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In Vitro and In Vivo Evaluation of Inactivated Rift Valley Fever Virus Vaccine

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In this study, 5 batches of inactivated rift valley fever (RVF) virus vaccine (ZH501) were evaluated according to *Office Internationale Epizooties* (OIE) RVF evaluation protocol. The tested batches proved to be sterile and safe when inoculated subcutaneously (S/C) and intraperitoneally(I/P) into mice (3-5 days old) and lambs without showing adverse post vaccinal reactions. Duration of immunity to RVF virus in vaccinated sheep has been determined by using both serum neutralization test (SNT) and enzyme-linked immunosorbent assay (ELISA) on sera collected weekly up to 7 weeks post vaccination, where the antibody titer elevated from 1st WPV till reach the protective level at 5th WPV.

Effective $dose_{50}$ in mice has been calculated in mice by inoculation of 5 groups of mice (10 each) with 2 doses of five fold diluted vaccine; inoculated mice have been challenged I/P with virulent RVF virus 10^3 to 10^4 mouse I/P lethal $dose_{50}$ ($MIPLD_{50}$), it was ranged from 0.006 - 0.0007. Using the Rt-PCR to confirm the efficacy of binary ethyleneamine as inactivator to RVF virus used in tested vaccine through applying RT-PCR on master seed RVF virus as positive control samples, RVF virus after inactivated with binary ethyleneamine and on the eluted RVF antigen from the tested vaccine, the master seed RVF virus gave positive band at 800bp but the RVF virus after inactivation and eluted antigen from vaccine didn't give any bands.

Keyword: RVF – vaccine evaluation protocol - inactivated vaccine - RT-PCR

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INTRODUCTION

Rift Valley Fever (RVF) is an acute infectious vector-transmitted viral disease of sheep and cattle which is abortifacient and causes high mortality in young lambs and calves and also is a zoonotic disease ***Easterday (1965)***. RVF virus is a member of Bunyaviridae family that derived its name from Bunyawera, the place of Uganda ***Daubney and Hudson (1931)***.

The virions measures 80-120 nm in diameter. They are spherical that display glycoprotein projections (10 to 11 nm) which are embedded in a lipid bilayered envelope ***Martin et al., (1995)***. The genome of the RVFV is composed of three single stranded RNA segments. The large one (L) codes for large polymerase protein, the medium sized segment (M) codes for the two major glycoproteins (G 1 and G2) while- the small segment (S) codes for the N protein ***Knipe et al. (2001)***.

Rift Valley Fever (RVF) was first reported in Egypt in 1977 in a massive outbreak among animals with extensive involvement of humans ***WHO (1978), Al-Akkad (1978) and Meegan (1979)***. The recent recurrence of RVF in 1993 ***Arthur et al. (1993)***, A revealed the need for a more practical and more efficient of quality control for vaccine.

This study focusing the evaluation of inactivated alum gel RVF vaccine ***Eman (1995)***. Using slandered inactivated alum gel RVF vaccine evaluation protocol OIE (2007). With further use of RT-PCR technique to insure the efficiency of inactivation process using Binnary ethylenamine that gives us an accurate results for safety test due to the inactivation process get red any living virus, and also the positive results of RT-PCR confirming the results of identity.

MATERIALS AND METHODS

1.Materials

1. Virus:

RVF virus used in this study was ZH501 and has a final titer $2X10^7$ MICLD50 / ml used in three forms master seed, inactivated and inactivated virus eluted from gel as recommended by ***El Nimr (1980) and Taha (1982)***.

2. Animals:

2.1. Mice:

Susceptible Swiss albino mice free from RVF antibodies were supplied by the breeding unit, Veterinary Serum and Vaccine

Research Institute, Abbassia, Cairo, Egypt.

2.1.1. Baby Mice:

3-5 days old Swiss albino mice (suckling mice) for the safety experiment of the local attenuated RVF vaccine before addition of adjuvant.

2.1.2. Adult Mice:

Susceptible Swiss albino mice 3-5 weeks old for testing the potency of the prepared vaccine and toxicity test.

2.2. Guinea. pig

Adult G. pigs, about 350 gm, free from RVF antibodies were supplied by the breeding unit, Veterinary Serum and Vaccine Research Institute, Abbassia, Cairo, Egypt. used for safety test.

