DEVELOPMENT AND EVALUATION OF ALTERNATIVE POTENCY TESTS FOR INACTIVATED RABIES AND INFECTIOUS BURSAL DISEASE VACCINES

Thesis

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IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

Master of Veterinary Science
(Veterinary Microbiology)

2023

Dedicated to....

My Beloved Parent's

and

Sister

भा.कृ.अनु.प.–भारतीय पशु चिकित्सा अनुसंधान संस्थान
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Dated: 2023

Certificate

This is to be certified that the research work embodied in this thesis entitled **"Development and evaluation of alternative potency tests for inactivated rabies and infectious bursal disease vaccines"** *submitted by* **Dr. Sudhir Singh, Roll No. M-6161,** *for the award of* **Master of Veterinary Science** *Degree in* **Veterinary Microbiology** *at Indian Veterinary Research Institute, Izatnagar, is the original work carried out by the candidate himself under my supervision and guidance.*

It is further certified that **Dr. Sudhir Singh, Roll No. M-6161,** *has worked for more than 21 months in the Institute and has put in more than 150 days attendance under me from the date of registration for the* **Master of Veterinary Science** *Degree in this Deemed University, as required under the relevant ordinance.*

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Certificate

We the undersigned members of Advisory Committee of **Dr. Sudhir Singh, Roll No. M-6161,** *a candidate for the degree of* **Master of Veterinary Science** *with the major discipline in* **Veterinary Microbiology**, *agree that the thesis entitled* **"Development and evaluation of alternative potency tests for inactivated rabies and infectious bursal disease vaccines"** *may be submitted in partial fulfilment of the requirement for the degree.*

We have gone through the contents of the thesis and are fully satisfied with the work carried out by the candidate, which is being presented for the award of **Master of Veterinary Science** *Degree of this Institute.*

It is further certified that the candidate has completed all the prescribed requirements governing the award of **Master of Veterinary Science** *Degree of the Deemed University, Indian Veterinary Research Institute, Izatnagar.*

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Place: *ICAR-IVRI, Izatnagar*

ABBREVIATIONS

%	:	Percentage
µg	:	Microgram
µl	:	Microliter
Ab	:	Antibody
AGE	:	Agarose Gel Electrophoresis
BHK-21	:	Baby Hamster Kidney-21
bp	:	Base pair
BSA	:	Bovine Serum Albumin
cELISA	:	Competitive Enzyme Linked Immuno sorbent Assay
CFR-9	:	Code federal regulation
cm	:	Centimeter
conc.	:	Concentration
CVS	:	Challenge Virus Standard
ddPCR	:	Droplet Digital PCR
DFA	:	Direct Fluorescent antibody
DNA	:	Deoxyribonucleic acid
dNTP	:	Deoxyribonucleotide
DW	:	Distilled water
EBS buffer	:	EDTA-borate-sucrose
EDTA	:	Ethylene-diamine-tetra-acetic acid
EID	:	Egg infectious dose
ELISA	:	Enzyme linked immune sorbent assay
EMEM	:	Eagles minimum essential media
et. al.	:	*et alia*
FA	:	Fluorescent antibody
FBS	:	Fetal Bovine Serum
FFID	:	Fluorescence Focus Infectious Dose
G/g	:	Relative centrifugal force
GMP	:	Good manufacturing practise
Hr(s)	:	Hours
HRPO	:	Horse reddish peroxidase
IAEC	:	Institutional Animal Ethics Committee
IBD	:	Infectious bursal disease
ICAR	:	Indian Council of Agriculral Research
IFA	:	Indirect fluorescent antibody

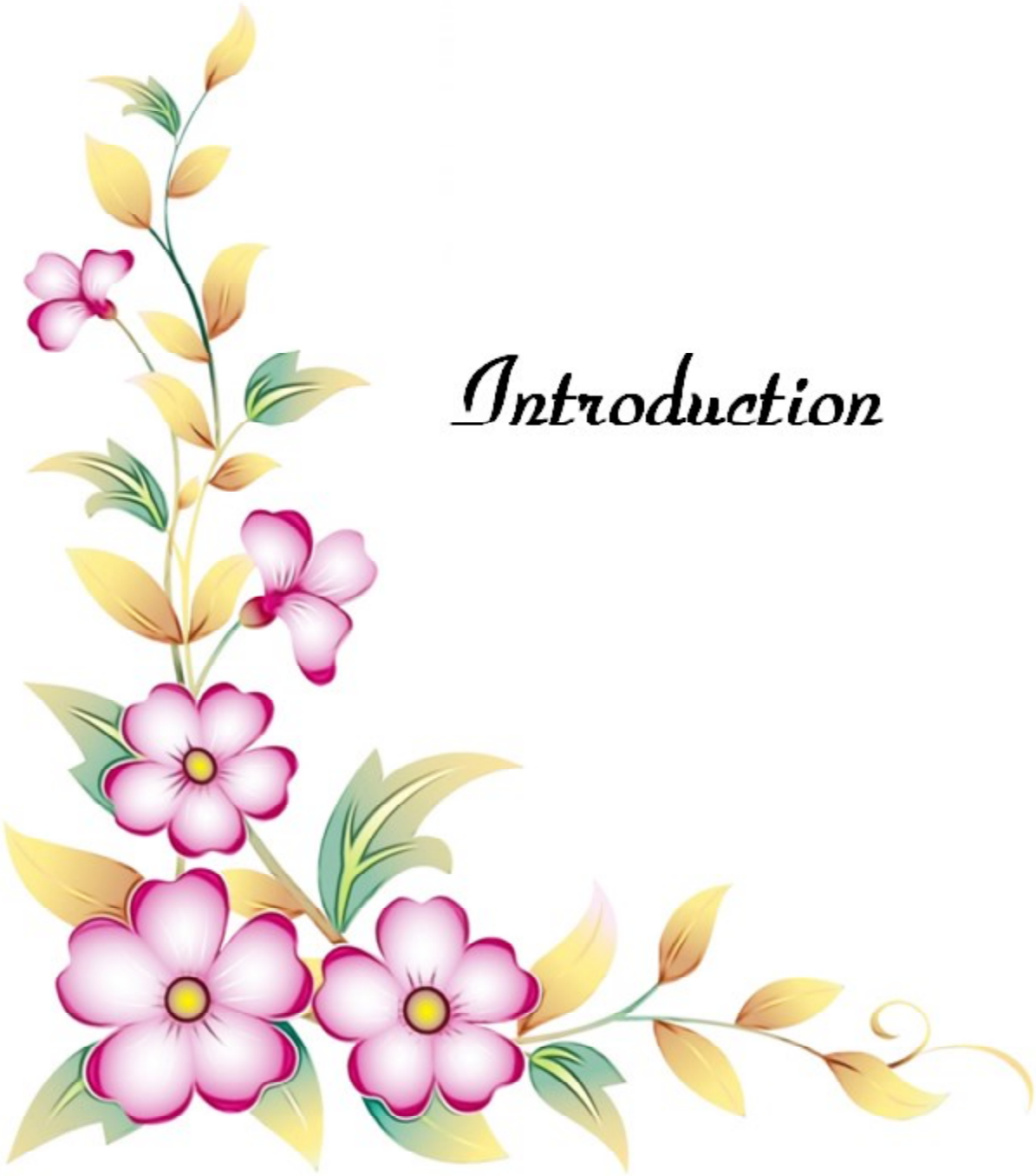
IgG	:	Immunoglobulin G
IgY	:	Immunoglobulin Y
IP	:	Indian Pharmacopoeia
IPM	:	Isopropyl myristate
IU	:	International unit
IVRI	:	Indian Veterinary Research Institute
Kb	:	Kilo base
kDa	:	Kilodalton
LD_{50}	:	Lethal dose 50
Lit	:	Litre
M	:	Mole
mA	:	Milliampere
mg	:	Milli gram
min (s)	:	Minute (s)
ml	:	Mililiter
mM	:	Millimole
NA	:	Neutralizing antibody
NCBI	:	National Centre for Biotechnology Information
ng	:	Nano gram
NIBSC	:	National Institute for Biological Standards and Control
nm	:	Nano meter
NTC	:	Non template control
$O.D_{450}$	:	Optical density at a wavelength of 450nm
^{o}C	:	Degree centigrade
OIE	:	World Organization for Animal Health
PBS	:	Phosphatebufferedsaline
PBS-T	:	Phosphate buffered saline with Tween 20
PCR	:	Polymerase chain reaction
QC	:	Quality control
qPCR	:	Quantitative PCR
q-RTPCR	:	Quantitative real-time PCR
RFFIT	:	Rapid fluorescent focus inhibition test
RNA	:	Ribonucleic acid
rpm	:	Rotation per minute
RVNA	:	Rabies virus neutralizing antibody
S/P ratio	:	Sample-positive ratio
Sec (s)	:	Second (s)
sELISA	:	Sandwich Enzyme Linked Immuno sorbent Assay

SMP	:	Skim Milk Powder
Taq	:	Thermus aquaticus
TMB	:	3,3',5,5'-Tetramethylbenzidine
U	:	Unit
UV	:	Ultra violet
V	:	Volt
viz	:	Videlicet
VP2	:	Viral protein 2
WHO	:	World Health Organization

LIST OF TABLES

LIST OF FIGURES

CONTENTS



Introduction

1 INTRODUCTION

Vaccination is an effective means for preventing and controlling of animal and poultry diseases. The success of vaccination program to control of any infectious disease is mainly depends on the release of a potent and safe vaccine for use in the target population by quality control (QC) laboratory as well as regulatory authorities. Animal and poultry vaccine are subjected to sterility, safety, and potency tests as per monographs mentioned in 9 CFR or European Pharmacopoeia (Ph. Eur.) or British Pharmacopoeia (BP) or Indian Pharmacopoeia (IP) or vaccine origin country's Pharmacopeia's. The potency test is one of the most important parameters to judge the quality of a vaccine. In India, potency assay of veterinary vaccines is conducted in animals and birds as per the standard of the Indian Pharmacopeia, 2018. Among the various animal vaccines, rabies and infectious bursal disease (IBD) vaccines plays an important role for control of these important diseases of livestock and poultry.

Potency testing of inactivated rabies vaccine is conducted by mouse challenge assay, commonly known as NIH test (Seligmann, 1973; WHO, 1984). This test evaluates the degree of protection of mice vaccinated with different dilutions (5-fold) of the test and standard rabies vaccine after being challenged with the rabies challenge virus standard (CVS). The number of mice (100-136), duration (28 days), intracerebral inoculation for challenge, and handling of rabies challenge virus standard are the major constrains of mouse challenge methods. It is well-known that NIH test being highly variable, leading to false results. For practical, economic, and ethical issues, there is an immediate need for replacement of mouse challenge assay (NIH) and its variants for batch release potency testing of rabies vaccine with some ideal *in vitro* alternatives.

Similarly, potency testing of inactivated IBD vaccine involves birds and is conducted by serological assays of vaccinated birds as per Indian pharmacopeia 2018. Requirement of 20 birds/batch of each vaccine (4 weeks of age), test duration of approximately 21-25 days and maintenance of birds for approximately 2 months in the animal shed has made test very cumbersome and time consuming. Therefore, limited number of IBD inactivated vaccine batches can be tested at time in the animal shed having limited bird rearing facilities.

Currently regulatory authorities and researcher are focusing towards the alternative tests to replace challenge method by serological methods (refinements) and completely replacement of animals by *in vitro* antigen quantification assays by sandwich ELISA or by genome copy number determination by real time PCR.

Various alternative serological methods for quantifying neutralizing antibodies from the serum of immunized laboratory and target animals have been developed for Rabies (Meslin *et al.,* 1996), NDV (Goddard *et al*., 1988; Horvath *et al*., 1999), FMD (Mackowiak *et al*., 1962; Pay and Hingley, 1992; Barnett *et al*., 2003), CPV (Larson *et al*., 1996), and PPR (Rossiter *et al*., 1985; Singh *et al*., 2004). Rapid fluorescent focus inhibition test (RFFIT) and fluorescent antibody virus neutralization test (FAVN) were found suitable alternative test for potency testing of rabies vaccine (Smith *et al.,* 1996; Cliquet *et al.,* 1998) as reference methods. As per the Ph. Eur., the RFFIT may be used for inactivated rabies veterinary vaccine after an appropriate correlation with the mouse vaccination-challenge test has been established. A study found a strong correlation between RFFIT and traditional *in vivo* immunization-challenge assay results (Kramer *et al.,* 2009). RFFIT & FAVN tests are time consuming, requiring expertise, costly and are carried out in reference laboratories because of requirement of live virus and, therefore, are not widely available. To overcome this limitation, ELISA-based approach will be used in the present study. Competitive ELISA has already been developed in the Division of Biological Standardization will be evaluated in the present study as an alternative to RFFIT for QC testing of rabies vaccine.

