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A Recent Approach for *In-vitro* Evaluation of the Inactivated Rift Valley Fever Vaccine

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Abstract

WITH THE GLOBAL movement toward more widespread implementation of the 3Rs principle (Replacement, Reduction, Refinement of animal use) in bioscience, substantial efforts have been made to advance alternative *in-vitro* potency assays thus eliminating the disadvantages of the animal-based models regarding the number of used animals, assay time, costs and variability of results. The present study aims to assess the use of sandwich ELISA as an alternative *in-vitro* potency assay for quality control and release testing of the inactivated Rift Valley Fever vaccine (ZH 501). Three inactivated Rift Valley fever vaccine batch samples and a reference sample were evaluated *in-vitro* and *in-vivo*. The results demonstrated the correlation between the antigenicity measured by the *in-vitro* potency assay and the immunogenicity measured by the current *in-vivo* assays. In conclusion, *in-vitro* potency assay proved to predict the *in-vivo* immunogenicity, it can be a reliable alternative not only providing a mean for ethical research by replacing animal use but also strongly impacting the assay time, cost and efforts and paving the way for the adoption of the consistency approach as a strategy for vaccine quality.

Keywords: Rift Valley Fever, 3Rs, *in-vivo*, *in-vitro*.

Introduction

One of the main causes of viral zoonosis that affects both domestic and wild ruminants is the Rift Valley Fever Virus (RVFV) [1]. Rift Valley fever virus (RVFV), a mosquito-borne virus, has been linked to major economic losses, trade restrictions, and possibly fatal diseases in cattle over the past 100 years. Spillover to humans occurs and can be fatal [2].

In regions where RVF is endemic, vaccination is the main method for preventing animal infections. Several types of RVF vaccines are produced including the inactivated vaccine which has been used extensively in controlling RVF [3].

Historically, in vivo potency assays were used for release testing of vaccines, necessitating the use of a large number of animals [4]. Over the past few decades, animal-based testing has demonstrated its utility in guaranteeing the safety and effectiveness of various vaccines that can save lives. They, however, have certain drawbacks as they include animal suffering, *in-vivo* potency studies (challenge and serology assays) are also costly, time-consuming, and morally dubious [5]. In 1959 Drs. William and Rex Burch first described the 3Rs principles in their book "The principles of Humane Experimental Technique" [6]. The 3Rs principles refer to the Replacement, Reduction and Refinement of animals used in research teaching and exhibition. Since then they have been involved in a widely acknowledged set of guidelines accepted and upheld by many regulatory agencies, legislations and private and public laboratories [7]. So the transition to the *in-vitro* alternative method is the most pressing need in the field of vaccines [8], This is due to the advantages of saving time, effort and reducing the use of laboratory animals which has a great impact on the quality control strategy [9].

So, the current study aims to advance alternative *in-vitro* potency assays for adopting the 3Rs principle in quality control of inactivated RVF vaccines.

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Material and Methods

Ethics approval

This work was accepted by the animal ethics Committee of the veterinary serum and vaccine research institute (VSVRI). All experiments agree with the VSVRI guidelines for animal research.

Experimental animals

Swiss Albino adult mice: For potency evaluation of vaccines, 200 adult mice (aged 21–28 days) were provided by the Lab Animal unit in VSVRI.

Sheep: Twenty-five Baladi sheep, estimated to be 3–4 months old, were found to be devoid of RVF antibodies as screened by Serum Neutralization Test(SNT). They were kept in insect-proof stables with proper sanitation protocols, a well-balanced diet, and adequate water. They were employed in the evaluation of vaccine humeral response.

Newly born Lambs: For safety assessment of different vaccine batches as well as the reference vaccine, five seemingly healthy newborn lambs (7–10 days old) were utilized for each vaccine batch sample.

Inactivated RVF vaccine

Locally prepared cell culture inactivated RVF vaccine was kindly provided by RVF Research Department, VSVRI, Abbassia, Cairo.

RVF Virus (ZH 501): It was kindly supplied by the RVF research department, VSVRI, and was used for a challenge in the mice potency test.

ELISA kits: **ELISA IDvet kit,** ID screen® Multi-species Rift Valley Fever Competition Multi-Species Double Antigen, REF RIFTC-4P, Lot F44 was employed to identify certain antibodies that fought the Rift Valley Fever virus.