2.4. Sheep:

Adult susceptible sheep 12 month **old, obtained from breeding unit of Central Laboratory for evaluation of vet. biologics with no history of RVF outbreaks and proved to be free from antibodies.**

2.5. Lambs:

Lambs for safety test of less than 10 days old obtained from breeding unit of Central Laboratory for evaluation of vet. Biologics, from susceptible ewes free from RVF antibody.

3. Antisera:

- Anti-sheep horseradish peroxidase labeled antispecies IgG, it was obtained from sigma company, USA, used for ELISA, diluted immediately in diluting buffer before use.
- Reference RVF anti-
- sera kindly supplied by NAMRU-3, Cairo. It was used as positive control serum.

4. Cell Culture:

Monolayer Baby Hamster Kidney cells (BHK_{21}), obtained from RVF department in Veterinary Serum and Vaccine Research Institute, Abbassia, Cairo, Egypt. The cells were grown and maintained according to ***Macpherson and Stocker (1962)***. The cells were used for virus titration, serum neutralization test and vaccine production.

5. Vaccine samples:

Five batches from the tested local inactivate RVF vaccine produced in Veterinary Serum and Vaccine Research Institute, Abassia, Cairo.

6- Materials for PCR:

6.1.Genomic RNA extraction Kit:

Viral genomic RNA extraction Kit (TALENT SEEK VIRAL RNA)

6.2. RT-PCR Kit:

The cDNA was created using (RevertAid ^{TM}, Cat. No., K 1622)

6.3. Reagents used for PCR amplification:

(Sambrook et al., 1989)

6.3.1. Native Taq DNA polymerase 5µg/ml (Stratagene,).

6.3.2. Deoxynucleotide mix (dNTPs): (Stratagene, Cat. No. 200415).

6.3.3. Primers: Biometra, Germany, It was designed based on the S gene sequence.

5' - ATG GAT TAC TTT CCT GTG ATA TCT – 3'

5' – CTA ATC AAC CTC AAC AAA TCC – 3'

7.Buffers and reagents used for agarose gel electrophoresis:

(Sambrook et al., 1989)

7.1. Ethedium bromide (EBr):

It was prepared in stock solution, 10mg/ml by dissolving one tablets (10mg/tab) in 1ml-distilled water, vortex, stored in dark at +4°C. To be used at 0.5 µg/ml final concentration.

7.2. 50X electrophoresis buffer:

(Tris-acetate EDTA, pH 8)

7.3. 6X gel loading buffer:

7.4. 1% agarose gel:

7.5. DNA ladder:

100 base pair ladder, obtained from Fermantas company, was used to size the PCR product from 100-1000pb.

2. Methods

1. Sterility test:

It was performed in accordance with the *US Code of Federal Regulation (1987), OIE (2007).*

2. Safety test:

According to *OIE (2007)*

2.1. Animal Inoculation:

- In mice:

Twenty baby Swiss albino mice 3-5 day old are used for each batch Ten of them inoculated

I/P with 0.2 ml of inactivated vaccine, another ten leaving as control, these mice kept under observation for 21 day.

- In G. pig and hamster:

Two adult G. pigs (about 350 gm) and two hamsters (150-200 gm) are inoculated I/P with 0.5 ml of inactivated vaccine, the same numbers of G. pigs and hamsters are kept as non-vaccinated controls, these group kept under observation for 21 day

- In lambs:

Four lambs (5-10 days old) for each batch, two of them inoculated with 5ml I/P and 5ml S/C from the tested vaccine. The inoculated lambs are kept under observation for 10 days and the temperature should be recorded daily, and kept under observation for 10 day.

2.2. Rt- PCR:

Ensure the safety of inactivation with binary ethyl amine vaccine through application of RT-PCR on three samples of virulent strain ZH-501, inactivated virus with binary ethyl amine and eluted antigen from alum hydroxide gel.