Antigen quantification could be a potential alternative for potency assays of inactivated veterinary vaccines. Antigen quantification using different methods such as sandwich ELISA,

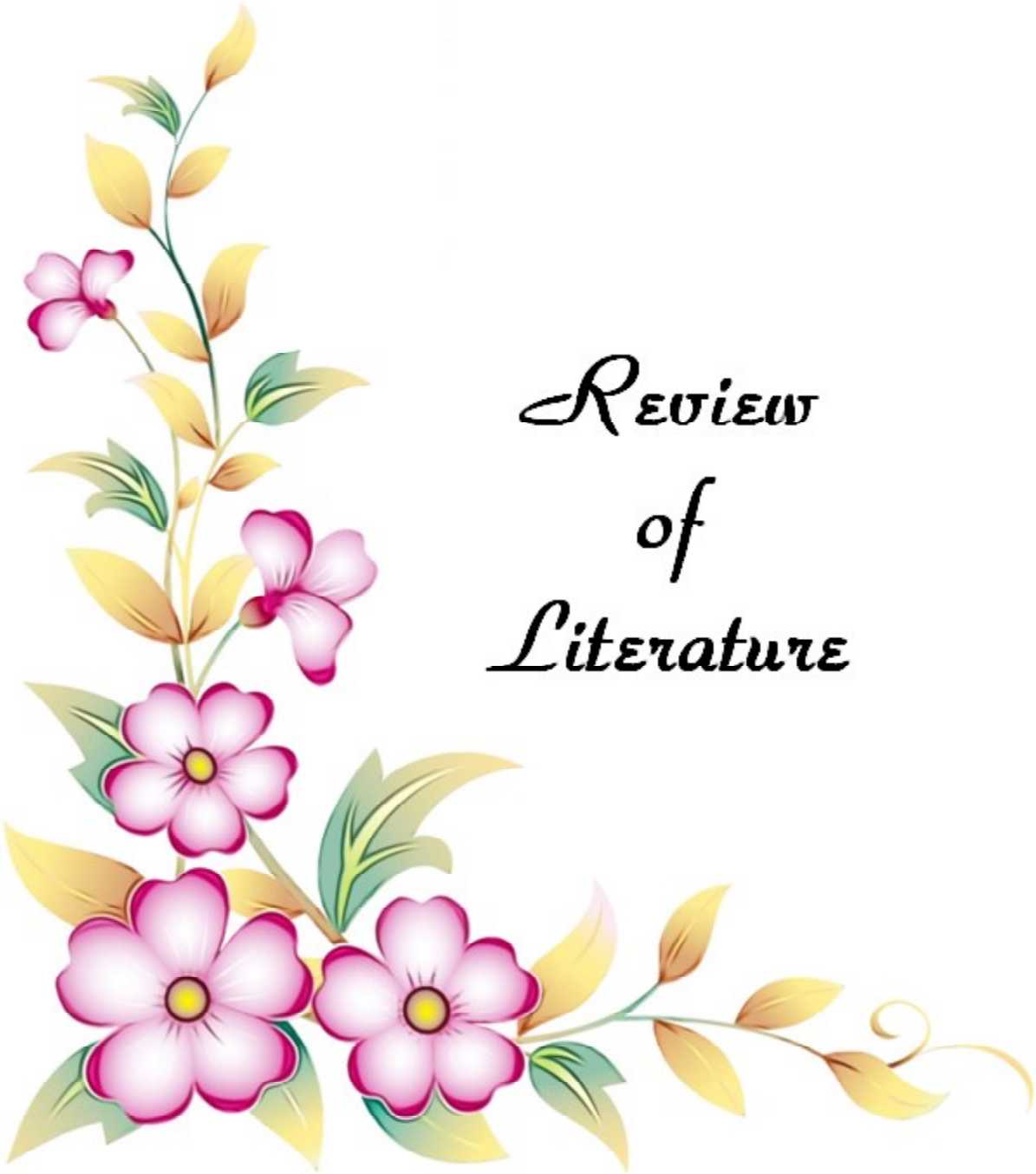
spectrophotometry, and surface plasmon resonance is used to determine *in vitro* potency for human and veterinary vaccines, including poultry vaccines. Single radial diffusion tests, antibody-binding tests, and ELISA methods are some of the antigen quantification tests currently being developed for killed rabies veterinary vaccines (Weisser *et al.,* 1997). Antigen quantification by sandwich ELISA is the most extensively used of these assays. ELISA methods are reproducible, economical and quantitative (Bruckner *et al.,* 2003). Animal-free potency testing of vaccines is an attractive feature of *in vitro* antigen quantification methods. The European Pharmacopeia has approved *in vitro* alternate potency testing methods for human vaccine such as inactivated polio vaccines, Hepatitis A and Hepatitis B. Similarly, antigen quantification based ELISAs have also been included in EP and 9 CFR for inactivated vaccines such as inactivated NDV (Ph. Eur. Monograph. 870), canine corona virus vaccine (USDASAM 322. 2007) and bovine respiratory disease vaccine (9CFR113.216 BRV; 9CFR113.115 BVD) etc. Two ELISAs for quantification of rabies and IBD antigen in inactivated vaccines have been developed in the Division of Biological Standardization, ICAR-IVRI. These ELISAs will be evaluated in the present study as an alternative *in vitro* assay for potency testing of these vaccines

Molecular method for the absolute quantification of nucleic acid present in vaccine virus which is expressed as copy number of nucleic acid per dose of vaccine can also be a potential alternative for potency testing of vaccine. Quantitative real-time PCR (q-RTPCR) and Droplet Digital PCR (ddPCR) offer the most accurate and sensitive molecular tool for copy number determination, and its use for *in vitro* alternate potency testing of vaccines is novel in our country. Potency estimation based on qPCR has been successfully implemented for Measles vaccine (Schalk *et al*., 2004), Rotavirus vaccine (Ranheim, 2006), Multivalent MMR vaccine (Schalk *et al*., 2005), Camelpox and Buffalopox vaccine (Prabhu *et al*., 2012), and Goatpox vaccine (Kallesh *et al*., 2009). Veach *et al*. (2015) reported the use of ddPCR for virus quantification during the preparation of influenza vaccine in cell culture.

Attempts are currently being made all over the world to follow the 4R concept such as reducing the number of animals per dose (reduction), humane endpoints (refinement), serology instead of immunization-challenge tests (combination of reduction and refinement), antigen

quantification models or molecular methods for absolute quantification of nucleic acid (replacement), and reuse of animals after experimentation (rehabilitation). In light of these considerations, the present study was designed to develop and evaluate alternative potency testing of Rabies and IBD vaccines with the following objectives.

1. **To develop quantitative PCR for determination of copy number of rabies and IBD virus genes in the inactivated vaccine.**
2. **To evaluate an ELISA based serological potency assay as an alternative test for inactivated rabies vaccine.**
3. **To evaluate sandwich ELISA based antigen quantification assay for potency testing of inactivated rabies and IBD vaccine.**
4. **To establish correlation of standard serological assay with copy number and antigen mass in inactivated rabies and IBD vaccine.**



Review of Literature

2 REVIEW OF LITERATURE

From the beginning of their establishment, some processes applied in biomedical research and vaccine testing have seldom changed. This was also applicable for the measurement of vaccine potency tests. Louis Pasteur was one of the first researchers to recognise the possibility of a vaccine to produce a protective immune response in the target animal or in a healthy susceptible animal species after immunisation and later challenged with virulent microorganisms in his experiments on the development of an anthrax and rabies vaccine. Immunization-challenge procedures in laboratory animals are commonly used to estimate the potency of vaccines. Potency testing is one of the most important aspects of assuring vaccine quality. The capacity to produce an effective degree of protection in the target species is known as potency. *In vivo* immunisation challenge for potency testing has been important for routine vaccine batch testing for a long time.

Animal and poultry vaccines require significant use of animals, including birds for quality control. It is recorded that over 8 to 9 million laboratory animals are continuously used globally for potency testing of the end product. For a variety of reasons, there is a growing interest in developing alternatives to the present *in vivo* immunization-challenge potency tests. Firstly, significant improvements in vaccine manufacturing process quality control, as well as compliance with good manufacturing practise (GMP) standards, have resulted in lower batch-to-batch fluctuations and a reduced risk of manufacturing unsafe or inefficient products. Secondly, the use of huge numbers of animals in experiments might cause suffering and pain, which is against the ethics of the experiment. Animal welfare is a key concern with *in vivo* challenge testing.

Currently, efforts are being taken globally to follow the 4R (Reduction, Replacement, Refinement, and Rehabilitation/Re-use) principles to establish and use *in vitro* alternate models to reduce or replace animal experiments in vaccine testing (Hendriksen. 2007; Jennings *et al.,* 2010; Woodlands R. 2011; Hans & Drayer, 2011; and Romberg *et al.,* 2012). Thirdly, the use of animal models is very expensive, laborious, time consuming (often taking six or more weeks), and there is also a risk for personnel when models involve exposure to live pathogenic organisms.

Requirements for veterinary vaccine potency testing

Veterinary vaccines must be safe, efficacious, pure, and potent. Because vaccines are so important for disease prevention, quality control (QC) testing of vaccines has become essential for successful immunization. The QC testing covers sterility, safety, and potency testing according to the Indian pharmacopoeia (IP, 2018). Of these, the potency test is the most crucial for quality control (QC) of the vaccine. Veterinary vaccines are important for both humans' and animals" health and well-being. Vaccines can provide a safe and efficient global food supply by controlling and preventing diseases in companion, poultry, and domestic animals. They help in the prevention of zoonotic and foodborne disease transmission from animals to humans.

Types of potency assays

Live (attenuated) and inactivated (killed) vaccines are mainly available for veterinary use. Subunit and genetically engineered vaccines are also present for animal uses. Vaccine manufacturers use the following types of potency tests:

- Immunization-challenge methods using either host animals (poultry) or laboratory animals (mice)
- Serological assay (*in vivo* to *in vitro*)
- *In vitro* assays like ELISA or other quantitative methods (qPCR, ddPCR)
- Titration of live organisms (*in vitro*)

For a typical U.S. veterinary vaccine manufacturer, 37% of tests use *in vitro* titration assays in cell culture using plaque formation or cytopathology, 22% use *in vitro* ELISAs, 12%

use some other *in vitro* method like qPCR or ddPCR, 8% use *in vivo* serological assays, and 21% use *in vivo* immunization–challenge methods.

The International Scientific Workshop on Potency Testing of Veterinary Vaccines for Animals held in Germany (December 2010) emphasized the need to move from *in vivo* to *in vitro* tests. The workshop prioritises the development of *in vitro* alternative replacement methods for vaccines that use a large number of either laboratory or target animals, cause pain and distress to the animals, and involve zoonotic microorganisms. Based on these criteria, the following vaccines were given high priority like rabies vaccine, Leptospira spp. vaccine, clostridium spp. vaccine, FMD and BT vaccine, and certain poultry vaccines such as NDV, IBDV, and IBV.

Potency testing of inactivated rabies vaccine

Potency testing of inactivated rabies vaccine is conducted by mouse challenge assay, commonly known as NIH test (Seligmann, 1973; WHO, 1984). This test evaluates the degree of protection of mice vaccinated with 5 different dilutions (5-fold) of the test and standard rabies vaccine after being challenged with the rabies challenge virus standard (CVS). This test utilizes 16 mice/dilution, with two vaccinations 7 days apart as recommended by the WHO expert committee on rabies (WHO, 1984). Alternatively, single immunisation with 10 mice per dilution is recommended by the Office International des Epizooties (OIE), Indian pharmacopoeia (IP), and European pharmacopoeia (EP). The effectiveness of the test is determined by comparing the number of animals protected from rabies in groups that received the test vaccine and the reference vaccine. The test is commonly referred to as the NIH test because it was created by the National Institutes of Health (USA; Wilbur *et al.,* 1996). The potency of the sample rabies vaccine was generally calculated in IU/ml in comparison with the reference vaccine. The NIH potency test (also referred to as the "mouse challenge assay") requires a huge number of mice and causes great suffering to them (Seligmann, 1973). The tests take a long time and put the laboratory staff in danger of infection (Rooijakkers *et al.,* 1996). It also produces frequent invalid results and high variability (Bruckner *et al*., 1986, Barth *et al*., 1988, Perrin *et al*., 1990, Wilber *et al*., 1997). A new reliable test that uses fewer animals, causes less suffering, and produces more consistent results is urgently needed.

