunosorbent assay (ELISA).

2. Material and methods

2.1. Virus

Locally isolated BEFV (BEFV/Abbasia/2000) strain from an outbreak during 2000-2001 (Soad et al., 2001) was used in the current study. The strain was tissue culture adapted on BHK-21 with end point titer of $10^{7.5}$TCID50/ml (Macpherson and Stocker, 1962 and Azab et al, 2002).

2.2. Preparation of live BEFV attenuated vaccine

Live attenuated BEFV vaccine was prepared from the lowest virus passage (3rdpassage) of BEFV strain on BHK21 clone13 with 10^6 $TCID_{50}$/ml and lyophilized to reconstitute in PBS containing saponin (Quillaja Saponaria Molina) obtained from ACROS ORGANICS, Code #: 419235000 (Department of Pet Animal Vaccine Research, Veterinary Serum and Vaccines Research Institute, Abbasia, Cairo, Egypt)

2.3. Quality control of the prepared vaccine

It was done according to *Code of Federal Regulation of USA (1987),* OIE*, 2006 and 2012 and Code of Regulation of Egypt (CLEVB) 2009.*

2.3.1. Sterility test: according to *Code of Regulation of Egypt (CLEVB) 2009.*

2.3.2. Safety test

Two calves were injected S/C with 10 doses (20ml) of the freshly reconstituted vaccine. No undesirable signs or conditions were observed for 10 days. according to *Code of Regulation of Egypt (CLEVB) 2009.*

2.3.3 Identity test

It was done using qRT-PCR. Briefly, the vaccine was reconstituted in TE buffer pH8 and the RNA extraction was done using Trizol according to the manufacture instruction and the QRT-PCR was done

using 5µL of the purified RNA, 50nM of each primers and 150nM of the probe (Table 1). The reaction was done in 50µL using of iProbeQPCR Master Mix-Low ROX, (Biomatik cat # A4219).

2.4. Animals and Vaccination

Nine mixed breed calves (1.5-2 years old) tested and free of BEFV antibodies were divided into 2 groups.The 1st group (*n=6*) was vaccinated (S/C)with 2ml of the freshly reconstituted live attenuated vaccine (*Daoud et al., 2001 and El-Behwar et al., 2010)*. The 2nd group (*n=3*) was kept as unvaccinated control group ($C_{1, 2 \text{ and } 3}$).

2.5. Sampling

Heparinized blood samples were taken from all animals in pyrogen free sterile vacutainers for the lymphocyte transformation assay and testing for γ-interferon production. Serum samples were also collected and stored at -20 °C for further serological screening. The samples were taken at day 0, 1, 3,5,7,14,21 and 28 post vaccinations.

2.6. Clinical investigation

The body temperatures were recorded daily and any clinical signs were also recorded for each animal separately.

2.7. Evaluation of the immune response after vaccination.

2.7.1. Humoral immune response.

2.7.1.1. Serum neutralization test (SNT):

Serum samples were tested for the BEFV antibodies using SNT(*Florence et al., 1992).* The antibody titer was estimated according to *Singh et al., (1967)*and expressed as Log_{10}.

2.7.1.2. Enzyme Linked Immunosorbent Assay (ELISA):

BEF antigen was prepared from the locally isolated BEFV according to *Brain and Hiller (1996)* to use in ELISA. The test was performed according to *Zakrzewski et al. (1992)* and the results wereexpressed as Log_{10}

2.7.2. Cell Mediated Immune Response:

The peripheral mononuclear cells (PMNCs) separation was done from heparinized blood samples by differential centrifugation onto Histopaqe lymphosep separation media (biowest Cat# L0560-500) according to (*Lucy 1974*). The PMNCs were incubated at 37°C /3 daysunder CO_2 tension of 5% with/without the stimulant (BEFV with titer of 10^6 $TCID_{50}$). The supernatant was collected for quantitation of IFN-γ usingID screen ruminant IFN-γ sandwich ELISA Kit (Innovative Diagnostics cat # IFNG-2P, USA). The cells were collected either on Trizol for QPCR experiment or subjected to live cell counting using cell proliferation XTT kit (AppliChem GmbH cat # A8088 Germany) according to the manufacture instruction. *(MacphersonandStocher, 1962 and Mayer et al., 1974).*

2.7.2.1. Lymphocyte Cell Proliferation Assay:

The cultured (PMNCs) after 3 days of incubation with or without the stimulate were inoculated with 50µl of Tetrazolium salts (XTT) reagent and further incubated for 5 h. The optic density (O.D) of the developed color was then measured by ELISA reader at 450 °A with reference of 630°A.