Sampling: Representative samples from three different batches of locally prepared inactivated RVF vaccine (A, B and C), in addition to a qualified standard reference vaccine were supplied by the RVF Research Department, VSVRI for evaluation purposes.

Quality assurance of different vaccine batches:

Sterility

In compliance with the recommendations of the Code of Federal Regulations [10] and WOAH [11], sterility testing of the three batch samples and the reference vaccine was conducted utilizing Sabouraud dextrose agar medium, soybean casein digest, thioglycolate, and mycoplasma solid and liquid media.

Safety

Safety in adult sheep

Single dose, repeated dose and overdose tests were conducted for safety assessment of the three different batch samples and the reference vaccine aligning with guidelines outlined before [11, 12].

Safety in lambs

A group of four lambs was used for each vaccine batch sample where each lamb was inoculated with 10 ml of each vaccine batch (five ml inoculated S/C and five ml inoculated I/P) and a 5th lamb was kept as control. Daily clinical observation was done for 2 weeks to detect any rise in body temperature or any abnormal clinical signs related to RVF as recommended before [13].

Potency assessment

Both in vitro tests and the conventional in vivo potency assays were conducted in a sequential evaluation as follows:

In vitro Potency assays

Antigen extraction

All vaccine samples were subjected to antigen extraction(whole antigen) according to the standard antigen extraction protocol [14].

After vortexing the vaccine sample for 1 minute at 5.5 rpm on a vortex , 0.3 mL of the sample was immediately transferred to an eppendorf microcentrifuge tube and 0.6 mL of extraction buffer (0.60 M sodium citrate dihydrate/0.55 M sodium phosphate dibasic, with 30 mM SDS pH 8.5) was added. The tube was mixed by inversion 10 times then incubated for 2.5 hours at 60°C with gentle mixing every 20 minutes during the incubation.

The tubes were then centrifuged at 425 g for 2 minutes at room temperature**.** Then extracted antigen was liquated for further analysis.

Characterization of the extracted antigen

Qualitative analysis

To confirm the integrity and identity of the extracted antigen of all vaccine samples, an agar gel precipitation test was conducted according to Gihan [15] using reference anti-RVF serum supplied by the Department of RVF Vaccine Research

Quantitative analysis

Sandwich ELISA was employed as antigen quantification assay as described before [16, 17] to quantify the antigen content of the three vaccine samples and reference vaccine.

In vivo Potency assays

Mouse potency assay (ED_{50})

It was conducted for each batch sample separately and the ED_{50} was calculated [18].

Serology based assays

Schedule of sheep vaccination Twenty-five sheep were screened for the absence of antibodies against

RVFV and assigned into five groups (five sheep/group) as follows:

Group (1) : Vaccinated with batch A

Group (2) Vaccinated with batch B

Group (3) Vaccinated with batch C

Group(4) Vaccinated with reference vaccine Each sheep received one dose of 1ml inoculated S/C in the inner thigh.

Group (5) Control non-vaccinated.

This step was followed by assessing humoral immune response**,** serum neutralization test (SNT) and competitive ELISA on serum samples obtained from all sheep groups on weekly intervals up to 4 weeks post vaccination.

Serum samples: Blood samples were obtained from all sheep groups through the jugular vein puncture weekly up to 4 weeks post vaccination in dry sterile screw-capped bottles kept for 30 minutes at 37°C and overnight in the refrigerator. The resulting serum was separated, spun for ten minutes at 3000 rpm, and then inactivated for thirty minutes at 56°C to break down any non-specific proteins then stored at -20°C until the subjected to the serological testing for monitoring of the sheep immune response.

Serum neutralization test (SNT)

RVFV antibody titer was determined in sheep sera using the SNT microtiter technique [19].

ELISA (competitive ELISA)

The test was conducted in compliance with the instructions sent by the manufacturer. The competition percentage (S/N %) was calculated for each sample. The samples less than or equal to 40% are considered positive, samples higher than 50% are considered negative, and samples greater than 40% and less than or equal to 50% are considered doubtful.

$$S/N\ \% = \text{OD samples} \times 100$$

Results and Discussion

The field of vaccines is experiencing notable progress in developing alternative methods to animal testing for quality control and release testing that has led to technological advancements in analytical methods and their application reflecting a growing momentum towards the application of the 3Rs principles (Replacement, Reduction, Refinement of animals use in bioscience) in certain animal-based tests and pave the way for the adoption of the consistency approach as a strategy for vaccine quality [5].