Potency test for inactivated IBD vaccine

Serological assays are used for potency testing of inactivated IBD vaccine (IP, 2018). In these assays, IBD specific antibodies are detected in 10 SPF or healthy 3- to 4-week-old susceptible chickens vaccinated with one dose of vaccine by intramuscular route. Blood from the vaccinated chicken is collected on 21 days post vaccination. By AGPT, the mean antibody titre of sera in the vaccinated group must be 1:8. Several commercial ELISA kits are also available to detect IBD specific antibodies in vaccinated chickens.

The 4 R's Approaches

The 4 R's approaches refer to reduction, refinement, replacement, and rehabilitation (Giridharan *et al.,* 2000). In human vaccine testing, the 4R principles are widely utilised and recommended (Isbrucker *et al.,* 2011).

A. Reduction

These are the methods that allow researchers to collect comparable levels of data from fewer animals by reducing target or laboratory animal usage, frequency of testing, and changing the test design to minimize the number of animals per group, as well as using single-dilution tests instead of multi-dilution tests.

B. Refinement

It's about improving processes to reduce pain, suffering, and discomfort while also allowing for a general improvement in animal welfare by use of anaesthesia/ analgesia for pain relief, improvement in housing systems of animals and replacement of challenge method by serological assays

Replacement of challenge test by serological assays

Instead of measuring protection against virulent microorganisms *in vivo,* the serological response can be measured *ex vivo in vitro*, leading to a significant reduction in both number of animals needed and the level of suffering. Time and money are saved by using a serological vaccination potency assay (Elisabeth Kamphuis, PEI, Germany). Animals are still used in serological assays, although the suffering and animal numbers have significantly reduced.

In terms of safety, animal welfare, efficiency, and test monitoring, serology has a lot of advantages. Firstly, it is no longer necessary to work with virulent pathogens. Secondly, because the animals and birds are no longer challenged with virulent pathogens, their levels of suffering and distress have reduced from severe to minimal. Thirdly, by skipping the observation period and doing a quantitative end point like antibody titre instead of using a qualitative end point like death or clinical signs, the test-performance time can be reduced. Finally, unlike challenge procedures, serology permits the storage of test serum samples. On the basis of this, serology is regarded as an alternative to using the challenge procedure.

As an alternative to the mouse challenge (NIH), according to the European pharmacopeia, a serological assay can be implemented for routine batch potency assay of veterinary rabies vaccines (Monograph 0451. Ph Eur. 2008). The primary goal of detecting and quantifying rabies antibodies is to check for rabies immunity or vaccine effectiveness of rabies vaccine. The serological assay is performed at 14 days after a single immunization of at least 5 mice each with one-fifth of the test vaccine's indicated dose. Blood samples are taken on day 14 after injection, and each serum is analysed for rabies virus neutralising antibodies (RVNA) using a rapid fluorescence focus inhibition test (RFFIT) (Zalan *et al*., 1979; Monograph 0723. Ph Eur. 2008). The RFFIT is one of the best current reference methods recommended by the WHO and OIE (World Organisation for Animal Health), as well as proposed by the EP. The RVNA titre generated by a vaccination meets the potency requirement if it is equal to or greater than the titre produced by a vaccine with proven potency. RFFIT requires a high level of expertise and is typically performed in reference laboratories, making them inaccessible for potency testing.

C. Replacement

It means implementing those methods that completely replace the use of laboratory animals. Models for antigen quantification and molecular methods for absolute nucleic acid quantification in terms of copy number per vaccine dose are the best examples of replacement.

Antigen quantification assays

Replacement of animal model potency tests with more quick and reliable *in vitro* alternate methods for potency assessment is highly desirable for practical, economic, and ethical reasons (Rooijakkers *et al.,* 1996). The consistency approach was developed as a strategy for enabling the transition from *in vivo* immunization-challenge to *in vitro* alternate batch vaccine testing. *In vitro* potency assays have several advantages over *in vivo* potency testing. *In vitro* assays normally take hours to days to complete, as compared to weeks to months for animal studies conducted by *in vivo* testing. They actually take minimal personnel time, generally requiring one operator, and they can be automated. This system also involves fewer costs as well as a reduction in the use of laboratory animals and birds, which are the most attractive features of alternate *in vitro* assays.

However, *in vitro* assays have several disadvantages also. The most significant disadvantage is the indirect relationship between the potency test result and the efficacy of the host animal. This correlation is determined by the antigen being quantified, the reliability of the host animal reference certification trial, and the similarity of the vaccine to the reference. Antigen processing by an immune system is not there, and the adjuvant action is usually not included in the test design and is not assessed. As a result, the sensitivity of the *in vitro* assay may be lower than that of the animal test. Adjuvanted vaccines add some difficulty to developing *in vitro* potency and safety assessments, although adsorption and extraction methods as well as the development of adjuvant-compatible procedures provide solutions to these.

Considering the fact that there has been increased in the number of inactivated veterinary vaccines produced, vaccine potency is of prime importance. However, most of the vaccines contain adjuvants like mineral oil (poultry vaccines like ND, IB, and IBD) and aluminium salts (Rabies), which can complicate the *in vitro* quantification methods. As a result, this adjuvant would have to be extracted from the antigen components of the vaccine before doing *in vitro* potency testing. Isopropyl myristate (IPM) has been used in several studies to extract antigen from mineral oil adjuvant vaccines without compromising antigenicity (Maas *et al.,* 2000; Claassen *et al.,* 2004; and Hendriksen 2007). However, such initiatives are not carried out in

our country. Because the adjuvant is a crucial element for generating an adequate protective response, additional *in vitro* testing may be required to confirm the quality of inactivated vaccines. Nevertheless, the impact of an adjuvant on the immunogenicity of the protective antigen will need to be examined when antigen quantification methods are developed (Claassen I., 2011). So one-to-one replacement with *in vitro* alternatives is not feasible, and a combination of approaches may be needed. There are several antigen quantified assays have been adopted by European and 9 CFR for potency testing of vaccines (Table 1 and 2)

Table 1: List of human vaccine potency assays which replace the use of animals

Vaccine (Disease)	4Rs Alternative	Traditional Test Procedure	Regulatory References
Hepatitis A vaccine (Hep A virus)	Antigen quantification by ELISA	Mice serology	Ph. Eur. Monograph (1935):20701 (2008)WHO TRS 858, (1995)
Hepatitis B Vaccine (Hep B virus)	Antigen quantification by ELISA	Mice serology	Ph. Eur. Monograph 1056 (2008)WHO TRS 889, (1999)
Inactivated polio vaccine (poliovirus)	Antigen quantification by ELISA	Rat serology	Ph. Eur. Monograph 214 (2008)WHO TRS 910, (2002)

Table 2: List of veterinary vaccine (inactivated) potency assays which replace the use of animals

Vaccine (Disease)	4Rs Alternative	Traditional Test Procedure	Regulatory References
Bovine respiratory viruses (BVD, BRV, PI3, BRSV) (Cattle respiratory disease)	Antigen quantification –*in vitro* ELISA	Immunization challenge test in bovines	USDA SAM 120 (1991)9CFR 113.216 (BRV)9CFR 113.115 (BVD)
Newcastle disease virus (Chicken respiratory disease)	Antigen quantification –*in vitro* ELISA or serology	Immunization challenge test in chickens; Serology	Ph. Eur. Monograph: 870Hendriksen 2007 Claassen *et al*., 2004

Nucleic acid quantification

The application of droplet digital PCR (ddPCR) for accurate, sensitive, and quick quality control testing of vaccines has been reported in a few recent studies. A study conducted

by Li *et al.,* 2019 suggested that ddPCR has 100 times more sensitivity than that of conventional PCR and 10 times that of real-time PCR. It has been reported that ddPCR was used to quantify viruses during the preparation of influenza vaccine in cell culture and subsequent downstream purifying steps (Veach *et al.,* 2015). Several studies focused on quantitative real-time PCR-based viral genome quantification for live vaccines such as Measles vaccine (Schalk *et al*., 2004), Rotavirus vaccine (Ranheim, 2006), Multivalent MMR vaccine (Schalk *et al*., 2005), Camelpox and Buffalopox vaccine (Prabhu *et al*., 2012), and Goatpox vaccine (Kallesh *et al*., 2009) but no attempts at absolute nucleic acid measurement in the extracted antigen of inactivated vaccines have been documented.

D. Rehabilitation

In India, rehabilitation is known as the 4th R, and the CPCSEA adopted it as an official policy in 2004.Rehabilitation and/or reuse of laboratory or target animals after completion of an experiment is important. The CPCSEA Amendment of 2006 mandates that the person conducting animal experiments and the institution where the experiments are performed are responsible for the aftercare and rehabilitation of the animals after the experiments are completed, and that they must not euthanize the animals unless the law specifies otherwise.

The aim of the present study to evaluate competitive ELISA as an alternative to RFFIT for potency testing of rabies vaccines and to evaluate sandwich ELISA for quantification of rabies and IBD antigen in the inactivated vaccine. Attempts was made to develop digital PCR based quantification of copy number in rabies and IBD immunogenic gene in each dose of vaccine and to correlate the copy number with gold standard methods mentioned in Indian Pharmacopoeia.

Materials and Methods

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Buffers, Chemicals, Molecular Reagents, and Media

All of the chemicals employed in the experiment were purchased from Sigma (USA), Difco (USA), Qiagen (USA), Amresco (USA), Thermo Scientific (USA), MP Biomedicals Pvt. Ltd. (India), Himedia (India), and SRL (India) etc. The main chemicals used in the present study are mentioned below.

- RiboZol RNA Extraction Reagent – Amresco, USA
- Chloroform – Amresco, USA
- Isopropyl alcohol – Amresco, USA
- Ethanol – Himedia, India
- Acetone – SRL, India
- Isopropyl myristate – Sigma-Aldrich, USA
- TMB Peroxidase ELISA Substrate – Bio-Rad, USA
- Eagle Minimum Essential Medium (EMEM) – Himedia, India
- Fetal Bovine Serum (FBS) – Himedia, India
- Trypsin-EDTA Solution – Himedia, India
- Antibiotic Antimycotic Solution 100X – Himedia, India
- 10X PBS – Himedia, India
- Horse Serum – Gibco, Invitrogen, USA
- Sodium bicarbonate Solution 7.5% - Himedia, India
- Anti-Chicken IgY (IgG) HRPO conjugate – Sigma, USA

- Anti-guinea pig HRPO conjugate – Sigma, USA
- Rabies DFA II Reagent – Sigma- Aldrich, USA
- PCR master mix – Takara, Japan
- NEX-GEN DNA Ladder – Genetix Biotech Asia Pvt. Ltd, India
- GeneRuler Low Range DNA Ladder – Thermo Fisher Scientific
- Molecular Biology Grade Water - Himedia, India
- Trypan blue 0.4% solution - Himedia, India
- QX200 ddPCR EvaGreen Supermix – Bio-Rad, USA
- Droplet Generation Oil for EvaGreen – Bio-Rad

The information related to the different buffers and media is provided in the appendix.

3.1.2 Commercial kits

The kits used in the experiment were procured from Thermo Fisher Scientific, USA (High Capacity cDNA Reverse Transcription Kit #4368814) and IDEXX Laboratories, USA (Infectious Bursal Disease Virus Antibody Test Kit IDEXX IBD-XR #99-09261).

3.1.3 CVS challenge virus and cell line

In the Division of Biological Standardization's Virology Lab II, fixed laboratory CVS-11 strain (challenge virus standard-11) of the rabies virus adapted into BHK-21 was used as the challenge virus. The Baby Hamster Kidney (BHK-21, clone-13) cells used in the present research were maintained at the Virology Lab II, Division of Biological Standardization, IVRI. Passage level ranged from 28 to 40. Using BHK cells, the CVS-11 seed virus was replicated to produce stock virus, which was then kept at -80°C for further use.

3.1.4 Primer

The various oligonucleotide primers were specifically synthesised by Eurofins Genomics Pvt. Ltd., Bengaluru, India, to amplify the glycoprotein and VP2 gene fragments of rabies and infectious bursal disease virus, respectively were used in the present study.