2.7.2.2. Estimation of IFN-γ by ELISA:

The test was performed by using ID Screen ®Ruminant IFN-ɣ Kit for the detection of bovine, ovine and caprine interferon gamma (IFN-ɣ), FRANCE.

2.7.2.3. Quantitation of IFN-γ transcripts by(qRT-PCR)

2.7.2.3.1. RNA extraction

RNA extraction was done from cultured PMNCs (stimulated or unstimulated) using Trizol reagent (life technologies USA) according to the manufacturer's instructions.

2.7.2.3.2. qRT-PCR

It was done using M-MuLV first strand cDNA synthesis kit (Biomatik cat # K5147). The extracted total RNA was converted to cDNA using hexamere random primers, cDNA was kept at -20 till used.The mRNA sequences of the key genes were obtained from NCBI database. Primers were designed using CLC main work bench V6 software and checked using Oligo Calculator (free on-line access) and Primer-Blast (NCBI database). Primers' sequences are listed in (Table 1).

Table (1) the nucleotide sequence of the primers and probes used in this study. dual labeled with the probes were FAM at 5' and TAMRA at 3' end of the sequence.

Gene	Forward primer	Reverse primer	Probe
GAPDH	GGCGTGAACCACGAGAAGTATAA	CCCTCCACGATGCCAAAGT	ATACCCTCAAGATTGTCAGCAATGCCTCCT
Bovine IFN-γ	GCGCAAAGCCATAAATGAAC	CTCAGAAAGCGGAAGAGAAG	CAAAGTGATGAATGACCTGTGCCA
BEFV	CAATGTTCCGGTGAATTGTG	GCGTCATCTTTCAACTGTGG	TCAAGCCCATCATAATCTTGCAAAGGA

GAPDH gene was used as non-regulated reference gene for normalization of target gene expression. QRT-PCR was performed using MX3005P (Agilent. USA). The results were analyzed using comparative *Ct* method. Relative transcript abundance of the gene equals Δ*Ct* values (Δ*Ct* = C_t GAPDH – C_t IFN-γ). Relative changes in transcript are expressed as ΔΔCt values (ΔΔCt = ΔC_t stimulated culture – ΔC_t unstimulated control). For performing the probe based QPCR, 2µl of the cDNA was mixed with 10 µl of iProbe qRT-PCR Master Mix-Low ROX, (Biomatik cat # A4219).

2.8. Testing for BEF virus replication after vaccination:

In order to test whether the reconstituted virus has any replicative activity in the animals after vaccination, heparinised blood samples was taken from each animal 24, 48, and 72 hr. post vaccination. The PMNCs were subjected to purification the total RNA was extracted. The RNA was examined for the presence of the BEF genome using qRT-PCR.

3. Results

3.1. Quality control of the prepared vaccine

3.1.1 Sterility test: No contamination was detected.

3.1.2 Safety test: No undesirable signs or conditions were observed for 10 days.

3.1.3 Vaccine identity:

The reconstituted vaccine was subjected to qRT-PCR. using a specific primers and probes detecting the glycoprotein G gene of the virus. The reconstituted vaccine gave a *Ct* of 14.43 (Fig 1) while the non-template control was completely negative.