3. Potency test:

3.1.Mice

Five-fold serial dilutions of the vaccine are prepared in suitable media, starting from 1:1 to 1:625. Five groups of adult mice (21-35 days old) are used. Each group containing 10 mice is used for each dilution and each mouse is inoculated with two doses 0.2 ml of the vaccine I/P, one week apart. Seven days after the second inoculation, all animals are challenged via I/P route with 0.1 ml. RVF virus containing 10^{3}-10^{4} $MIPLD_{50}$/ml

This is in addition to other two groups of mice, one group is inoculated with challenge virus as positive control and the other group is kept as non vaccinated non-challenged negative control. All groups of mice are kept under observation for 21 days and deaths are recorded daily. The ED_{50}/ml is calculated according to the method *Reed and*

Muench (1938). Deaths occurring during the first day are considered non-specific.

3.2. In Sheep

Six susceptible sheep, 3-4 months old age of a neutralizing index (NI) less than 0.3 are used. Four sheep are vaccinated S/C with one field dose. The remaining two sheep are kept separately as non-vaccinated (control groups). Sera sample were collected weekly from all lambs for estimation the antibody response by SNT, ELISA.

3.2.1. Serum neutralization test (SNT):

According to ***OIE (2007)***

- Inactivate the test sera for 30 minutes in a water bath at 56°C.
- Add 25 µl of cell culture medium with 5% RVF-negative serum and antibiotics to each well of a 96-well cell culture plate.
- Add 25 µl of test serum to the first well of each row and make two fold dilutions. Known positive and negative control sera should be included.
- Add 25 µl of RVF virus (diluted in cell culture medium and calculated to provide 100 $TCID_{50}$ per well) to each well that contains diluted test serum and to wells in rows containing negative and positive control serum.
- Incubate for 30 minutes at 37°C.
- Add 50 µl / well of BHK cell at a dilution known to produce a confluent monolayer within 12 hours.
- Incubate the plates in an atmosphere of 3-5% CO_2 for 3-5 days.
- Using an inverted microscope, the mono layers are examined daily for evidence of CPE.

3.2.2. Enzyme-linked immunosorbent assay:

According to ***OIE (2007)***

- Coat half the plate with 50 µl/well positive antigen and half with negative antigen in

carbonate-bicarbonate buffer pH 9.6 at a predetermined dilution. Incubate overnight at 4°C. Wash the plate.

- Add 100 µl/well blocking buffer, incubate for 1 hour at 37°C. Wash the plate.
- Add test and control sera at a predetermined dilution in duplicate wells to positive and negative antigen. Incubate for 1 hour at 37°C. Wash the plate.
- Add antispecies-peroxidase conjugate of the tested sera at a working dilution. Incubate for 1 hour at 37°C. Wash the plate.
- Add suitable substrate and leave the plate for 20 minutes at room temperature (22°C) in the dark (developing time).
- Add stop solution (2 M sulphuric acid) and read the plate at optical density 450/650 nm.
- The ELISA titer was calculated through this equation:

$$\text{ELISA titer} = \frac{\text{OD of samples} - 50}{\text{OD of -ve control} - \text{OD of +ve control}} \times \text{ELISA titer of reference positive}$$

OD (mean of two wells)

4. Identity test:

According to *the US Code of Federal Regulation (1987), OIE (2007)*. Obtained from results of SNT and ELISA on serum samples of potency test and results of RT-PCR of safety test.

4.1. Rt- PCR:

4.1.1. Propagation of RVF virus

RVF the Egyptian isolate ZH-501 used to infect BHK-21 cells. Thirty six hours post inoculation, the cells gives almost full cytopathic effects (CPE). A total of 1×10^7 infected cells was harvested to be used in virus genome extraction.

4.1.2. RVF-RNA extraction:

The virus genome was extracted using Talent Seek viral RNA kit.

The viral samples were 1st concentrated by dialysis against PEG 6000 and then treated with proteinas K (10mg/ml).

Then 100µl of solution 1 was added (lysis buffer), after incubation for 5 min at room temperature the whole solution was transferred to the spin column provided with kit and 100µl of binding buffer was added and the column was spined at 13000rpm/1min. The column was then washed twice with the washing solution and sepined as before. The RNA was then elutes with worm 55C elution buffer provided with the kit and after incubation for 1 min the column was spinede as before. The RNA sample was kept at -20C till used

4.1.3. cDNA synthesis:

1- Prepare the following reaction mixture in a tube on ice:

* Template RNA:
Total RNA
* Primer:
Oligo $(dT)_{18}$ primer (0.5µg /µl) 1 µl
DEPC-treated water to 12 µl

Mix gently and spin down for 3-5sec. in a microcentrifuge.