3.1.5 Glassware and plastic ware

The glassware's utilised in present work were purchased from Schott Duran (Germany) and Borosil India Pvt. Ltd. (New Delhi, India). All of the plastic items used in work were purchased from Tarson (India), Corning (USA), Millipore (USA), and Thermo Fisher Scientific (USA). They were issued with a certificate claiming that they lacked DNase and RNase activity. Tarson (India) and Genaxy (India) provided thin-walled PCR tubes, micropipette tips, and microfuge tubes.

3.1.6 Experimental animals

Mice: Healthy Swiss albino mice aged 3 weeks and weighing 10 to 15 g were procured from the Division of Laboratory Animal Research, ICAR-IVRI, Izatnagar. These mice were used to test the potency of the rabies vaccine after approval of Institutional Animal Ethics Committee guidelines.

Chicks: Healthy SPF chicks of one day old were obtained from Central Avian Research Institute (ICAR-CARI) used for testing of IBD vaccine after approval of Institutional Animal Ethics Committee guidelines.

3.1.7 Reference vaccine (Standard Vaccines)

In the present work, the internal reference standard developed by the Division of Biological Standardization was utilised. This standard was validated using the 6th international rabies vaccine standard, which was obtained from NIBSC, UK. The antigenic mass per vial was 5.8 IU. The non-adjuvanted, freeze-dried standards were kept at -80°C and reconstituted with 5.8 mL of sterile distilled water or PBS, the final concentration was fixed at 1 IU/mL.

3.1.8 Commercially available vaccines

Rabies vaccines: The five batches of commercially accessible aluminium hydroxide/ phosphate adsorbed inactivated rabies vaccines (A-E) for veterinary use from five different manufacturers were used in the present study. These vaccines were received in the Division of Biological Standardization, ICAR-IVRI for testing/certification of COA and stored in cold room facility of Division of Biological products, ICAR-IVRI. These vaccines were coded as

A, B, C, D, and E. These inactivated rabies vaccines were licensed in India for use in animals.

IBD vaccines: The five batches of commercially available oil adjuvanted IBD vaccines from two different manufacturers received for testing/certification of COA were used in the present study. These vaccines were coded as I, II, III, IV and V. These five inactivated IBD vaccines were licensed in India for use in birds.

3.1.9 Serum samples

After immunizing mice and chicks with commercially available inactivated rabies vaccine along with standard vaccine and IBD vaccines, respectively neutralising antibodies were produced, and these were subsequently conformed by serological assays.

3.1.10 Reference serum

In the present work, the in-house developed reference serum (2 IU/mL) was used, which was calibrated against the 2nd international WHO standard for anti-rabies immunoglobulin (NIBSC, UK).

3.1.11 Equipments

The equipments used in this study are given as under-

- Water bath (Narang Scientific, India)
- Electronic weighing balance (Wensar, China)
- Hot air oven (Narang Scientific, India)
- Autoclave (Genist, India)
- Vertical Laminar flow system (Klenzaids, India)
- Shaker Incubator (Orbitek, Scigenics Biotech, India)
- Centrifuge (HermleZ326K, Germany)
- Dry bath (Helix Biosciences, India)
- Deep freezer (-20°C) (Vestfrost, India)
- Ultra-low freezer (-70 °C) (Krispcold, India)

- Ultracentrifuge (Sorvall UltraPro 80,USA)
- Power pack (BioRad, USA, Bangalore Genei, India and Tarson, India)
- Gel Doc system (Azure Biosystems, Dublin)
- pH meter (edgepH, Hanna, India)
- SpectrostarNano (BMG Labtech, Germany)
- Multimode reader (Synergy HTX, Gen5v3.05, USA)
- QX200™ Droplet Digital PCR (ddPCR) system (Droplet Reader) and QuantaSoft™ Software (Bio-Rad Laboratories, USA)
- QX200™ Droplet Generator (Bio-Rad Laboratories, USA)
- DG8™ cartridge (Bio-Rad).
- PX1 PCR Plate Sealer and corresponding Plate support Block (Bio-Rad)
- C1000 Touch™ Thermal Cycler (Bio-Rad)
- Pierceable Foil Heat Seal (Bio-Rad)
- ddPCR 96-well plate (Bio-Rad)
- Vortex mixer (Spinix, India)
- Ice maker machine (Blue Star, India)
- Inverted binocular microscope-CK30 (Olympus, India)
- Fluorescent microscope (Nikon, Japan)
- Gradient PCR (Sure Cycler 8800, Aligent, USA)
- Micropipettes (0.5-1000µl.) (Eppendorf, Germany and Thermo Scientific, USA)
- Horizontal electrophoresis apparatus (Bangalore Genei, India)
- Haemocytometer (Shah and Company, India)
- Freezer (-80°C) (Panasonic)
- Magnetic stirrer (Baird and Tatlok, England)
- Refrigerators (Samsung, India) etc. were used in the present study.

3.2 Methods

3.2.1 Development of quantitative PCR for determination of copy number of rabies and IBD virus genes in the inactivated vaccine

3.2.1.1 Standardization of reverse transcriptase–PCR (RT-PCR) for amplification of glycoprotein gene of rabies and VP2 gene of IBDV

A: Designing of oligonucleotide primers

The oligonucleotide primers for PCR amplification of the glycoprotein gene of rabies virus and VP2 gene fragments of IBD virus were designed using the glycoprotein and VP2 gene sequences available in NCBI **(www.ncbi.nlm.nih.gov)** using Gene Tool software.

Table 3: Details of oligonucleotide primers used for amplification of glycoprotein and VP2 gene fragments

Primer combination	Sequence 5' to 3'	Length	Annealing Temp. (°C)	Amplicon size (bp)
rabg240_F	GYACAGGTGTBGTGACAGAG	20	55	116
rabg356_R	AGTTRTAYGCGGCTCTACAT	20		
IBDFVP2-472	CAGGGGATCAGATGTCATGGTC	22	58.8	125
IBDRVP2-597	CGACGGATCCTGTTGCCACTC	21		

B: Gradient PCR for optimization of annealing temperature of glycoprotein and VP2 gene fragments

Gradient PCR at different annealing temperatures (50-62°C) was conducted using rabies and IBD virus cDNA available in the Virology Lab-II, Division of Biological Standardization, ICAR-IVRI to optimize annealing temperature of primers as mentioned in the table 3. Total nine reaction mixture for eight different temperatures gradient (50-62 °C) for each gene was prepared. To exclude the possibility of contamination a non-template control (NTC) was also included. The quantity of all components in a single reaction is mentioned in the table 4 and amplifications conditions in table 5. Amplified products were analysed by 2.0% agarose gel electrophoresis.

Table 4: PCR reaction mix for the amplification of glycoprotein and VP2 gene fragments

S. No.	Reaction components	Quantity	
		GP 116	VP2 125
1	2X Sapphire Master Mix	12.5 µL	12.5 µL
2	Forward primer (10 pM/µL)	0.5 µL (rabg240_F)	0.5 µL (IBDFVP2-472)
3	Reverse primer (10 pM/µL)	0.5 µL (rabg356_R)	0.5 µL (IBDRVP2-597)
4	Nuclease Free Water	9.5 µL	9.5 µL
5	Template DNA (cDNA)	2 µL	2 µL
	Total Volume	25 µL	25 µL

Table 5: PCR reaction conditions for the amplification of glycoprotein and VP2 gene fragments

S. No.	Reaction parameter	Reaction condition	
		Temp (°C)	Time (min)
1	Hot start	95	5
2	Denaturation	95	0.30
3	Annealing	50-62	0.30
4	Extension	72	0.45
5	Repeat Step 2 to 4 (35 cycles)		
6	Final extension	72	10
7	Hold	4	Infinite

3.2.1.2 Optimization of ddPCR for amplification of glycoprotein and VP2 gene

To find out the optimum annealing temperature, a gradient from 50 to 60°C was performed to optimize the separation between positive and negative partitions of the immunogenic glycoprotein gene of rabies virus and the VP2 gene of the IBD virus. The details procedure of digital PCR is mentioned in the section 3.2.1.3v. In a ddPCR assay, the amount of cDNA template is crucial for calculating absolute nucleic acid concentration (copy number). cDNA input was tested between 80 and 2.5 ng in order to determine the appropriate cDNA template amount.

3.2.1.3 Determination of copy number of glycoprotein gene in the inactivated rabies and VP2 gene in the inactivated IBDV vaccine

All of the selected commercial rabies and IBD vaccines were in a liquid presentation with antigen adsorbed onto the adjuvant, so an extraction procedure was done before quantifying the absolute copy number in the selected inactivated vaccines.

3.2.1.3i Antigen extraction from inactivated vaccine

To increase immunogenicity, inactivated rabies and IBD vaccines are typically prepared with aluminium hydroxide/phosphate and mineral oil adjuvants, respectively. The *in vitro* assay should be capable of quantifying the amount or mass of antigen in the vaccine after it has been extracted from the aluminium adjuvant or oil adjuvant using chemical methods. For inactivated rabies vaccine (A-E), antigen was extracted from the 5 different doses of the vaccine (2 dose, 1 dose, $1/5^{th}$, $1/25^{th}$, and $1/125^{th}$), and for inactivated IBD vaccine (I-V), antigen was extracted from the 5 different doses of the vaccine (2 dose, 1 dose, $1/2^{th,}$ $1/4^{th}$, and $1/8^{th}$) by the method mentioned in the table 6 and 7.

Table 6: Procedure for rabies vaccine antigen extraction

S.No.	Steps
1	Rabies vaccines were mixed with an extraction buffer (0.60 M sodium citrate dihydrate; 30 mM SDS; 100 mM EDTA) in 1:2 ratios.
2	To ensure adequate mixing, the mixture was incubated at 60°C for 120 minutes on a rotor or shaker.
3	After incubation, the mixture was centrifuged at 3000 rpm for 10 minutes for precipitating the Al-EDTA complex.
4	The supernatant was taken and concentrated by 10 kDa centrifuged tube at 6000 rpm for 15 minutes.
5	Concentrated part was collected and used directly (RNA isolation and antigen quantification assay) or stored at 4°C until use.

Table 7: Procedure for IBD vaccine antigen extraction

S.No.	Steps
1	To create a homogeneous emulsion, the vaccines were vigorously shaken for 10 seconds.
2	Two mL of vaccine were added to 6 mL IPM in a 15 mL tube. (1:3)
3	In a mini shaker, the mixture was agitated vigorously for 2 minutes at 2500/min.
4	The tubes were centrifuged at 2000 rpm for 10 minutes at 4°C.
5	The upper phase, which contained IPM and oil, was removed.
6	The lower phase was the aqueous-phase, which contained IBD antigen. The colour and clarity of this la can vary between different vaccine manufacturing firms.
7	Aqueous-phase was collected and used directly (preferably for RNA isolation or antigen quantification assay) or stored at 4°C until use.

3.2.1.3ii. RNA isolation from extracted antigen of rabies and IBD vaccine

Before RNA isolation, extracted antigen volume from different doses was made up to 250 µL by nuclease free distilled water. RNA isolation was conducted as per the procedure mentioned in the table 8.

Table 8: Procedure of RNA isolation

S.No.	Steps
1	750 µL of RiboZol was added to 250 µL of extracted antigen and mixed well by inverting the tube. The mixture was incubated at room temperature for 10 minutes.
2	200 µL of chloroform was added to the above mixture and mixed vigorously. The mixture was kept at room temperature for 10 minutes and centrifuged at 12000 rpm for 15 minutes at 4ºC for phase separation.
3	The RNA-containing upper aqueous phase was transferred to a fresh 1.5 mL microcentrifuge tube.
4	An equal volume of isopropanol was added to the aqueous phase. The content inside the tube was mixed by inversion and incubated at -20°C for 1 h to precipitate the RNA.

5	The mixture was centrifuged at 12000 rpm for 15 minutes at 4ºC and the supernatant was discarded.
6	The RNA pellet was washed twice with 500 µL of 70% ethanol, gently mixed, and centrifuged for 10 minutes at 4°C at 12000 rpm.
7	After drying for 10 minutes, the RNA pellet was dissolved in 20 µL of pre-warmed NFW (37°C) and stored at -80°C until further usage.
8	The purity of the RNA was tested by measuring the optical density (OD) at 260/ 280 nm using a NanoDrop (SpectrostarNano, BMG Labtech, Germany)

3.2.1.3iii. Synthesis of cDNA from RABV ssRNA

The total RNAs were reverse transcribed to cDNAs using High-Capacity cDNA Reverse Transcription Kits. (Cat. No. 4368814, Thermo Fisher Scientific, USA). The reaction mixture procedure is mentioned in the table 9.

