Application of RT-PCR as A Rapid and Accurate Quality Assurance Tool for Quantification of Rift Valley Fever Vaccinal Strain

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Abstract

The Rift Valley Fever Virus (RVFV) is a zoonotic disease that is spread by mosquitoes. The potential effects of RVFV on both public health and agriculture have led the CDC and the USDA to designate it as a Category "A" priority disease that overlaps with select agents. Vaccination plays a significant role in controlling RVF disease, as it does with other viral infections. Rapid and sensitive quality assurance procedures tailored to the vaccination strain are crucial for ensuring the vaccine performs as expected. In light of the difficulties caused by the present outbreaks, these protocols will prove useful. Using real-time PCR with SYBR Green and low ROX, this work attempted to produce a more accurate and efficient procedure for measuring the Rift Valley Fever virus vaccinal strain than the traditional tissue culture-based titration method. The amount of virus in the vaccinal strain and seven different samples of RVFV ZH-501 were measured using both the usual tissue culture method and real-time PCR. Afterwards, the outcomes were contrasted. The Real-time PCR standard curve that was created displays the mean CT values, the linear equation (y = -3.4639x + 38.506) and the r-squared value (0.996). A correlation coefficient of 0.996 indicated that the amplification was 94.4 percent effective. Results from the Real-time PCR were comparable to those from tissue culture titration and there was no statistically significant difference ($p > 0.05$). In cases of viral quantification, the results showed that the real-time

PCR assay is the way to go because it is quick, easy, accurate, simple, and cheap.

Keywords: RVFV, Virus quantification, Real time PCR, Syber Green.

Introduction

The Rift Valley fever virus (genus Phlebovirus) is responsible for causing Rift Valley fever, a zoonotic disease that is spread by vectors. Based on its potential for an outbreak, the World Health Organization Blueprint has listed RVFV as one of the top eight priority infections ***(Lapa et al., 2024).***

RVFV has received a lot of focus as of late. In 2024, RVF disease was officially recognized as a notifiable disease by the World Organization for Animal Health ***(WOAH, 2024)***. Both the CDC and the USDA have included it to their lists of overlapping select agents ***(CDC, 2021).***

RVFV is a member of the Phenuiviridae family and the Bunyavirales order of phleboviruses ***(International Committee on Taxonomy of Viruses, (2008)***. A small (S) segment with ambience polarity, a medium (M) segment, and a large (L) segment - all of negative sense - make up this virus's single-stranded RNA genome***. Ikegami, (2012)*** identified the S segment's anti-genomic RNA as a key virulence component that codes for the non-structural NSs protein. Nucleoproteins make up the N protein. Membrane fusion and cell tropism are regulated by the glycoprotein precursors Gc and Gn, which are encoded by the M segment. Viral RNA-dependent RNA polymerase (RdRp) is encoded by the L segment ***(Ikegami and Makino, (2011)***. The presence of genomic reassortment among various strains of RVFV has been established through experimental evidence ***(Gaudreault et al., 2019).***

During a sheep epidemic in 1930, Kenya reported the first incidence of RVF. Prior to the year 2000, the illness was only found in Africa ***(WHO, 2018).*** The ancient geographic distribution of RVF disease has been extended in the last quarter of a century into the Middle East (Saudi Arabia and Yemen) and the livestock-raising regions of eastern and southern Africa. Infected cattle were traded from the Horn of Africa, which caused multiple epidemics and epizootics, which led to this growth ***(Zouaghi et al., 2021).*** The World Health Organization is concerned that the disease could spread to other regions of Asia and Europe after its first recorded case outside of Africa ***(WHO, 2023).***

According to ***Tercero and Makino (2024),*** there are currently no anti-RVFV medicines or vaccinations that have been

approved for use in humans. The only surefire way to stop the spread of RVFV and stop the transmission chain in its tracks is to vaccinate animals ***(CDC, 2020).***