2- Incubate the mixture at 70°C for 5min, chill on ice and collect drops by brief centrifugation.

3- Place the tube on ice and add the following components in the indicated order:

5x reaction buffer
RiboLock™ Ribonuclease inhibitor (20u/µl)
10mM dNTP mix

Mix gently and collect drops by brief centrifugation.

4- Incubate at 37°C for 5min (at 25°C for 5min if random hexamer primer is used).

4.1.4. PCR amplification of S segment:

According to *Sambrook et al. (1989)*

In a 0.2 ml PCR tubes the following **reaction mixture** was **added:**

DNA tamplet (100 ng/µl).....0.1-3µl
taq polymerase (1u/µl. .....1 µl
10x enzyme buffer..... 5 µl
dNTPs..... 2 µl
Primer 1..... 1 µl
Primer 2.....1 µl
Bidist.water.....to .....50 µl

The mixture was spined and placed in the thermal cycler (T gardiant , biometra, Germany), **which was programmed as follow:**

- Initial denaturing..... ..... 95°C/2 minute
- Cycles (40 cycles) denaturing....95°C/1 minute annealing.....50°C/30 seconds extension 74°C/45seconds
- Final xtension.....74°C/10 minutes

4.1.5. Agarose gel electrophoresis:

According to ***Sambrook et al. (1989)***

To assess the PCR product, 4 mm thickness of 1% agarose containing 0.5µg/ml EBr was poured in mini-gel and left till solidify before submarine loading of 8µl of PCR-product mixed with 2µl of DNA loading buffer. 100 bp DNA ladder was used 3 µl in 1µl loading buffer were used as DNA marker. DNA was electrophoresed at 80 v/15 minutes and finally examined using UV transilluminator.

Results

The five batches of binary inactivated alum gel RVF vaccine were evaluated and the results were as following:

Purity test:

The vaccines batches were sterile, free from aerobic, anaerobic and fungal contamination as shown in table (1).

Safety test:

- In mice: the vaccine was safe and non specific deaths were occurred allover period of observation as shown in table (1).
- In lamb: the vaccine was safe and no elevation or specific variation in body temperature as shown.

Potency test:

- The vaccine was potent following challenge with pantropic RVF virus as the ED_{50} in mice was less than 0.02/ml in all batches as shown in table (2).

- The neutralizing antibody titers was measured by SNT as shown in table (٣), figure (1) where the neutralizing antibody titers are more than 1.5 with in 5th WPV according to the ***OIE (2007)***.
- The ELISA test were done on the same sera sample and compared its results with SNT result at the same week to defined the protective level measured by ELISA as shown in table (4), figure (2). The antibody titer in 5th WPV consider protective from SNT titer, So ELISA titer in same week consider protective.

RT-PCR

- Both identity and safety test was confirmed by using RT-PCR technique, The nucleic acid of RVF virus (ZH 501) has been extracted using (TALENT SEEK VIRAL RNA) according to the protocol described in section (4.1.2.) filful by RT-PCR. The amplified piece of DNA has been run on Agarose containing ethidium bromide gal and detected by the trans illumination under UV detection. The bands of the amplified gene are illustrated in figure (3) and represent the amplification reaction obtained from master seed virus before and after inactivation as well as after elution.

Discussion

Rift Valley Fever (RVF) is an arthropod borne viral disease of an economic importance, characterized by high mortality rate among lambs and calves and abortion of pregnant ewes and cows ***Easterday et al., (1962)*** and it causes economic losses in life stock. As well as different complications in humans like hemorrhages, encephalitis and blindness and in some cases lead to death, ***Besselaar and Black born (1992)***.

This study deals with the evaluation of BEI inactivated RVF vaccine (ZH501) by different methods:

• The traditional methods for detection of residual living virus by either means of injection of vaccine in mice and observe the no of deaths or injection in lambs to observe rise in temperature and clinical signs and also the way of blind passage in tissue culture.

• But in this study we used one of the recent bio-techniques for detection of living residual virus (Rt-PCR).