Table 9: Reaction mixture for the cDNA synthesis of RABV ssRNA

S. No.	Reaction components	Quantity
1	10X RT Buffer	2.0 µL
2	25X dNTP Mix (100 mM)	0.8 µL
3	10X RT Random Primers	2.0 µL
4	MultiScribe Reverse Transcriptase	1.0 µL
5	RNase Inhibitor	1.0 µL
6	Nuclease-free water	3.2 µL
	Total per reaction	10.0 µL

The components were mixed gently and centrifuged briefly. An equal quantity of RNA sample (10.0 µL) was added into the tube, pipetting twice up and down to mix. The tube was briefly centrifuged to spin down its contents and remove any air bubbles. The thermal cycler was programmed using the conditions listed below.

Settings	Step 1	Step 2	Step 3	Step 4
Temp.	25°C	37°C	85°C	4°C
Time	10 minutes	120 minutes	5 minutes	infinite

3.2.1.3iv. Synthesis of cDNA from IBD dsRNA

This was performed in two steps. In first step, the total RNA-random primer mixtures was incubated in a thermocycler at 65°C for 5 minutes and immediately snap chilled on ice bed for 5 minutes.

Reagents	Volume (µL)
Total dsRNA	10
10X RT Random Primers	2
Total	12

Upon completion of the first step of primer annealing, the RT reaction was carried out by adding the following reagents to the primer annealed RNA.

Reagents	Volume (µL)
10X RT Buffer	2.0
25X dNTP Mix (100 mM)	0.8
MultiScribe Reverse Transcriptase	1.0
RNase Inhibitor	1.0
Nuclease-free water	3.2
Total	20

The above 20 µL reaction was then incubated at 25ºC for 10 minutes followed by 42ºC for 60 minutes and finally at 70ºC for 5 minutes.

3.2.1.3v Determination of copy number of glycoprotein and VP2 gene in the cDNA generated from different doses of rabies and IBD inactivated vaccine by ddPCR

After conformation of band in agarose gel electrophoresis, ddPCR was carried out to know the absolute quantity of nucleic acid (DNA) present in sample. Absolute quantification of DNA was carried out in different doses of commercially available rabies and IBD vaccine with the help of QX200 Droplet Digital PCR (ddPCR) system (Bio-Rad Laboratories, USA). Based on the results, a correlation was established between different doses and copy number of the immunogenic gene of the rabies and IBD viruses. The steps followed are briefly outlined below.

3.2.1.3va Preparation of ddPCR master mix

For each sample, a reaction was prepared using QX200 ddPCR EvaGreen Supermix (Cat. #1864034), primer (forward and reverse), template cDNA, and nuclease free water (NFW) to make total volume of 20 µL.

Table 10: ddPCR master mix for absolute quantification of glycoprotein and VP2 gene

S. No.	Reaction components	Quantity	
		GP 116	VP2 125
1	QX200 ddPCR EvaGreen Supermix	10 µL	10 µL
2	Forward primer (10pM/µL)	0.4 µL (rabg240_F)	0.4 µL (IBDFVP2-472)
3	Reverse primer (10pM/µL)	0.4 µL (rabg356_R)	0.4 µL (IBDRVP2-597)
4	Nuclease Free Water	7.2 or 4.2 µL	7.2 or 4.2 µL
5	Template DNA (cDNA)	2 µL or 5 µL	2 µl or 5 µL
	Total Volume	20 µL	20 µL

3.2.1.3vb: Droplet Generation

Following that, each assembled ddPCR reaction master mix was loaded into a sample well of an eight-channel disposable droplet generator DG8 cartridge (Bio-Rad). For each channel, a volume of 70 µL of droplet generation oil #1864006 (Bio-Rad) was loaded into the

oil well. The sample was split into thousands of individually isolated droplets, each having little or no target sequence (glycoprotein and VP2). After the cartridge was taken out of the droplet generator, the droplets that had collected in the droplet well were manually transferred using a multichannel pipet to a fresh 96-well PCR plate. The plate was heat-sealed with a foil seal by the help of PX1 PCR Plate Sealer (Bio-Rad) at 180°C for 5 seconds.

3.2.1.3vc: PCR Amplification

The heat-sealed plate was placed on a C1000 Touch Thermal Cycler (Bio-Rad) for amplification of glycoprotein and VP2 gene of rabies and IBD virus respectively with the following thermal cyclic conditions:

Table 11: ddPCR thermal cycling conditions for amplification of glycoprotein and VP2 gene

Cycling Step	Temp (°C)	Time (min)	Number of Cycles
Enzyme activation	95	5	1
Denaturation	94	0.30	40
Annealing/extension	55 (GP), 58.8 (VP2)	1	40
Enzyme deactivation	90	5	1
Hold (optional)	4	Infinite	1

Each droplet had its own PCR reaction, and droplets containing the target sequence were amplified.

3.2.1.3vd Droplet Reading and Data Analysis

After the PCR amplification cycle was completed, the 96-well PCR plate was loaded onto the QX200 Droplet Reader (Bio-Rad), which automatically reads the droplets from each well of the plate. Analysis of the ddPCR data was performed with QuantaSoft analysis software (Bio-Rad) that accompanied the droplet reader. The droplets were fractionized into positive (contain target sequence) and negative (lack target sequence) fraction. Thresholds were placed manually, and the fractions of positive and negative droplets were used to calculate the concentration and copy number quantification of target DNA sequence.

3.2.1.4 ddPCR quantification of titrated rabies and IBD virus

RNA was extracted from $10^{5.6}$ to $10^{1.6}$ FFD_{50}/mL of the CVS-11 strain of rabies virus and from $10^{5.6}$ to $10^{1.6}$ $TCID_{50}$/mL of the D78 strain of IBD virus by using the TRIzol reagent except 100 µL of samples was used instead of 250 µL as mentioned in the table 8. cDNA was synthesized from the total RNA by using High-Capacity cDNA Reverse Transcription Kits (Table 9). DNA was quantified at 260 nm/280 nm ratios using a NanoDrop (SpectrostarNano, BMG Labtech, Germany). Droplet digital PCR was performed according to the manufacturer's instructions by using a QX200 droplet generator and a QX200 droplet reader (Bio-Rad). Based on the results, a correlation was established between the virus titre and the copy number of the immunogenic gene of the rabies and IBD viruses. The detailed protocol for ddPCR is mentioned in the section 3.2.1.3v.

3.2.2 Evaluation of ELISA based serological potency assay as an alternative potency test for inactivated rabies vaccine

3.2.2.1 Immunization and bleeding

Immunization and bleeding in mice were carried out as per the IP, 2018 for the inactivated rabies vaccine. *Ad libitum* supplies of a balanced diet and pure water were given. Prior to immunization, mice remained healthy. The details of the procedure are described below.

- Total 60 mice of 3 weeks age were procured from the Division of Laboratory Animal Research, IVRI
- Mice were housed for 1 week to acclimatized the environment before immunization
- Mice were randomized into 6 groups (5 test vaccine and 1standard vaccine), with 10 mice each.
- Mice were immunized with 1/5th dose volume (500 µL) of the test vaccine (A-E) and 1/5th dose volume (500 µL) of standard vaccine (1 IU/mL) by intra-peritoneal route.
- The mouse was restrained and held supine. The needle and syringe were held parallel to the animal's vertebral column, and the injection was done at an angle of about 10° in the lower left quadrant of the abdomen.

- On day 14 after the immunization, blood was drawn from all mice from the retro-orbital sinus, and the sera were tested individually for RVNA by means RFFIT and cELISA. The volume of serum got from the immunized mice was varies from 75-100 µL, therefore all the serum were diluted (1:1) with sterile PBS before using these in RFFIT.
- After blood collection on day 14, all the mice were challenged with 39 LD_{50} of rabies CVS by intracerebral route (i.c.), and observations were made from the 5th to the 14th days of challenge.
- A correlation with RFFIT titre, cELISA percentage inhibition, and the mouse protection test was established.

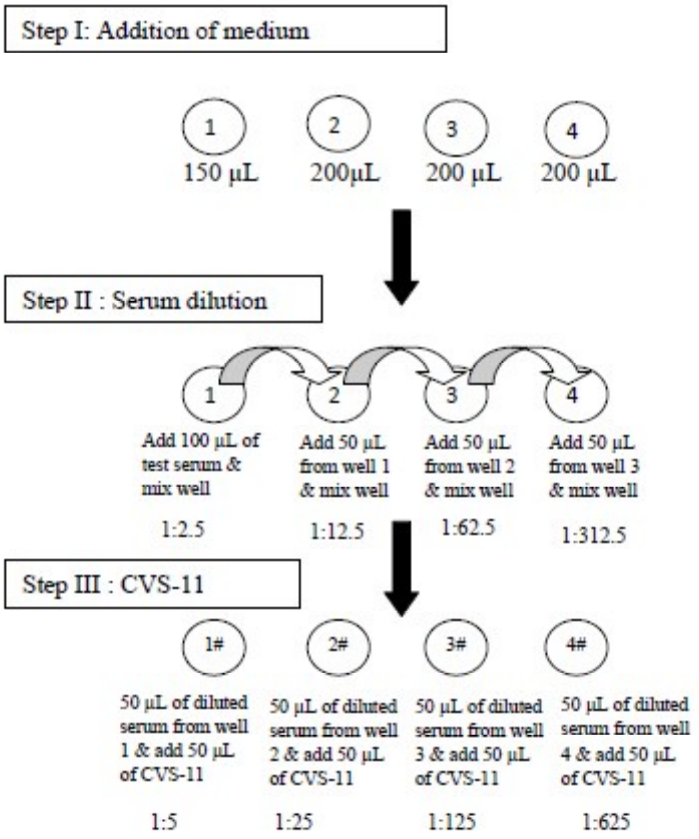
3.2.2.2 Determination of RABV-neutralizing antibodies

A: RFFIT (Rapid fluorescent focus inhibition test)

RFFIT was carried out in accordance with the WHO-recommended protocol and as per the standard methods mentioned in the OIE manual, with minor modifications. The RFFIT titres in IU/mL of the total test serum along with the reference standard serum were determined. The details of the procedure are described in the table 12.

Table 12: Details of different steps involved in RFFIT

S.No.	Steps
1	Serum samples were heated at 56°C for 30 minutes before the test in order to inactivate complement.
2	1:2.5 dilution of serum was made by adding 100 µL of inactivated serum with 150 µL of EMEM-10 to one well of the 96-well tissue culture plate (Falcon, Corning Incorporated, USA).
3	Afterwards, 50 µL of the 1:2.5 dilutions was transferred to the second well containing 200 µL of EMEM-10 to make a 1:12.5 dilution. Like-wise, serial 5 fold dilutions were carried out to make 1:62.5 and 1:312.5.
4	50 µL of a CVS-11 suspension (containing 100 FFD_{50}/50 µL) was added to all serum dilutions, yielding final dilutions of 1:5, 1:25, 1:125, 1:625, and 1:3125. Steps for RFFIT endpoint 96-well tissue culture plate dilution is mentioned below



5 Incubation was done at 37°C in humidified CO2 incubator for 90 minutes.

6 After incubation, 100 µL of a healthy freshly prepared BHK 21 cell suspension (5×10^5 cells/mL) were added to each well. (5×10^4 cells/well)

7 The plate was further incubated at 37°C in humidified CO2 incubator for another 24 h.

8 After incubation, the media was decanted and cell monolayer was washed one time with 1XPBS (pH 7.4).

9 1XPBS was decanted gently and 100 µL of chilled acetone (80/20 in PBS) was added and kept for incubation at -20°C for 30 min. After that, acetone was aspirated completely and the plate was air dried for 30 minutes at 37°C.

10 After complete drying, FITC conjugated anti-rabies antibody (Rabies DFA II Reagent, anti-N, Millipore Sigma, USA #5500) solution (1:100) was added @30 µL to each well in the dark.