A long-term vaccination campaign for animals can stop RVF outbreaks. Vaccines for animals can be either inactivated or modified live attenuated virus ***(WHO, 2023).*** The development of a highly sensitive quantification assay that is suitable for rapid selective titration is a topic of concern since the quantification of the vaccinal strain is vital for quality testing during vaccine manufacture. For the detection and quantification of nucleic acid, real-time PCR assays are the way to go. They are more quick, sensitive, and reproducible. As a result of operating in closed vessel systems, the process is inexpensive, and the absence of post-PCR processing reduces the possibility of contamination. All of these things combine to make it an ideal tool for making a very sensitive diagnostic in a hurry ***(Valasek and Repa, 2005).***

This study contrasts the traditional tissue culture-based titration method with a real-time PCR assay that utilizes SYBR Green with low ROX to guarantee the vaccination strain's quality and amount.

Material and methods

Virus

The Rift Valley Fever virus vaccinal strain ZH-501 was adapted for tissue culture, and after that, it was propagated five times in Vero cells ***(WOAH, 2018).*** In addition to seven separate samples of RVFV ZH-50 were generously provided by Rift Valley Fever Department (RVFRD), Veterinary Serum and Vaccine Research Institute (VSVRI), Cairo, Egypt.

Cell culture

Vero cell culture (African green monkey kidney) was maintained in Dulbecco's modified Eagle's medium (DMEM) obtained from Gibco Company, Lot No. 055 K 83032, supplemented with 2% heat-inactivated fetal bovine serum (FBS), as described by ***Macpherson and Stocker, (1962)***.

Virus titration

Microtiter technique was used to titrate the vaccinal strain of RVFV ZH-501 on Vero cell cultures and estimation of the $TCID_{50}$/mL value as reported by ***Reed and Muench's (1938).*** To track the progression of the cytopathic effect (CPE), the microtiter plates were left to incubate for 72 hours and then examined every day. Seven more RVFV ZH-501 samples were tested using the same approach for comparison. The $TCID_{50}$/mL value represents the infectivity level of each sample as calculated according to ***Reed and Muench's (1938)*** method.

The extraction of viral RNA

The Patho Gene-spin™ DNA/RNA Extraction Kit (INtRON BIOTECHNOLOGY,

Korea) was utilized to extract the vaccinal ZH-501 strain of RVFV which contains 10^8 $TCID_{50}$/ml and other different seven virus samples. A standard curve was developed by serially diluting the isolated RNA of the vaccinal strain by a factor of 10. The lower limit of the assay linear dynamic range was determined by triplicate testing of each dilution using SYBR Green one step qRT-PCR.

Conventional RT-PCR

For a conventional one-step RT-PCR on the RVFV RNA extract, we followed the Trans one step RNA PCR instructions (Transgenbiotech, China) and utilized Forward (5'-CCTTAACCTCTAATCAAC-3') and Reverse (5'-TATCATGGATTACTTTCC-3') primers as described by **Sall et al. (2001).** Each 20 µl reaction tube contained 10 µl of one-step Reaction Mix 2xE, 0.4 µl of one-step Enzyme Mix, 1 µl of each primer (10 µM), and 1 µgm of RNA extract and up to 20 µl of nuclease-free water. The mixture went through a series of thermal cycling conditions using MJ Mini thermal cycler (BIO RAD), starting at 45°C for 30 minutes and then increasing to 94°C for 5 minutes. After that, it went through 40 cycles of denaturation at 94°C for 30 seconds, annealing at 44°C for 30 seconds, extension at 72°C for 1 minute, and ultimately, 72°C for 10 minutes. A 1.2% agarose gel was loaded with 20 µl of amplification products and then stained with ethidium bromide to allow visualization.