3.2. Clinical examination:

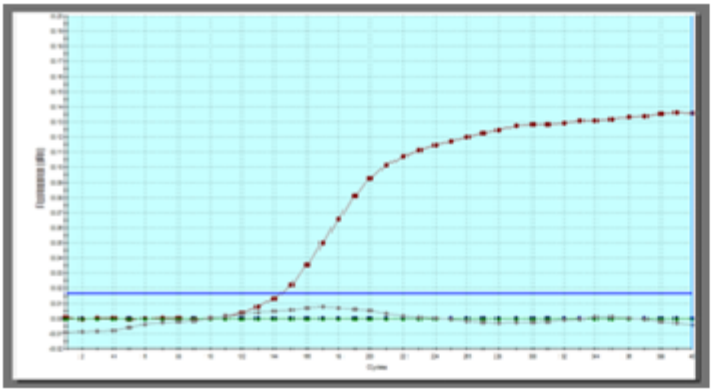
No difference was observed between rectal temperatures of the vaccinated and control groups and also no adverse effects were observed after administration of live attenuated BEFV vaccine (Table 2, Figure 2).

3.3. Evaluation of the immune response after vaccination.

3.3.1. Humoral immune response:

Serum Neutralization Test (SNT) & Enzyme Linked Immunosorbent Assay (ELISA):

Results of ELISA came in a parallel manner with that of SNT confirming each other, showing that the mean specific BEF antibody titers were detectable by the 1st week post vaccination in vaccinated animal group with the highest antibody titers were recorded by the 4th week post vaccination (SNT =1.83, ELISA = 2.48) (**Table 3, Figure 3**).



Well	Well Name	Threshold(dRn)	Ct (dRn)
C3	BEFV	0.0163	14.4
F2	NTC	0.0163	No Ct
C3	BEFV	Reference	Reference
F2	NTC	Reference	Reference

Fig. (1) Amplification plot of the BEFV reconstructed in TE Buffer.

Table (2): Clinical Signs and Body Temperatures of vaccinated and Control calves

Animal Groups	Clinical Signs and Body Temperatures / DPV*																	
	Pre Vac.		1st		3rd		5th		7th		10th		14th		21st		28th	
	CS	Temp.	CS	Temp.	CS	Temp.	CS	Temp.	CS	Temp	CS	Temp	CS	Temp	CS	Temp	CS	Temp
GP1 Vacc. Anim. (n=6)	N	38.1	N	38.6	N	38.8	N	38.9	N	38.6	N	38.4	N	38.3	N	38.1	N	37.9
GP2 Cont. Anim. (n=3)	N	37.9	N	37.9	N	38.1	N	38.1	N	38.1	N	38.1	N	38.0	N	37.9	N	37.9

CS = Clinical Signs N = Normal Temp = Body Temperature
*DPV = Days post vaccination Normal Cattle Temperature range (37.5–39.0 °C)

Table (3): Mean BEF Serum Neutralization & ELISA antibody titers in Vaccinated and control calves (expressed as Log_{10})

Animal Groups	BEFSNT and ELISA antibody titers / DPV*													
	3rd		5th		7th		10th		14th		21st		28th	
	SNT	ELISA	SNT	ELISA	SNT	ELISA	SNT	ELISA	SNT	ELISA	SNT	ELISA	SNT	ELISA
GP1 Vaccin. Animals (n=6)	0.07	0.14	0.25	0.71	0.68	1.18	1.02	1.58	1.33	1.94	1.61	2.38	1.83	2.48
GP2 Control Animals (n=3)	0	0.04	0	0.05	0	0.05	0	0.05	0	0.06	0	0.07	0	0.07

*DPV = Days post vaccination

NB: 1- The SN protective antibody titers against BEFV are (1.2 log_{10} equivalents 16 titer) reached to more (1.8 log_{10} equivalents 64titer) as the peak of Protective titer according to Mellor (2001) & ELISA reading protective antibody titers against BEFV are (1.5 log_{10} equivalents 32 titer) reached to more (2.1 log_{10} equivalents 128 titer) the peak of Protective titer according to *Zakrzewski et al. (1992)*

3.3.2. Cell Mediated Immune Response

3.3.2.1. Lymphocyte Cell Proliferation Assay (XTT):

Table (4) and Figure (4): Show elevation of Cell mediated immune response in samples of vaccinated calves from 3rd day post vaccination (DPV) to 14th DPV then stars to decrease gradually to 28th DPV.