11 Incubation at 37°C for 30 min within a humid incubator was done.

12 After incubation, the FITC solution was decanted and washed 3 times with 1XPBS.

13 Finally, mounting with 50% glycerol added @50 µL/well was done and observation was conducted in 20 microscopic fields per well for each serum dilution under an inverted fluorescence microscope to determine the number of wells showing fluorescent cells

14 The 50 % endpoint titres of serum were calculated according to the Reed-Muench method. The values were normalized to IU/mL by comparing them to the values obtained with the 2nd international reference serum (NIBSC, UK).

$$\text{Antibody titre (IU/mL)} = \frac{\text{End-point titre of test serum}}{\text{end-point titre of reference serum}} \times \text{titre of reference serum (IU/mL)}$$

B: cELISA (Competitive ELISA)

The in-house developed cELISA was used in in the present study for determination of immune response in the immunized mice in-term of percentage inhibition (PI value). This in-house cELISA was already optimized and validated in three different institutes in the earlier study. Based on the 130 pre-immune and post-immune human samples tested by cELISA and commercial ELISA (Platelia Rabies II kit, Bio-Rad Laboratories Inc) in the earlier study, the relative sensitivity and specificity of in-house cELISA was claimed to 100 %. After screening of all the mice serum by optimized in-house cELISA protocol (undisclosed), the PI value of the serum samples was calculated by the following formula.

$$PI = (\text{Control OD} - (\text{Test OD}/\text{Control OD})) \times 100.$$

C: Mouse protection test

Potency testing of inactivated veterinary rabies vaccine was conducted by mouse challenge assay as per OIE, IP 2018 and European pharmacopoeia (EP). A single intra-peritoneal immunisation was given to a group of 10 mice, followed by an intra-cerebral challenge 14 days later using the Challenge Virus Standard (CVS virus). The effectiveness of the test was determined by comparing the number of animals protected from rabies in groups that received commercially available inactivated rabies test vaccine and the standard vaccine. The details of the procedure are described in the table 13.

Table 13: Details of different steps involved in Mouse protection test

S. No.	Steps	Procedure
1	**Immunization of mice**	Five-fold (1/5) dilution of inactivated rabies test vaccines (A-E) and reference vaccine were made in PBS (1 mL of neat vaccine and 4 mL of PBS). The detailed procedures for immunization were the same as those described in the immunization and bleeding section**.**
2	**Challenge**	Fourteen days after the immunization, all mice were inoculated through the intracranial route with a challenge dose of CVS virus providing about 39 LD_{50} per 30 µL (syringe 1 mL; needle 26G/1/2)**.** CVS ampoule was taken from -80°C and thawed rapidly under cold running water. In order to prepare the challenge dose, the CVS virus was pre-diluted in sterile de-ionized water supplemented with 2% of heat-inactivated horse serum (Gibco, Invitrogen, USA), and antibiotics (Benzyl penicillin 500000 units, Streptomycin 1 g). Mice were handled on their neck scruff by finger and thumb. The syringe was pushed through the skull with a quick thrust.
3	**Monitoring**	All mice were monitored daily for 14 days for signs of rabies (convulsions, paralysis) and the numbers were recorded in each group that showed signs of rabies in the period of 5 to 14 days after CVS challenge. The test is not valid if more than 2 mice of any group die within the first 4 days after challenge (non-specific death).

3.2.3 Evaluation of sandwich ELISA based antigen quantification assay for potency testing of inactivated rabies and IBD vaccine

Antigen extracted from the different doses of inactivated rabies (A-E) and IBD (I-V) vaccines as mentioned in the section (3.2.1.3i) was tested by an in-house developed Sandwich ELISAs for rabies and IBD, respectively, and the OD_{450} values of the different doses of vaccine were determined.

A. The details of the procedure of IBD sELISA is described in the table 14.

Table 14: Steps and procedure for IBD Sandwich ELISA

S. No.	Steps and procedure
1	**Coating** ELISA plate (Polysorp, Nunc, Denmark) wells were coated with 100 µL of anti-IBDV rabbit serum in coating buffer (Appendix) at 1:8000 dilutions. Plate was kept for 1 hour at 37°C.
2	**Blocking** After incubation, all the wells were washed 3 times with wash buffer (Appendix) and subsequently, 300 µL of blocking buffer (5% SMP) was added in the all wells. Plate was kept for 1 hour at 37°C.
3	**Antigen binding** After 3 washing with washing buffer, 100 µL of extracted antigen from the 5 different doses (2, 1, 1/2th, 1/4th, 1/8th) of IBD vaccines were added in the appropriate wells. Plate was incubated for 1 hour at 37°C. (made total volume of 100 µL by adding PBS). Besides this, antigen was also extracted from different doses ranging from 1 dose, 1/2.5th dose, 1/5th dose, 1/10th dose, 1/20th dose, 1/40th dose, 1/50th dose, and 1/100th dose of new batch of IBD vaccine III and added in the appropriate wells.
4	**Detection binding** After 3 washing with washing buffer, wells were filled with 100 µL of anti-IBDV guinea pig serum in blocking buffer at 1:5000 dilutions. Plate was incubated for 1 hour at 37°C.
5	**Conjugate binding** After incubation, all the wells were washed 5 times with wash buffer. Dilution of anti-guinea pig IgG HRPO conjugate was made in blocking buffer (1:5000) and subsequently 100 µL of diluted conjugate was added in the all wells. Plate was incubated for 1 hour at 37°C.
6	**Peroxidase reaction** After the plates were washed 5 times, 50 µL of TMB substrate (cat # 1721072, Bio-Rad) was added and the plates were incubated for 10 minutes. Then 50 µL of 1.0 M H_2SO_4 was added to each well to stop the reaction and OD was measured at 450 nm with the help of ELISA reader (SYNERGY HTX, Gen5v3.05, USA).

B. Steps and procedure for rabies vaccine sELISA

Anti-rabies goat polyclonal antibody (1:5000 dilutions) was used as coating antibody in sELISA for the detection of extracted rabies antigen, and anti-rabies chicken antibody (1:400 dilution) was used as detector antibody. Antigen extracted from the 5 different doses (2, 1, 1/5th, 1/25th, and 1/125th) of rabies vaccines was added in the respective wells. Sandwich ELISA of freeze-dried standard rabies vaccine was also performed at different concentrations ranging from 2 IU, 1 IU, 0.2 IU, 0.04 IU, 0.008 IU, and 0.0016 IU. Dilution of anti-chicken IgY (IgG) HRPO conjugate was made in blocking buffer (1:5000), and subsequently 100 µL of the diluted conjugate was added to all wells. Other steps and procedures were the same as described in the above table 14**.**

3.2.4 Correlation of standard serological assay of rabies and IBD vaccine with copy number and antigen mass of respective vaccine

3.2.4.1 Correlation of standard serological assay of IBD vaccine with copy number and antigen mass

3.2.4.1i Immunization and bleeding

Immunization and bleeding in chicks were carried out as per the IP, 2018 for the inactivated IBD vaccine. *Ad libitum* supplies of a balanced diet and pure water were given. Prior to immunization, chicks remained healthy. The details of the procedure are described below.

- Total 75 SPF chicks of one day old were received from the CARI hatchery.
- Chicks were housed till 21 days before immunization.
- After 3 weeks of acclimatization, all chicks were immunized with 5 commercially available inactivated IBD vaccines (I-V).
- Chicks were immunized with three different dose volumes of each inactivated IBD vaccine (1 dose, 1/2nd, and 1/4th dose) in five chicks for each dose volume by intramuscular route.
- In a different experiment, total 24 chicks were immunized with different dose volumes ranging from 1 dose, 1/2.5th dose, 1/5th dose, 1/10th dose, 1/20th dose, 1/40th dose, 1/50th dose, and 1/100th in three chicks for each dose volume using new batch of commercial IBD vaccine.

- The chick was restrained manually by hand. The needle and syringe were kept at an angle of 90° and injection was made in the thigh muscle of leg.
- Pre-immune serum from all chicks was collected. On 21 days post-immunization, serum samples from all the chicks were collected from the heart to assess antibody response in different groups by indirect ELISA (IDEXX kit).

3.2.4.1ii Determination of IBDV-neutralizing antibodies by IDEXX ELISA

An ELISA kit (IDEXX IBD-XR, IDEXX Laboratory, Inc., USA) was used to detect the presence of neutralizing IBDV antibodies in the serum of vaccinated chicks. This technique was developed to assess the relative level of IBD antibodies in chick serum. The manufacturer's instructions were strictly followed throughout the experimentation.

Using an ELISA reader, the absorbance value was calculated at 650 nm. The manufacturer's recommendation that the mean OD value of NCX must be $\leq$0.150 and that the outcome of subtracting it from the mean OD of PCX must be >0.075 is provided as the basis for validating the result.

The endpoint titre of the samples was calculated using the formula

$$\text{Log}_{10}\text{Titre} = 1.09\ (\text{Log}_{10}\text{S/P}) + \text{C}.$$

Where, S/P (sample to positive ratio) = (Sample mean-NCX)/ (PCX-NCX)

C is 3.36

According to the kit, the presence of IBD antibody was reported as positive when S/P ratio is >0.2 and negative when S/P ratio is <0.2. Furthermore, the antilogarithm of Log_{10} titre greater than 396 indicates immunization or other exposure to IBD.

3.2.4.1iii Correlation of results of IDEXX ELISA, sELISA, and ddPCR for inactivated IBDV vaccine

Antibody titres obtained from standard serological assays (IDEXX ELISA) were correlated with the copy number and antigenic mass of the inactivated IBD vaccine doses. Serum collected from the different groups of chicks vaccinated with full, 1/2nd, and 1/4th dose

as well as 1, 1/2.5th, 1/5th, 1/10th, 1/20th, 1/40th , 1/50th, and 1/100th dose of inactivated vaccine and antigen quantification by in-house sELISA from same doses were established. Based on the result of antibody titre, amount of antigen (proportional to the OD_{450} value), and copy numbers of the VP2 gene, a rank was established among the selected IBD vaccine.

3.2.4.2 Correlation of rabies virus neutralizing antibody titre (RFFIT titre), antigenic mass, and copy number

Antibody titres obtained from the mice vaccinated with 1/5th dose of different test rabies vaccines by standard serological assays (RFFIT) was correlated with the copy number and antigenic mass by in-house sELISA from the same dose of the inactivated rabies vaccine. Based on the result of antibody titre (IU/mL), amount of antigen (proportional to the OD_{450} value), and copy numbers of the glycoprotein gene, a rank was established among the selected rabies vaccine.

3.2.4.3 Statistical Analysis

The effectiveness of in-house ELISAs (Competitive ELISA and Sandwich ELISA) was evaluated using two-tailed Spearman correlation analysis by comparing it with antibody titres obtained by RFFIT or IDEXX-XR IBD ELISA kit. The absolute copy number present in selected rabies and IBD vaccines was also evaluated. All statistical analysis and correlation fittings were performed using GraphPad Prism Version 8.0 for Windows, accessed on December 21, 2022.

Results

4 RESULTS

4.1 Development of quantitative PCR for determination of copy number of immunogenic gene in inactivated rabies and IBD vaccine

4.1.1 Optimization of annealing temperature by gradient RT-PCR and ddPCR

Initially, optimum annealing temperatures for amplification of rabies glycoprotein and IBD VP2 gene were determined by gradient PCR from 50-62°C. Amplification of both rabies glycoprotein and IBD VP2 gene were observed in all the temperature (Fig 1 and Fig 2).