Quantify Rift Valley virus using real-time PCR

QRT-PCR assays were conducted utilizing a thermal cycler (Bio-Rad, USA) in 96-well plates with SYBR Green and low ROX (enzynomics, Korea), employing selected primers for the amplification of the S-segment of RVFV using the Forward primer (5'-AAGGCAAAGCAACTGTGGAG'3) and the Reverse primer (5'-CAGTGACAGGAAGCCACTCA'3) were described by ***Jonas et al. (2008).*** The isolated RNA was analyzed utilizing the TOP realTM One-step RT qPCR Kit, SYBR Green with Low ROX (enzynomics, Korea). In a 20 µl reaction mixture, there was 1 µg of extracted viral nucleic acid, 10 pmol of each primer, 1 µl of qPCR Enzyme MIX, and 10 µl of qPCR Reaction MIX. Each reaction was conducted using the temperature profile (Bio-Rad, USA): 50°C for 30 minutes, 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 20 seconds, annealing at 62°C for 20 seconds, and extension at 72°C for 40 seconds. We defined the cycle threshold number (Ct value) as the PCR cycle count at which fluorescence is detected in the reaction. Any reaction that registered a Ct value was deemed positive, whereas any reaction that

did not register a Ct value was classified as negative and utilized to build standard curves.

Statistical analysis:

The data was obtained from the result of tissue culture titration and the qRT-PCR titration for the various RVF virus samples and analyzed using Wilcoxon's test to calculate the p value for detecting the statistically significant difference.

Results

ZH501-TC virus propagation and titration

The vaccinal strain and seven different samples of RVFV ZH-501, which were grown in Vero cell cultures, demonstrated a clear cytopathic effect (CPE) 48–72 hours after inoculation, as evidenced by cell rounding, followed by the destruction of the whole cell sheet within 12–24 hours, as shown in **Fig. (1)**. The infectivity titer was determined as illustrated in **Table (1).** The infectivity titration of the seven RVF virus samples on tissue culture were respectively $10^{5.5}$, 10^{4}, $10^{6.25}$, $10^{6.75}$, $10^{4.5}$, $10^{3.25}$ and $10^{4.25}$, while the vaccinal strain had an infectivity titer of 10^{8} $TCID_{50}$ /ml.

3.2. Conventional PCR

The PCR product of the reference ZH-501 RVF virus strain using specific primers for conventional PCR was detected at 810 bp by separating it on 1.2% agarose gel electrophoresis (**Fig. 2A)**. The conventional PCR product using primers from real-time qPCR, was detected at 155 bp and separated on 1.5% agarose gel electrophoresis, as illustrated in **Fig. (2B)**.

3.3. SYBR Green I real time RT-qPCR

The reliability and reproducibility of the QRT-PCR assay were assessed by employing 10-fold dilutions of standard RVFV RNA. Cycle Threshold (CT) values were graphed against the logarithmic concentrations of the successive dilutions (**Fig. 3)**.

$y = -3.4639x + 38.506$

$R^2 = 0.9961$

Generation of standard curve

The generated standard curve of RT-qPCR for quantification of the reference ZH-501 RVF virus strain was developed to examine the relationship between 10-fold serial dilution of the extract RNA and Ct values. A linear regression relationship was noted with slope of −3.464, a reaction efficiency percentage of 94.4%, and a coefficient of determination (R2) of 0.996. **Figure (4) and Table (2)** demonstrate that the developed standard curve was linear across the whole quantification range, which spanned seven orders of magnitude.

The comparison between the titration of RVFV using qRT-PCR and the titration of different RVF virus samples using tissue culture.

Table (3) shows that there was no statistically significant difference between the tissue culture titration findings and the qRT-PCR titration results for the various RVF virus samples (p value = 0.433).