Table (4): Cell mediated immune response of Vaccinated and control calves (XTT results Expressed as optical density)

Animals #	Cell XTT / DPV*						
	3rd	5th	7th	10th	14th	21st	28th
1	0.785	1.326	1.585	1.765	1.937	1.379	0.980
2	0.781	1.324	1.525	1.738	1.935	1.374	0.974
3	0.763	1.220	1.418	1.616	1.848	1.348	0.932
4	0.774	1.217	1.483	1.687	1.836	1.341	0.921
5	0.727	1.292	1.496	1.666	1.825	1.343	0.947
6	0.773	1.263	1.467	1.686	1.832	1.340	0.939
Mean of Samples	0.767	1.274	1.496	1.693	1.869	1.354	0.949
C1	0.133	0.125	0.123	0.121	0.119	0.115	0.113
C2	0.136	0.133	0.131	0.125	0.120	0.118	0.114
C3	0.140	0.138	0.135	0.132	0.127	0.126	0.124
Mean of Controls	0.136	0.132	0.130	0.126	0.122	0.120	0.117

*DPV = Days post vaccination

Mean OD results of control calves (C1,2,3) between 0.117 – 0.136 all over the experiment

Fig.2

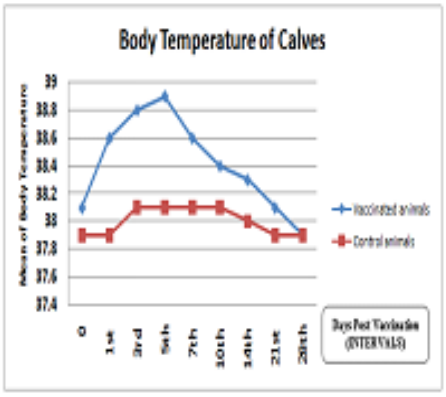


Fig.3

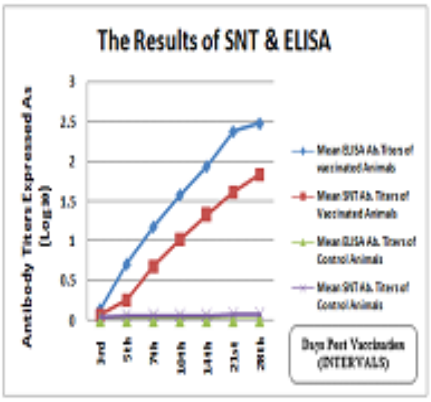
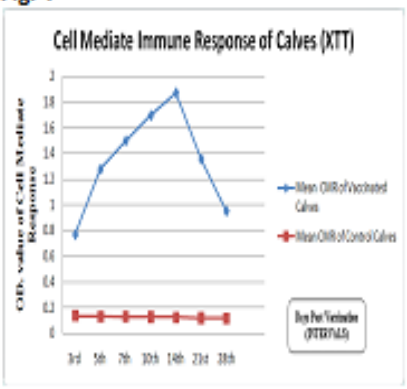


Fig. 4



Figures 2, 3, and 4: Clinical, Serological, and Cellular responses of vaccinated and control calves.

3.3.2.2. Estimation of IFN-γ by ELISA:

Table (5): Detection of bovine INF-γ by ID Screen® Ruminant IFN-ˠ Kit - sandwich ELISA (Expressed as mean S/P %)

Animals#	MEANIFN-γ ELISA (S/P %) / DPV*						
	3rd	5th	7th	10th	14th	21st	28th
GP1 Vaccin. Animals (n=6)	22% (Negative)	2o% (Negative)	19% (Negative)	18% (Negative)	16% (Negative)	15% (Negative)	15% (Negative)
GP2 Control Animals (n=3)	3% (Neg.)	3% (Neg.)	2% (Neg.)	1% (Neg.)	1% (Neg.)	1% (Neg.)	1% (Neg.)