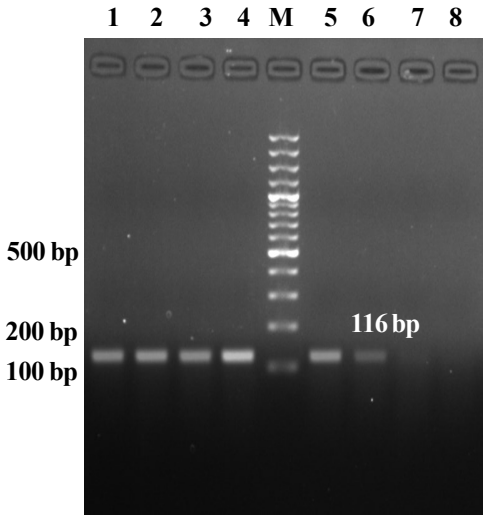


Fig. 1: Gradient PCR for amplification of RABV glycoprotein gene
Lane M : 100-3000bp DNA Ladder
Lanes 1-8 : 116bp amplified glycoprotein gene at 50°C, 52°C, 54°C, 55°C, 56°C, 58°C, 60°C and 62°C

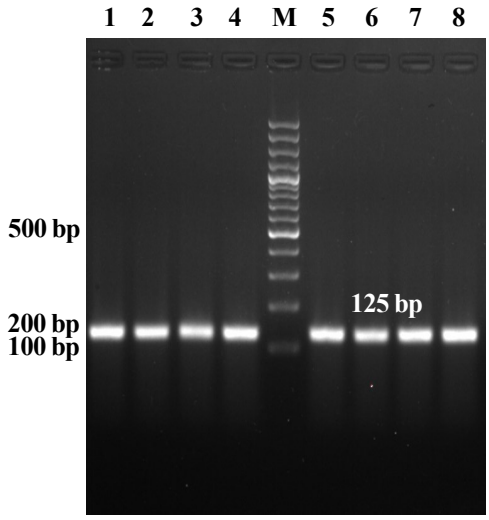


Fig. 2: Gradient PCR for amplification of IBDV VP2 gene
Lane M : 100-3000bp DNA Ladder
Lanes 1-8 : 125bp amplified VP2 gene at 50°C, 51.6°C, 53°C, 54.5°C, 56°C, 58.8°C, 60.2°C and 62°C

Subsequently, annealing temperature gradients from 50-62°C were performed to obtain maximum separation between positive and negative partitions in droplet digital PCR for both RABV glycoprotein gene and IBDV VP2 protein gene. The distinction in signals between the fluorescent channels were found maximum at 55°C annealing temperature for rabies virus (Fig 3) and at 58.8°C annealing temperature for IBD virus (not shown). Optimized annealing temperatures for both rabies glycoprotein gene and IBDV VP2 protein gene was used for determination of copy number by ddPCR.

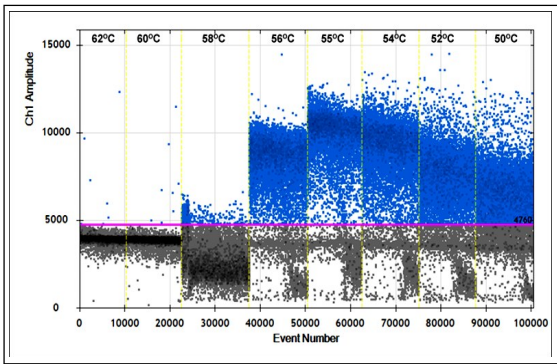


Fig. 3: Optimal annealing temperature for rabies glycoprotein gene by ddPCR
Channel 1 amplitude showing glycoprotein gene positive (blue) and negative (black) droplets, separated by the threshold (pink line). Yellow dashed lines separate different reaction wells. A temperature gradient from 62°C to 50°C was used, and annealing temperature of 55°C was selected as optimal

4.1.2. Determination of copy numbers of immunogenic gene in different doses of inactivated vaccine

4.1.2i Quantification of glycoprotein gene in inactivated rabies vaccine

The different batches of inactivated rabies vaccines selected in the present studies from different manufacturers (A, B, C, D, E). The rabies virus in these vaccines was inactivated by beta-propiolactone (BPL) or acetylethylamine, or binary ethylenimine (BEI). The antigen was extracted from different doses and cDNA was synthesized as per the section 3.2.1.3i–iii of material & methods. Before ddPCR, RT-PCRs of different doses of vaccines were conducted. Amplifications of 116bp glycoprotein gene fragments were observed in all the vaccines except vaccine D. Based on the intensity of bands in RT-PCR samples, cDNA from different doses of vaccines were further diluted uniformly to achieve a range of copy number suitable for ddPCR. Different doses of vaccine A, cDNA was diluted 2 times; vaccine B, cDNA diluted to 200 times; vaccine C, cDNA diluted to 800 times whereas, undiluted cDNA of vaccine E were taken as template for ddPCR. The distribution of positive (blue) and negative droplets (black) in different doses of inactivated rabies vaccine (A. B, C, E) was observed in the ddPCR (Fig 4). The copy numbers of glycoprotein gene in the different doses (2, 1, 1/5th,

1/25th, and 1/125th) of all the vaccines were calculated after considering amount of RNA isolated from each dose and used for synthesis of cDNA, dilution factor of cDNA, volume of cDNA taken for the ddPCR. The copy number of each dose of the all the vaccines (A, B, C, E) is mentioned in the table 15.

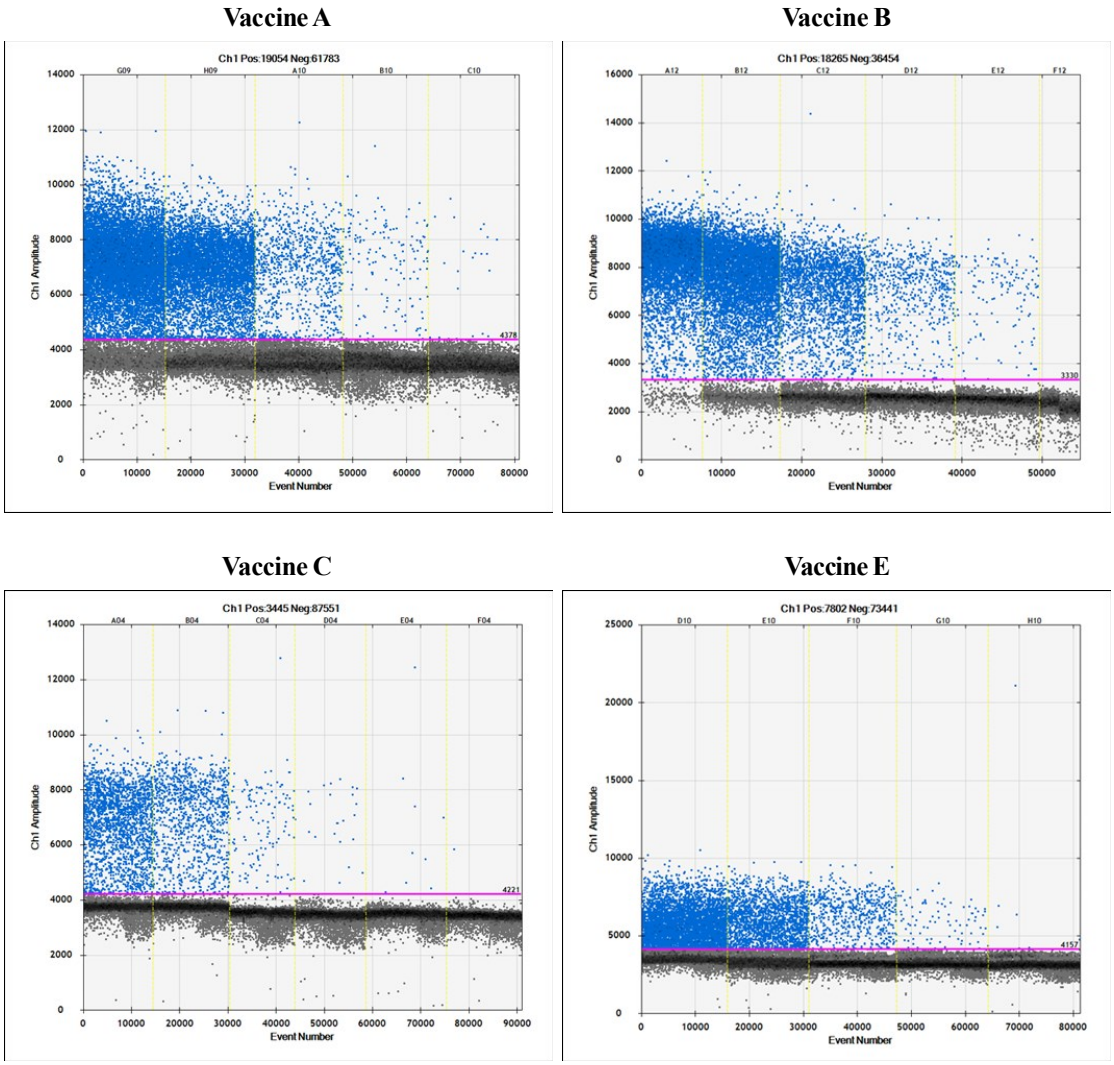


Fig. 4: Distribution of positive and negative droplets in different doses of rabies vaccine by ddPCR

In a single dose, highest copy number was detected in vaccine B (55552000) followed by vaccine C (51200000), A (487200) and lowest in E (66800). The ddPCR of vaccine D was not conducted as any amplification was not observed in the RT-PCR from the cDNA generated from vaccine D.

Table 15: Copy number of glycoprotein gene in the different doses of inactivated rabies vaccines

	Doses	Copy number/ reaction	RNA & cDNA factor	Dilution factor	Copies number/ dose
Vaccine A	Double	30440	20	2	1217600
	Full	12180	20	2	487200
	1/5th	1388	20	2	27760
	1/25th	234	20	2	9360
	1/125th	30	20	2	1200
Vaccine B	Double	83600	8	200	133760000
	Full	34720	8	200	55552000
	1/5th	6440	8	200	10304000
	1/25th	1366	8	200	2185600
	1/125th	316	8	200	505600
Vaccine C	Double	3840	40	800	122880000
	Full	1600	40	800	51200000
	1/5th	300	40	800	9600000
	1/25th	40	40	800	1280000
	1/125th	12	40	800	384000
Vaccine E	Double	9060	20	1	181200
	Full	3340	20	1	66800
	1/5th	858	20	1	17160
	1/25th	118	20	1	2360
	1/125th	10	20	1	200

4.1.2ii Quantification of VP2 gene in inactivated IBD vaccine

Different batches of inactivated IBD vaccines were selected in the present studies from different manufacturers (I, II, III, IV, V). IBDV virus in these vaccines was inactivated by formalin. The antigen was extracted from different doses and cDNA was synthesized as per the section 3.2.1.3i, ii, iv of material & methods. Before ddPCR, RT-PCRs of different doses of vaccines were conducted. Amplifications of 125bp glycoprotein gene fragments were observed in all the vaccines. Based on the intensity of bands in RT-PCR samples, cDNA from different doses of vaccines were further diluted 1:20 times to achieve a range of copy number suitable for ddPCR. The distribution of positive (blue) and negative droplets (black) in different doses of inactivated IBDV was observed in the ddPCR (Fig 5). The copies numbers of IBDV gene in the different doses (2, 1, 1/2nd, 1/4th, 1/8th) of all the vaccines were calculated after considering amount of RNA isolated from each dose and used for synthesis of cDNA, dilution

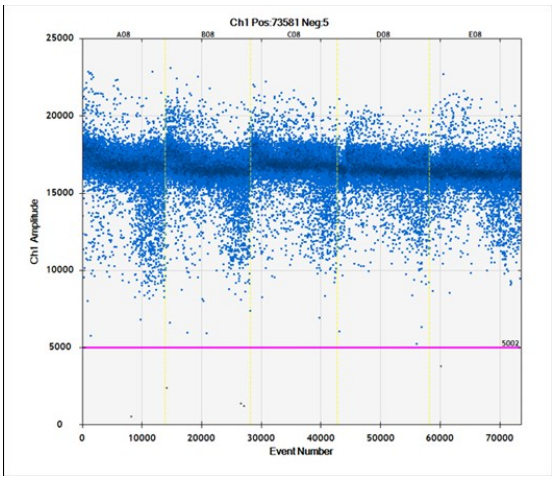
factor of cDNA, volume of cDNA taken for the ddPCR. The copy number of each dose of the all the vaccines (I, II, III, IV, V) is mentioned in the table 16.

Table 16: Copy number of VP2 gene in the different doses of inactivated IBD vaccines

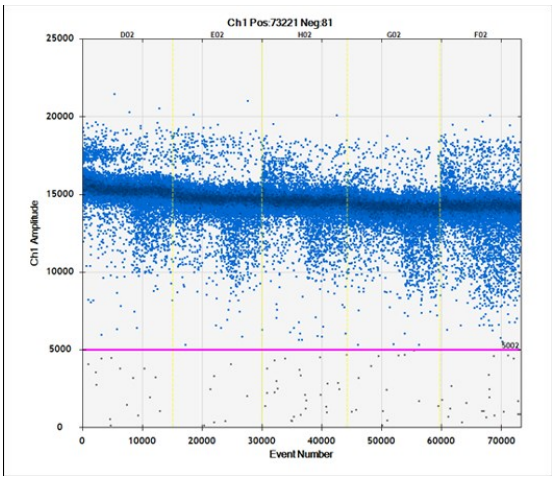
	Doses	Copy number/ reaction	RNA & cDNA factor	Dilution factor	Copy number/ dose
Vaccine I	Double	184000	20	1	3680000
	Full	176000	20	1	3520000
	1/2nd	172000	20	1	3440000
	1/4th	166000	20	1	3320000
	1/8th	145000	20	1	2900000
Vaccine II	Double	147200	20	1	2944000
	Full	140000	20	1	2800000
	1/2nd	132600	20	1	2652000
	1/4th	122000	20	1	2440000
	1/8th	102600	20	1	2052000
Vaccine III	Double	188000	20	1	3760000
	Full	164000	20	1	3280000
	1/2nd	159200	20	1	3184000
	1/4th	141400	20	1	2828000
	1/8th	81800	20	1	1636000
Vaccine IV	Double	198000	20	1	3960000
	Full	174000	20	1	3480000
	1/2nd	170000	20	1	3400000
	1/4th	163000	20	1	3260000
	1/8th	155400	20	1	3108000
Vaccine V	Double	200000	20	1	4000000
	Full	198000	20	1	3960000
	1/2nd	188000	20	1	3760000
	1/4th	180000	20	1	3600000
	1/8th	174000	20	1	3480000

In a single dose, highest copy number was detected in the vaccine V (3960000) followed by vaccine I (3520000), vaccine IV (3480000), vaccine III (3280000) and lowest in vaccine II (2800000).