Five batches of the vaccine were tested for sterility using different medium to insure that the batches are free from bacteria, fungus or mycoplasma. In regard to table no. (1), shows that the vaccine batches were sterile i.e. no bacterial or fungal contamination, standardized as the evaluated protocol ***Randall et al. (1962)***, ***El-Nime (1980)*** and ***OIE (2007)***.

We applied the safety test in either lambs or mice.

In lambs, which injected with 5 ml I/P and 5 ml S/C from tested vaccine, showed no obvious thermal reaction during the period of observation (10 day). The body temperature was within normal in all tested groups of animals in addition there is no clinical manifestation appeared in lambs allover the period of observation, this result agreed with ***Binn et al., (1963)***, ***Iman and El-Karamany (1978)*** and ***OIE (2007)***.

In mice, which injected with 0.2 ml I/P for 10 mice and kept under observation for 21 day, no. of deaths in mice were occurred, this result agreed with ***Binn et al., (1963)***, ***Iman and El-Karamany (1978)*** and ***OIE (2007)***.

Secondly testing of the vaccine for immunogenicity through ED_{50} in mice, Serum neutralization test and ELISA test.

The results of potency of the vaccine by

the calculation of ED_{50} which obtained by observation with counting the dead mice in inoculated group allover 21 day, and then calculated according to ***Reed and Muench(1938)***, in which the permissible limited of ED50 of tested vaccine must not exceed 0.02/ml to considered potent according to ***OIE (2007)***.

In our experiments ED_{50} all of five batches of tested vaccine are not exceed 0.02/ml as shown in table (2), our results are agreed with ***OIE (2007)***.

Serum neutralization test (SNT) of serum samples from vaccinated sheep showed arise in the mean neutralizing antibodies started from the first week post vaccination at 0.75 log_{10} and increased gradually till reached to the protective level at the 5th week post vaccination at 1.575 log_{10} as revealed in table no. (3), and figure no. (1).

Our results agreed with ***Abdel Ghaffar et al., (1981)*** and ***Torky et al., (1991)*** who reported that the serum neutralizing antibodies were detected 7 days after vaccination with killed vaccine. Also agree with ***OIE (2007)*** which approved that the lowest protective level to RVF inactivated vaccine must be not less than 1.5 log_{10}. But our results disagreed with ***Marcoss (1992)*** who found that serum neutralizing antibodies started by 4th day post vaccination and continued to increase before declining to reach the figure of 1.7 after 25 days. Also disagree with ***Gihan (1990)*** who found that serum neutralizing antibodies reached to 2.75 log_{10} at the 2nd week post vaccination. And disagree with ***Ibrahim (2002)*** revealed that the protective level was achieved in the 3rd week post-vaccination using SNT.

This disagree due to the type of vaccine used in experiment either (inactivated or live attenuated), adjuvant either (oil or alum hydroxide gel) and animal factor either

(age or sex or animal species) as mentioned by ***Muntiu et al. (1971)***.

Enzyme Linked Immunosorbent Assay (ELISA) of serum samples from vaccinated sheep showed arise in the mean antibody titers started from the first week post vaccination at 320 and increased gradually till reached the higher level at the 5th week post vaccination at 2005.5 as revealed in table no. (4) and figure no. (2).

Our results agreed with ***Taha et al., (2002)*** found that ELISA gave higher figure of sensitivity than AGPT. Also agree with ***Guilherne et al. (1996)*** examined blood samples by ELISA for the presence of antibodies to RVF.

The ELISA titers ran parallel course with those of SNT where the antibody titers started to rise after the 1st week post vaccination and continued to rise till reached to the protective level after 5th week post vaccination.

Results of comparison between both mean of SNT and ELISA to determine the protective level of ELISA which approved that the lowest protective level of RVF inactivated vaccine by means of ELISA must be not less than 2000, and By SNT not less than 1.5, this results agreed with ***OIE (2007)*** which recorded that the protective level of RVF inactivated vaccine by means of SNT must be not less than 1.5.

The most recent technique to determine the accuracy of inactivation process to ensure there is no residual living virus is the RT-PCR.