The objective of the present study is to review aspects related to establishing a well-designed *in-vitro* assay implementing the 3Rs principle in quality control and batch release testing of the licensed inactivated RVF vaccine.

The alternative *in-vitro* procedures were designed employing sandwich ELISA as antigen quantification assay, including using a reference vaccine sample for comparing the test vaccine potency and demonstrating batch-to-batch consistency.

The reference sample (Homologous sample) is a qualified standard sample produced by the same line of vaccine production and proven to be safe and potent. As a part of consistency approach the use of the reference sample in such a methodological quantification provides a powerful tool for demonstrating the vaccine is consistent in quality across different batches to confirm that batches are as similar as possible in the criteria defined by the regulatory authorities, thus provide an additional advantage of improved assay precision as described by Hendriksen et al. [20] who addressed the use of consistency testing as a substitute method for lot release testing to demonstrate any deviation from consistency of vaccine production. Also Bruysters et al. [21] put forth the consistency method as a tactic to facilitate the shift of vaccination batch testing from *in-vivo* to *in-vitro*.

To assess the reliability of the *in-vitro* testing as a potency assay, our study compares the two different *in-vivo* potency assays and the alternative *in-vitro* assay, to demonstrate its equality or superiority to the traditional *in-vivo* assays.

After affirming the samples compliance to rigorous sterility and safety standards (Table 1), the samples were assessed for potency by both *in-vivo* and *in-vitro* assays and data obtained from each assay were compared.

The vaccine potency was initially assessed by the *in-vitro* assay to provide insight into the effectiveness of the *in-vitro* assay as a tool for evaluating vaccine potency as follow:

The standard antigen extraction protocol described by Zho et al. [14] was performed for recovery of the protective antigen from aluminum hydroxide gels.

Qualitative analysis of extracted antigen was carefully considered as a critical step for accurate result to avoid incomplete recovery or alteration of the antigen structure during the process. AGPT was conducted to confirm identity of the extracted antigen as carried out by Gihan [15] where a positive result was demonstrated by immune precipitin line of the tested samples against the reference RVF antibodies (Table 2).

To assess the potential of extracted antigen to elicit the desired immune response, sandwich ELISA was conducted, two different antibodies targeting the extracted antigen of the test sample to directly

compare the antigen content of the test sample and the reference. As illustrated in Table (2) the results show that sample A had an OD value of 0.126, sample B had an OD value of 0.125, and sample C had an OD value of 0.128 while the reference sample had an OD value of 0.125. These findings demonstrated that the three batch samples possess potent neutralizing properties, indicating their efficacy in eliciting a robust immune response.

The use of sandwich ELISA as an *in-vitro* potency test (IVRP) for the vaccination against hepatitis B was examined by Descamps et al. [22] comparing it to WHO TRS 978's description of the Immunogenicity assay and demonstrated that compared to the immunogenicity assay, it was far more reliable and discriminating in identifying sub-potent batches. Also Sanyal [23] stated that *in-vitro* potency assays, employing ELISA have been approved by US and European regulatory agencies for use in vector-borne polyvalent vaccinations against human papillomavirus (HPV) and hepatitis B lately. Recently Sandwich ELISA was proved by Szeto et al. [24] to demonstrate its accuracy and specificity as an *in-vitro* substitute for the *in-vivo* PRN potency test in formulations based on DTaP-IPV.

The *in-vivo* data were obtained from both mouse potency assay and serology based assays for a comprehensive comparison.

Mouse potency assay determined the ED_{50} of three vaccine batch samples in addition to the reference sample as shown in Table (1). The results showed that sample A had an ED_{50} value of 0.002, sample B had an ED_{50} value of 0.0013, and sample C had an ED_{50} value of 0.003, while the reference sample had an ED_{50} value of 0.002. These findings suggest that the four samples are within the permissible limit as cited by Randall et al. [18] who stated that ED_{50} must not exceed 0.02/ml thus the lower ED50 value indicates the higher sample potency.

Our initial findings provide valuable insight into the effectiveness of the vaccine samples, serology based assays were conducted to assess the antibody response. The serological data were obtained over four consecutive weeks following a single dose vaccination to evaluate the onset and intensity of the immune response for each batch sample separately including the reference vaccine sample.