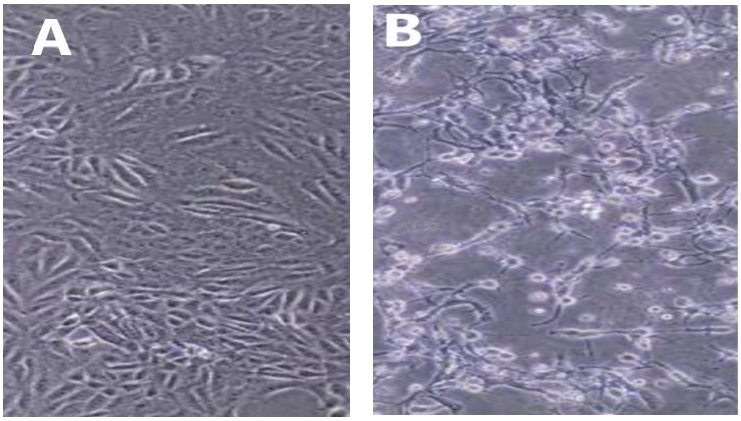

Fig. (1): Normal and RVFV (ZH-501) infected Vero cells
(A) Spindle cells are visible in a monolayer sheet of normal Vero cells.
(B) Cell rounding and detachment were seen as specific CPE of RVFV (ZH-501) 48-hour RVFV (ZH-501) post Vero cell infection.

Table (1): *Infectivity titration of tested RVFV samples on tissue culture*

Sample	Infectivity titer (Log_{10} $TCID_{50}$ /ml)
The vaccinal strain	8.00
Sample 1	5.50
Sample 2	4.00
Sample 3	6.25
Sample 4	6.75
Sample 5	4.50
Sample 6	3.25
Sample 7	4.25

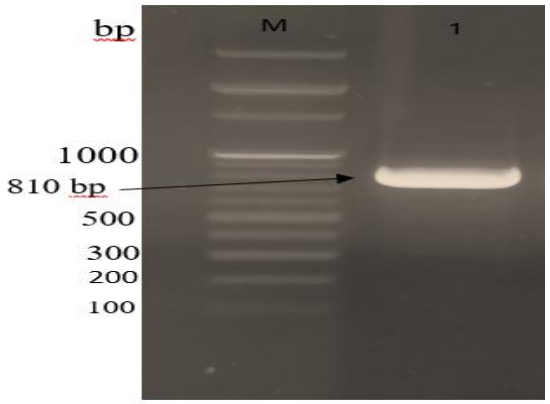

Fig. 2 -A: Conventional PCR performed using the specific primers to identify the amplification of the reference RVFV ZH-501; Marker (M) 100 bp DNA ladder (Trans, China); Lane-1 (PCR product, 810 bp). PCR product is separated by 1.2 % agarose gel electrophoresis and visualized by ethidium bromide staining.

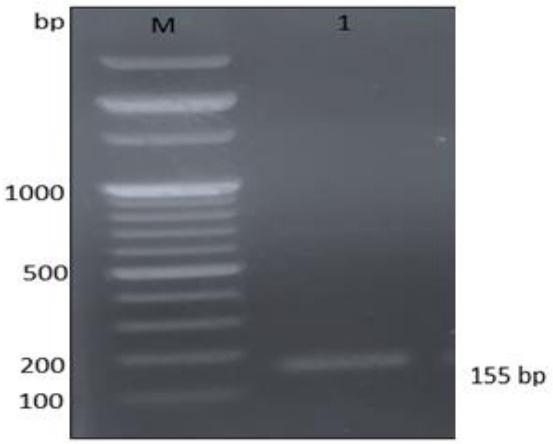

Fig. (2B): Conventional PCR performed using the real time primers for amplification of the reference RVFV ZH-501; Marker (M) 100 bp DNA ladder (Trans, China); Lane 1 (PCR product, 155 bp). PCR product is separated by 1.5 % agarose gel electrophoresis and visualized by ethidium bromide staining.