The virus nucleic acid was successfully extracted and purified from the infected BHK cells using (TALENT SEEK VIRAL RNA) kit. When the gene order on the S-segment is NS, The extracted genomic RNA was used as a template to amplify the target gene.

The primed RT-PCR of S segment gene came to a conclusion that its size was about

800 bp ***Collet et al., (1985)***.

The results of application of RT-PCR on live seed virus showed positive band on gel electrophoresis at molecular weigh 800bp. These results agree with ***Saad et al., (2002)***.

The inactivated RVF virus sample give negative result, this result confirmed that the effect of binary ethylenemine on the nucleic acid, so it make complete inactivation to the virus, this results agreed with ***Habib et al., (2006)*** who mentioned that Binary ethylenimine reacts very little with proteins and more specific for the nucleic acid and it produces antigenically superior vaccine.

Table (1): Purity, identity, safety of five batches of inactivated alum gel RVF (ZH 501) vaccine

Purity	Identity (RT-PCR)	Safety	
		Baby mice	Lamb
Sterile	RVF antigen	Safe	Safe

Table (2): Potency results following challenge with RVF virus in mice

Batch No. of vaccine	ED_{50}
1	0.006
2	0.0007
3	0.005
4	0.002
5	0.0009

The Permissible limit of ED_{50} should be less than 0.02.

ED_{50}/ml = Effective dose fifty

Table (3): Mean antibody titers of vaccinated sheep with inactivated RVF vaccine (ZH501) Batches by SNT

No. of Batch	Before vaccination	Weeks post vaccination						
		1st	2nd	3rd	4th	5th	6th	7th
1	0.375	0.75	1.05	1.2	1.35	1.575	1.8	2.1
2	0.45	0.75	0.9	1.05	1.35	1.5	1.8	2.175
3	0.3	0.6	0.75	0.975	1.125	1.425	1.65	1.95
4	0.45	0.675	0.9	1.2	1.35	1.65	2.025	2.325
5	0.375	0.675	0.9	1.125	1.35	1.575	1.875	2.25

* Figures represent antibody titers measured by serum neutralization test and expressed as $\log_{10}$.
* The mean antibody titers began to rise in 1st WPV (0.675 $\log_{10}$) and continued to rise till reach to protective level at 5th WPV (1.575 $\log_{10}$).

Table (4): Mean evaluation of inactivated RVF vaccines (ZH 501) batches in sheep sera by the means of ELISA

No. of batch	Before vaccination	Weeks post vaccination						
		1st	2nd	3rd	4th	5th	6th	7th
1	272.5	320	555	1570	1792.5	2005.5	2225.5	2553
2	295	350	562.5	1610	1895	2012.5	2203	2562.5
3	317.5	367.5	582.5	1655	1925	1997.5	2247.5	2617.5
4	265	330	545	1635	1885	2015	2193.2	2590.5
5	257.5	315	525	1585.7	1832.5	2055	2263.7	2603

Figures represent reading obtained from ELISA reader followed by statistical conversion using the equation described under materials and methods section

(3. 2.2.)

Fig. (1): Mean antibody titers of vaccinated sheep with inactivated RVF vaccine (ZH501) Batches by SNT

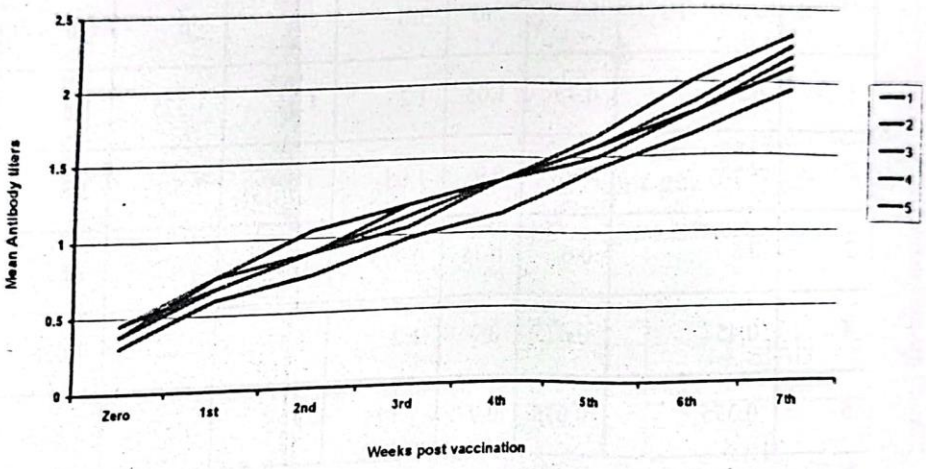


Fig. (2): Mean evaluation of inactivated RVF vaccines (ZH 501) batches in sheep sera by the means of ELISA