SNT was conducted according to Swanepoel et al. [19]. The results showed that batch A induced a neutralizing index of (1.5), batch B induced a neutralizing index of (1.2), sample C induced a neutralizing index of (1.6), and the reference sample induced a neutralizing index of (1.8) in the second week post vaccination. Based on the neutralizing indices the four samples demonstrated a protective level of antibodies in vaccinated sheep as early as two weeks post vaccination as shown in Table (3).

To confirm these findings, competitive ELISA was conducted. Optical density OD readings were obtained at a wavelength of 450 nm. ELISA results are shown in Table (4) were corresponding to SNT.

Data obtained from serological assays strongly suggest that the vaccine samples have significant potency and effectively stimulate a robust immune response.

The obtained results indicate that the alternative *in-vitro* potency assay has been correlated with the *in-vivo* measurement of antibodies induction assessed by the serological based assays and the protectivity assessed by the mouse potency assay.

The mouse potency test requires five dilutions of the test sample and at least 10 mice for each dilution (50 mice / sample).

As detailed here, the mouse potency test requires five dilutions of the test sample and at least 10 mice for each dilution (50 mice / sample).Vaccinated and unvaccinated mice are challenged with the live RVFV. This method results in severe suffering that is how the test is in conflict with the 3Rs and Animal welfare. The use of a lethal challenge in the mouse potency assay poses significant biosafety concerns; strict biosafety protocols must be conducted to prevent potential risks to both laboratory personnel and environment. Thus the implementation of appropriate containment measures, biosafety equipment, facilities, and well trained personnel are required leading to high costs besides the long test period (21 days) that delays the batch release.

In serological assays animals experience less discomfort but the assay is associated with significant costs owing to the necessity of utilizing large number of sheep in addition to an extended test period (at least 4 weeks).Also SNT entails the use of live RVFV, which necessitates involvement of specialized biosafety personnel , biosafety equipment and containment measures.

Furthermore, both mouse potency test and the serology based assays are animal based models that can exhibit natural variations in biological response leading to inherent variability that strongly impact the result as monitored by Coen et al. [25] who quantified the variability of *in-vivo* potency release tests for four different manufacturers' DTaP (diphtheria, tetanus, and a cellular pertussis) products. With relatively high coefficients of variance (CV), which range from 16% to 132%.

The recent European Pharmacopoeia General text on the replacement of *in-vivo* assays discussed the inherent variability of *in-vivo* assays that poses a challenge to their replacement with more precise *in-vitro* assays [26].

Results obtained from comparing the *in-vivo* assays to the *in-vitro* assays demonstrate a clear correlation between antigenicity measured by *in-vitro* assay and the immunogenicity assessed through the *in-vivo* assays, suggesting that the *in-vitro* assay is a valuable tool for predicting the *in-vivo* outcome.

In addition to eliminating the disadvantages of *in-vivo* methods regarding assay time (the assay can be completed in 3 days) and costs, number of animals used and variability of results. It also provides a useful tool for rapid testing of large numbers of samples and can avoid the need for costly biosafety facilities and eliminate potential health related issues associated with handling the live organism. Based on these results, the *in-vitro* potency assays proved to be a suitable alternative to the *in-vivo* potency assays and can be an appropriate method for release testing.

For lot release, a number of manufacturers and regulatory agencies favour *in-vitro* potency testing. In recent instances, in compliance with guidelines from the European Medicines Agency (EMA) and World Health Organization (WHO), mRNA and viral vector-based COVID-19 vaccines were released in Europe and the USA based on *in-vitro* potency assays [26].

Conclusion

From these findings, it can be inferred that the *in-vitro* potency assay proved to be a suitable alternative potency evaluation system not only aligns with ethical considerations in research by replacing animals, it also significantly affects the assay costs and time which is a critical measure in such emergencies, beside the fact that they are subject to methodological assessment that is much more consistent leading to more accurate and reliable data making it an excellent platform for applying the consistency approach, in addition to abridging the health related threats regarding the use of the biologically hazardous RVFV to lab worker and environment. Therefore, *in-vitro* potency assays represent a safe, timesaving, precise and cost-effective ethical alternative.

Conflict of interest

The authors declare that they have no conflict of interest.