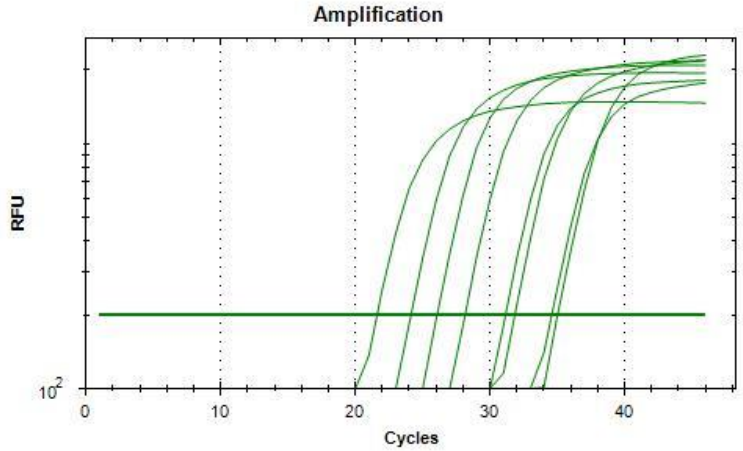

Fig. (3): Amplification plot of the reference ZH-501 RVF virus strain (from 10^7 $TCID_{50}$/ml to 10^2 $TCID_{50}$/ml)

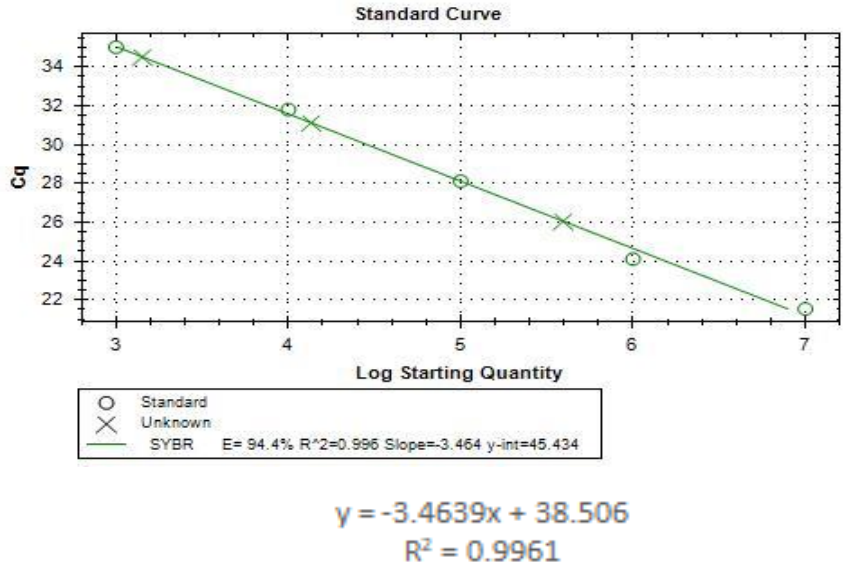

Fig. (4): The developed standard curve and the calculation equation for the reference ZH-501 RVF virus strain

Table (2): *An overview of the linear equations, R-squared values, and mean (cycle threshold) CT values from the real-time PCR for the RVF virus*

Virus Dilution	Cycle threshold (CT) values
10^7	21.54
10^6	24.10
10^5	28.11
10^4	31.80
10^3	35.01
10^2	38.15
Linear equation y=-3.4639x+38.506	
R-squared= 0.996	

Table (3): Evaluation of the various RVF virus samples by comparing their q-PCR titers with their (infectivity) titrations on tissue cultures

Samples no.	Ct	Equation	qrt-PCR	Infectivity titer (TCID 50 /ml)
1	**26.05**	**y= -3.4639x+38.506**	**5.6**	**5.50**
2	**31.12**		**4.1**	**4.00**
3	**23.90**		**6.20**	**6.25**
4	**21.54**		**7**	**6.75**
5	**29.40**		**4.60**	**4.50**
6	**34.70**		**3.4**	**3.25**
7	**30.80**		**4.4**	**4.25**
			There is no significant difference The *p*-value is 0.433	

Discussion

In order to keep making headway in the fight against the virus, it is urgently needed to establish protocols for rapid, highly sensitive quality assurance in process testing to guarantee that vaccines work as intended ***(Situma et al., 2024).***