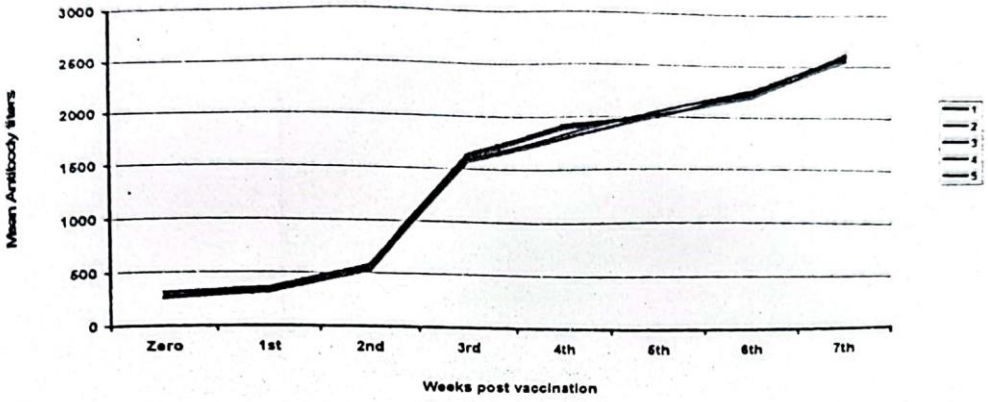
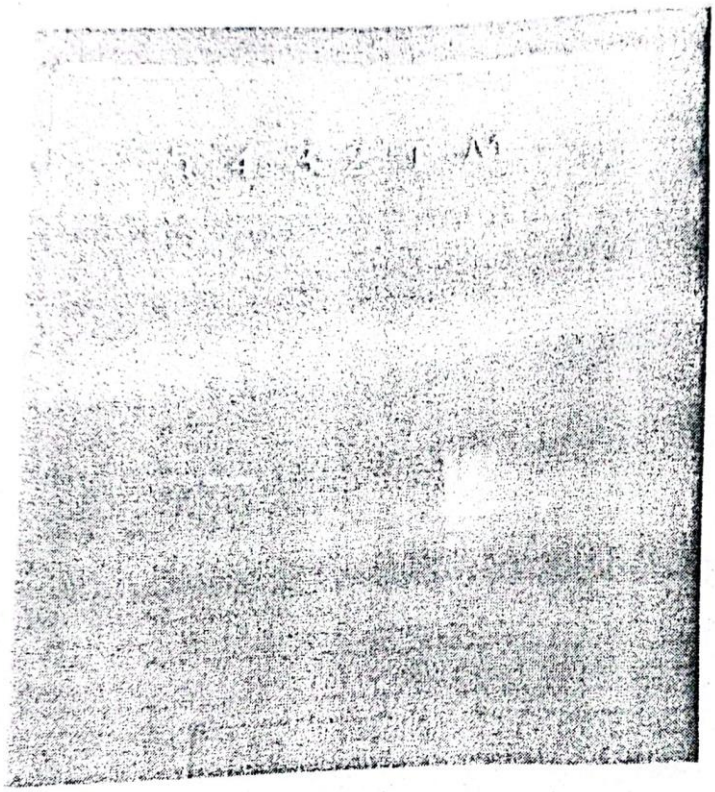


Figure (3) Rt-PCR



M: Marker
Lan No. (1) : Represent the inactivated RVF (ZH501) eluted from aluminium hydroxide gel
Lan No. (2,3). Represent the inactivated RVF (ZH501) before adding aluminium hydroxidegel
Lan No. (4,5). Represent virulent RVF (ZH501)
Lan No. (1,2,3) : -ve
Lan No. (4,5): +ve
The band represented +ve result located in opposite to 800bp band of marker

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