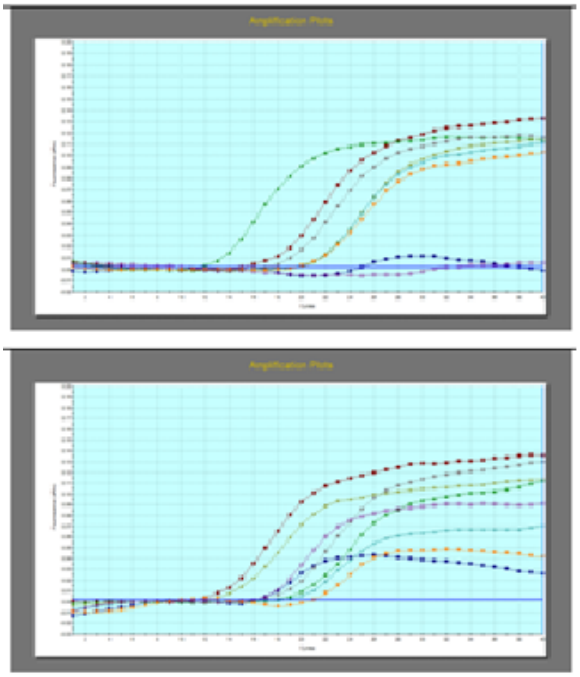
*DPV = Days post vaccination

S/P% Less than 35% are considered negative (no IFN-ˠ)

S/P% Greater than or equal to 35% are considered positive (IFN-ˠ is induced)

3.4. Quantitation of gamma interferon using qPCR:

Fold change in IFN-γ production from stimulated cultured PMNCs revealed that the transcripts was greatly detected by the first day post vaccination (the fold change was 4.04 up regulated when compared to the calibrator which is the day 0 unstimulated culture) , by day 3 post vaccination the level of IFN-γ transcript was greatly reduce (0.3fold change up regulated) . The level of the transcript was generally near to zero by day 5 on word indicating the down regulation of the transcript.



Well Comment	Well Type	Threshold(dRn)	Ct (dRn)	Rel Quant to Cal. Avg. (dRn)
00 DPV	Samples	0.0019	31.91	2.73e-006
01 DPV			11.42	4.04
03 DPV			15.00	0.338
05 DPV			19.40	1.59e-002
07 DPV			19.73	1.27e-002
14 DPV			19.83	1.19e-002
21 DPV			17.01	8.40e-002
28 DPV			25.00	3.29e-004
00 DPV	Calibrator	0.0019	13.43	Calibrator
01 DPV				
03 DPV				
05 DPV				
07 DPV				
14 DPV				
21 DPV				
28 DPV				

Fig. (5) Amplification plot of gamma interferon

3.5. Testing for BEF virus replication after vaccination using qRT-PCR:

The virus replication after reconstitution and vaccination of the animals in comparison to the positive control (virus reconstituted in TE buffer) showed that vaccinated animals showed negative results while positive control showed ***Ct***= 22 (Fig. 6).

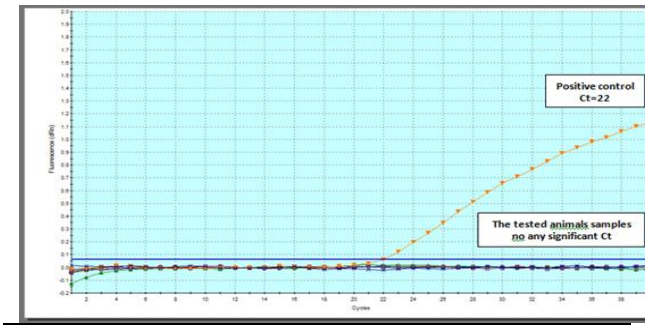


Fig (6) Amplification plot of the BEFV reconstructed in PBS containing Saponin .