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TABLE 1. Quality control of different vaccine samples

Vaccines	Sterility	Safety		Mouse Potency (ED_{50}/ml)
		In adult sheep	In lamb	
Batch A	Sterile	Safe	Safe	0.002
Batch B	Sterile	Safe	Safe	0.0013
Batch C	Sterile	Safe	Safe	0.003
Reference sample	Sterile	Safe	Safe	0.002

*The permissible limit of ED_{50} does not exceed (0.02) as reported by Randall et al., (1964)[19].

TABLE 2. Characterization of the extracted RVF Antigen

Vaccines	AGPT	Sandwich ELISA
Batch A	+ve	0.126
Batch B	+ve	0.125
Batch C	+ve	0.128
Reference sample	+ve	0.125

- Cut off value (0.03).
- +ve: immune precipitin line of tested samples against RVF antibodies.

TABLE 3. Mean values of neutralizing indices of sera of sheep vaccinated with different RVF vaccine samples

Sheep groups	SNT Antibody titer Log_{10}			
	Weeks post vaccination			
	W1	W2	W3	W4
Gp 1	*0.9	1.5	1.8	2.22
Gp 2	0.6	1.2	1.62	2.04
Gp 3	1.05	1.6	1.8	2.4
Gp 4	1.2	1.8	2.1	2.4
Gp 5	0.60	0.44	0.44	0.74

Gp (1): Vaccinated with batch A
Gp (2): Vaccinated with batch C
Gp (3): Vaccinated with batch B
Gp (4): Vaccinated with reference vaccine.
Gp (5): Control non-vaccinated
* Protective neutralizing antibody titer 1.5 according to Randall et al. (1964)[18].

TABLE 4. Mean OD value of ELISA in sheep sera vaccinated with different inactivated RVF vaccine batches

Sheep groups	ELISA S/N%			
	Weeks post vaccination			
	W1	W2	W3	W4
Gp 1	*54	37	32	29.6
Gp 2	57	52	33.5	30.5
Gp 3	52	35.5	33	24.6
Gp 4	51.5	35.5	31.8	24
Gp 5	72	70	72	69

Gp (1): Vaccinated with batch A
Gp (3): Vaccinated with batch B
Gp (2): Vaccinated with batch C
Gp (4): Vaccinated with reference vaccine.
Gp (5): Control non vaccinated
*S/N percentage (S/N %) ≤ 40% is considered positive.

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الملخص

مع الاتجاه العالمى نحو تنفيذ أكثر انتشارا لمبادىء (الاستبدال ، الحد ، التحسين) فى استخدام الحيوانات فى العلوم الحيوية تم بذل جهود كبيرة للتقدم في تطوير إختبارات كفاءة معملية بديلة ، للتخلص من عيوب النماذج القائمة على الحيوانات بالنسبة لعدد الحيوانات المستخدمة، وزمن الاختبار، والتكاليف، وتباين النتائج. الهدف من الدراسة الحالية هو تقييم فاعلية استخدام تقنية الساندويتش اليزا كتقنية معملية بديلة لتقييم جودة و كفاءة اللقاح المثبط لحمى الوادى المتصدع. تم تقييم ثلاث عينات من ثلاث دفعات مختلفة من اللقاح المثبط لحمى الوادى المتصدع ، بالإضافة إلى عينة مرجعية، باستخدام كل من الأساليب المعملية وأساليب التقييم في الجسم الحي. اظهرت النتائج التناسب الطردى بين النشاط الأنتيجينى الذى تم تقييمه بواسطة اختبار المعملى لكفاءة اللقاح والمناعة التي تم تقييمها بواسطة إختبارات تقييم المناعة في الجسم الحي، وبذلك أثبت الإختبار المعملى لكفاءة اللقاح كفاءته التنبؤية للمناعة في الجسم الحي، ويمكن أن يكون بديلاً موثوقًا ليس فقط لتوفير وسيلة للبحث الأخلاقي من خلال استبدال استخدام الحيوانات ولكن أيضًا للتأثير بشكل قوي على زمن الاختبار والتكاليف والجهود، وفتح الطريق لاعتماد نهج الاتساق كاستراتيجية لتقييم جودة اللقاح.

الكلمات الدالة: حمي الوادي المتصدع ، الاليزا ، المناعة ، لقاح اكثر كفاءة.