As a part of standard quality control procedures, vaccine manufacturers must quantify the vaccinal strain. Endpoint titration in cell cultures and the determination of $TCID_{50}$/mL are long-established ways to measure infectious titers ***(Lei et al., 2021).*** Extensive expertise in cell culture propagation and maintenance is required for this tedious and time-consuming operation, which typically takes three to four days to finish. In addition, a Biosafety Level-3 (BSL-3) laboratory is required for cell infection. In addition to the challenge of consistently plating the same amount of cells, the inherent

unpredictability of cell cultures as biological systems makes it challenging to standardize this type of quantification ***(Brandolini et al., 2021).*** Quantitative reverse-transcription polymerase chain reaction and other nucleic acid detection and amplification technologies have the potential to reduce the analysis time and eliminate the need for biocontainment measures while still yielding a highly sensitive viral quantification ***(Brandolini et al., 2021).*** This study's overarching goal is to find out how RT-PCR stacks up against the tried-and-true cell culture-based titration method when it comes to quantifying the vaccinal strain.

The infectivity titer was firstly established by conducting infectivity titration of the vaccinal strain and the seven separate samples of RVFV ZH-501in tissue culture, as shown in **Table (1)** and demonstrated in **Figure (1).** The results illustrated that the infectivity titration of the seven RVF virus samples on tissue culture were respectively $10^{5.5}$, 10^{4}, $10^{6.25}$, $10^{6.75}$, $10^{4.5}$, $10^{3.25}$ and $10^{4.25}$, while the vaccinal strain had an infectivity titer of 10^{8} $TCID_{50}$ /ml.

The genetic identification was done using conventional PCR, as illustrated in **Figures (2 A, B)** and agreed with the results that were reported by ***Sall et al. (2001)*** and ***Jonas et al. (2008).*** SYBR Green-based QRT-PCR assay was carried out in this study due to its advantage of SYBR Green I chemistry with optimization procedures comparing with Taqman in the previous approach reported by ***Jonas et al., (2008)*** **and** ***Almajhdi, (2011).*** The standard curve of QRT PCR assay for the reference ZH-501RVFV strain contain 10^{8} $TCID_{50}$/ml was generated and demonstrated the linear equation (y = -3.4639x + 38.506), slop (-3.464), the r-squared value (0.996) and the mean CT values; 21.54, 24.1, 31.8, 35.01, 38.15 that related with their dilution as mentioned in **Table (2).** A correlation coefficient of 0.996 indicated that the amplification was 94.4 percent effective. This is in agreement with the previously published real-time SYBR Green I RT-PCR assay for the same gene target of RVFV by ***Jonas et al. (2008)***.

The reference ZH-501RVFV strain and seven virus samples that were quantified through SYBR Green-based QRT-PCR approach and compared with their infectivity titer on tissue culture. The finding results of infectivity titer of seven RVF virus samples on tissue culture were $10^{5.5}$, 10^{4}, $10^{6.25}$, $10^{6.75}$, $10^{4.5}$, $10^{3.25}$ and $10^{4.25}$, while the titer of the same RVF virus samples by RT-qPCR were $10^{5.6}$, $10^{4.1}$, $10^{6.2}$, 10^{7}, $10^{4.6}$, $10^{3.4}$ and $10^{4.4}$ respectively. The p-value of 0.433 was calculated by applying Wilcoxon's test, so there was no statistically significant difference

between the tissue culture titration findings and the qRT-PCR titration results for the various RVF virus samples. This finding is in line with that of a prior study carried by ***Abousenna et al. (2020),*** who found no statistically significant difference between the two approaches to titrate sheep pox virus, as a p-value was 0.3662, which is greater than the significance level of 0.05.

Finally, and according to the finding results, the newly developed SYBR Green I based real time RT-PCR assay was useful and applicable for quantification of RVF virus during in process of vaccine production and emergency case reducing time and efforts.