4. Discussion

In the current study, the formal quality control measures of vaccine were applied in addition to further evaluation by identity test as the vaccine was reconstituted in TE buffer and subjected to QRT-PCR using a specific primers and probes detecting the glycoprotein G gene of the virus. The reconstituted vaccine gave a Ct of 14.43 (fig 1) while the non-template control was completely showed negative to confirm viability of live attenuated BEFV before reconstitution in PBS containing saponin. This result is agreeable with Stram et al., (2004) who citied that a real-time RT-PCR assay was developed as a rapid, precise and sensitive test for identifying BEFV and

Abdel Baky, et.al, (2008) who used Real time - PCR for detection of BEF virus. Also, qRT-PCR was used for confirmation of complete inactivation of live attenuated BEFV after reconstitution in PBS containing Saponin. qRT-PCR showed no any virus replicative activity of BEFV in the heparinised blood samples taken from animals on 24, 48, and 72 hr. post vaccination as shown in (fig.6). The results agreeable with (Lee et al., 2012) who tested inhibitory effect of saponin on Hepatitis C virus (HCV) replication by qRT-PCR. Also, no any clinical signs of BEF were observed during the whole examination intervals, the body temperature was elevated in all vaccinated animals in range 0.5^{o}C after one-day post inoculation and then return to the normal temperature. It was clear that the temperature of the control and vaccinated cattle within the normal range indicated no viremia as shown in (Table 2 and Figure 2) to confirm the safety and complete inactivation of live attenuated BEFV vaccine and agreeable with (OIE, 2012) procedures for evaluation of viral vaccines.

Different assays for evaluation of the immune response after vaccination were applied to study both humoral immune response by SNT and ELISA and cell mediated immune response by lymphocyte proliferation assay and estimation of IFN-γ by ELISA kit and quantitation of gamma interferon using qPCR

SN antibody titers reached the protective level (1.2 $\log_{10}$) at the 10th DPV, reached the highest level (1.8 $\log_{10}$) at the 28th DPV. The results of ELISA revealed that the protective antibody titers elevated to reach more than the protective level (1.5 $\log_{10}$) at the 10th DPV, reached more than the highest protective level (2.1 $\log_{10}$) at the 28th DPV as shown in (Table 3and Figure 3) agreeable with results of El-Bagoury et, al (2014). That greatly confirming the adjuvant effects of saponin mentioned by Oda et al., (2000) and Song, and Hu (2009) who recorded that saponin based adjuvants have the ability to enhance antibody production. Also, it has the advantage that only a low dose is needed for adjuvant activity. There was acell mediated immune responsein samples of vaccinated calves from 3rd DPV (OD. 0.767) to 14th DPV(OD.1.869) and decreasing at 28th DPV (OD.0.949) as shown in (Table 4 and Figure 4) agreeable with Delmas et al., (2000) ; Haridas et al., (2001) ; Oliveira et al., (2001) and Yui et al., (2001) who mentioned that saponin not only has stimulatory effects on the components of specific immunity and monocyte proliferation but also induce a strong adjuvant effect to T-dependent as well as T-independent antigens and CD8+ lymphocyte responses . The results of IFN-γ estimation by ELISA (S/P%) shown in (Table 5) were equivalent with that of qRT-PCR for Quantitation of IFN-γ (Figure 5) and supported by the results of qRT-PCR of heparinised blood samples taken from each animal 24, 48, and 72 hr. post vaccination (Fig 6) to confirm that none of the samples gave a positive (Ct) at any stage indicating loss of the transcription efficacy of the virus after reconstitution with PBS containing saponin but the presence of saponin explain that the fold change was 4.04 up regulated when compared to the calibrator which is the day 0 unstimulated culture , by day 3 post vaccination the level of IFN-γ transcript was greatly reduced (0.3fold change up regulated) .As the level of the transcript was generally near to zero by day 5 on word indicating the down regulation of the transcript that revealed to the presence of saponin agreeable with Jie et al., (1984)and Kensil, (1996) who reported that saponins reportedly induce production of cytokines such as interleukins and interferons that might mediate their immunostimulant effects.

4. Conclusion

As the results gained from the current study in addition to previous researches in the field of vaccines quality control we could recommended the use of already locally produced live attenuated BEFV vaccine safely with Solvent PBS containing saponin, evaluated by qRT-PCR as a rapid, precise and sensitive test for identifying BEFV vaccine content beside the use of different serological and cellular assays to evaluate the potency of vaccine and tray to use another advanced methods for quality control of the vaccine.

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