Conclusion

Given this context, molecular methods offer a viable alternative that can have a positive impact on the sector due to their sensitivity, reproducibility, wider laboratory application, time- and cost-effectiveness, and ability to quickly and accurately determine the infectious viral titer.

Conflict of interest:

The authors have no conflict of interest to declare.

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إستخدام (RT PCR) كأداة سريعة ودقيقة لضبط الجودة فى تحديد عيارية عترة لقاح حمى الوادي المتصدع

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الملخص

الخلفية:حمى الوادي المتصدع (RVF) هي مرض فيروسي حيواني المنشأ ينتقل عن طريق البعوض ويُسببه فيروس .RVFV يتم تصنيف فيروس حمى الوادي المتصدع (RVFV) كعامل مسبب للأمراض من الفئة أ وعامل مختار متداخل من قبل مركز السيطرة على الأمراض (CDC) ووزارة الزراعة الأمريكية (USDA) بسبب تأثيره المحتمل على الصحة العامة والزراعة. مثل الأمراض الفيروسية الأخرى، يعتمد التحكم في حمى الوادي المتصدع بشكل كبير على التطعيم. لذا، فإن تصميم بروتوكولات سريعة وفعالة لضمان جودة سلالة اللقاح هو أولوية قصوى لضمان أن اللقاح يلبي أعلى معايير الفعالية، وستكون هذه البروتوكولات مفيدة في مواجهة التحديات المستمرة التي تطرحها التفشي الحالي. هدفت الدراسة الحالية إلى تطوير طريقة سريعة للتقييم العيارى لسلالة الفيرس المستخدم لانتاج لقاح حمى الوادي المتصدع عن طريق RT-PCR باستخدام SYBR Greenمع ROX للمقارنة بالطريقة التقليدية، حيث تم التقييم العيارى للفيروس باستخدام تقنية الزرع النسيجى.

النتيجة: تم انشاء منحنى معيار (Standard Curve) باستخدام تفاعل البوليميراز المتسلسل في الوقت الحقيقي (**SYBR Green I real time RT- qPCR**) لسلالة فيروس الحمى الوادى المتصدع RVFV ZH-501 ذو عيارية 10^8 جرعة معدية من زراعة الأنسجة 50/مل، وكانت متوسط قيم التصوير المقطعي (CT) المحوسب كالاتى 21.54 ،24.1، 31.8، 35.01 و38.15 بالتناوب مع التخفيف التسلسلى المضاعف عشرة 10^7، 10^6، 10^5، 10^4 ، 10^3، 10^2 ترتيبا؛ والمعادلة الخطية (y = -3.4639x + 38.506) وقيمة 0.996 (r-squared). وهذا يشير معامل الارتباط البالغ 0.996 إلى أن التضخيم كان فعالاً بنسبة 94.4 بالمائة بكفائة ودقة .

ثم تم قياس ومقارنة كمية الفيروس في سلالة اللقاح وسبع عينات مختلفة من RVFV ZH-501 باستخدام كل من طريقة زراعة الأنسجة المعتادة(Tissue Culture) وتفاعل البوليميراز المتسلسل في الوقت الحقيقي (qRT-PCR) و كانت نتائج بينهما مماثلة بالتناوب مرتبا كالاتى [$10^{5.5}$ ، 10^4، $10^{6.25}$، $10^{6.75}$ ، $10^{4.5}$، $10^{3.25}$، $10^{4.25}$ ،[$10^{5.6}$، $10^{4.1}$، $10^{6.2}$، 10^7 ، $10^{4.6}$، $10^{3.4}$، $10^{4.4}$] ولم يكن هناك فرق كبير إحصائيًا ($p> 0.05$) بنسبة p تبلغ قيمة 0.433 فقط. **الاستنتاج**: اختبار تفاعل البوليميراز المتسلسل في الوقت الحقيقي RT-PCR باستخدام SYBR Greenمع ROX هو اختبار كمي سريع وبسيط ومنخفض التكلفة وعالي الحساسية، وبالتالي فهو مناسب تمامًا للحالات الطارئة.