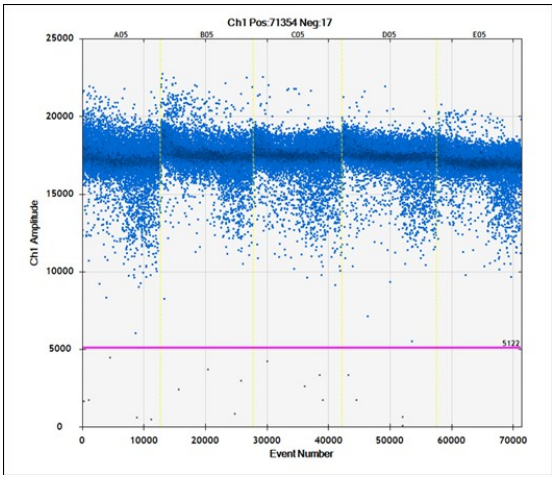
Vaccine I



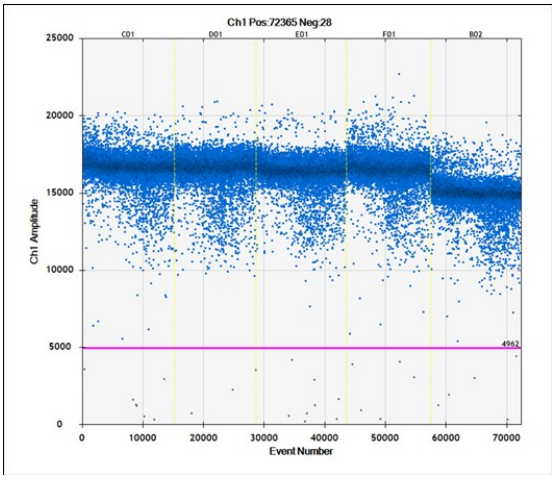
Vaccine II



Vaccine III



Vaccine IV



Vaccine V

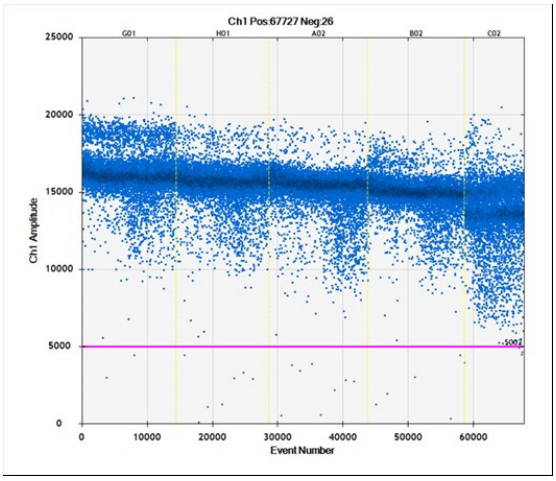


Fig. 5: Distribution of positive and negative droplets in different doses of IBDV vaccine by ddPCR

4.1.3. Correlation between copy number and virus titration

4.1.3i. Correlation between copy number of glycoprotein gene and rabies virus titre

RT-PCR and ddPCR was conducted from the cDNA generated from live CVS-11 titrated viruses ranging from $10^{5.6}$ to $10^{1.6}$ $FFID_{50}$/mL. RNA was isolated from 0.25 mL of 10-fold serial dilutions of stock CVS-11 virus ($10^{5.6}$ $FFID_{50}$/mL) and was used for PCR analysis followed by agarose gel electrophoresis (AGE) and ethidium bromide staining. A gradual reduction of band intensity of amplified product was observed as titre of the virus reduced from $10^{3.7}$ to $10^{0.07}$ $FFID_{50}$ per sample. Sample containing less than $10^{2.7}$ $FFID_{50}$ was not detected in AGE analysis (Fig 6). The distribution of positive (blue) and negative droplets (black) in 10 fold diluted rabies virus ($10^{3.7}$ to $10^{0.07}$ $FFID_{50}$) was observed in the ddPCR (Fig 7). In ddPCR, an excellent correlation (r = 0.999) was found between virus titre ($FFID_{50}$) and the copy number/reaction of the immunogenic gene of the rabies virus (Fig 8). The correlation (r = 0.999) was also calculated between virus titre ($FFID_{50}$/mL) and the copy number/ml of glycoprotein gene (Fig 9).

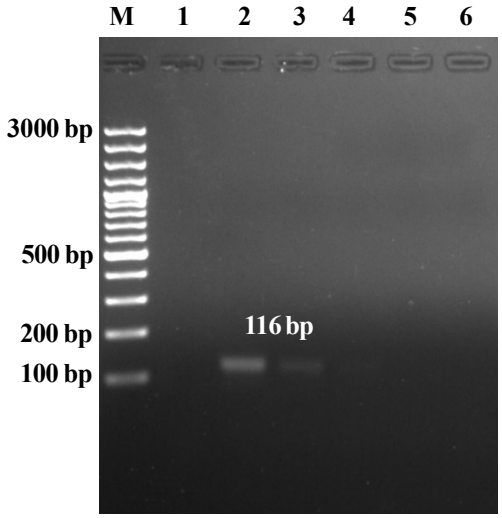


Fig. 6: Amplified glycoprotein gene from different FFID50 of CVS-11 virus

Lane M : 100-3000bp DNA Ladder
Lane 1 : Negative control
Lanes 2-6 : Dilution series representing the equivalent of $10^{3.7}$ to $10^{0.07}$ FFID50 per sample

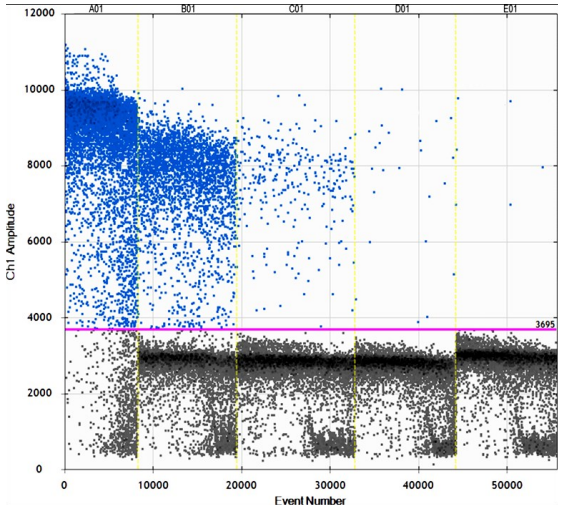


Fig. 7: Distribution of positive and negative droplets in 10 fold diluted live rabies virus by ddPCR

Reaction wells A01 to E01, dilution series representing the $10^{3.7}$ to $10^{0.07}$ FFID50 per 20 µL reaction mixture

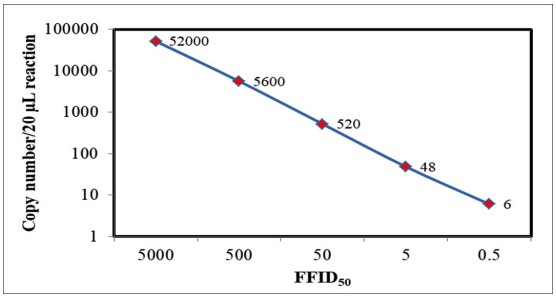


Fig. 8: Correlation between the ddPCR copy number/ 20 µL reaction and the titre of CVS virus ($FFID_{50}$) (r = 0.999)

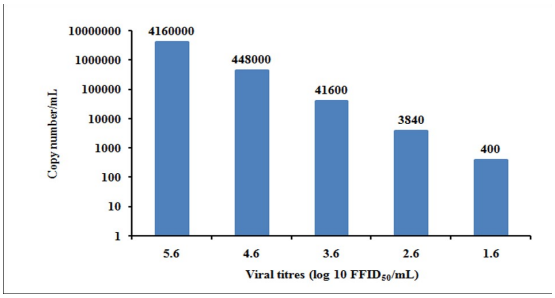


Fig. 9: Correlation between the ddPCR copy number and the titre of CVS virus ranging from $10^{5.6}$ $FFID_{50}$/mL to $10^{1.6}$ $FFID_{50}$/mL (r = 0.999)

4.1.3ii. Correlation between copy number of VP2 gene and IBD virus titre

RT-PCR and ddPCR was conducted from the cDNA generated from live IBDV virus having ranging from $10^{5.6}$ to $10^{1.6}$ $TCID_{50}$/mL. RNA was isolated from 0.10 mL of 10-fold serial dilutions of stock IBD virus ($10^{5.6}$ $TCID_{50}$/mL) and was used for PCR analysis followed by agarose gel electrophoresis (AGE) and ethidium bromide staining. A gradual reduction of band intensity of amplified product was observed as titre of the virus reduced from $10^{5.6}$ to $10^{1.6}$ $TCID_{50}$ /mL (Fig 10). The distribution of positive (blue) and negative droplets (black) in 10 fold diluted IBD virus ($10^{5.6}$ to $10^{1.6}$ $TCID_{50}$ /mL) was observed in the ddPCR (Fig 11). In

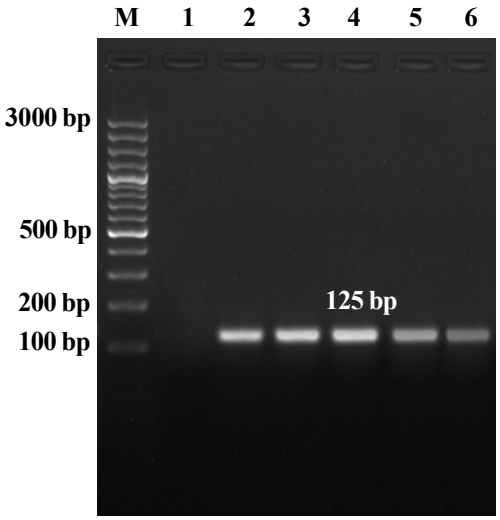


Fig. 10: Amplified VP2 gene from cDNA prepared from different dilution of stock IBD virus (10^5 EID_{50} /mL)

Lane M : 100-3000bp DNA Ladder
Lane 1 : Negative control
Lanes 2-6 : Dilution series representing the equivalent of $10^{5.6}$ to $10^{1.6}$ $TCID_{50}$/mL

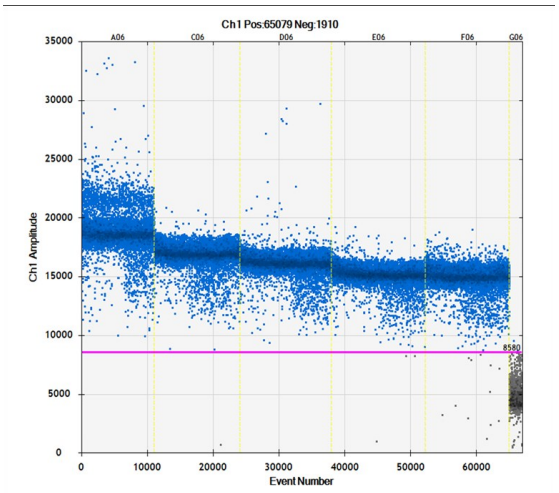


Fig. 11: Distribution of positive and negative droplets in 10 fold diluted live IBD virus (10^5 EID_{50}/mL) by ddPCR.
Reaction wells A01 to E01, dilution series representing the equivalent of $10^{5.6}$ to $10^{1.6}$ $TCID_{50}$/mL

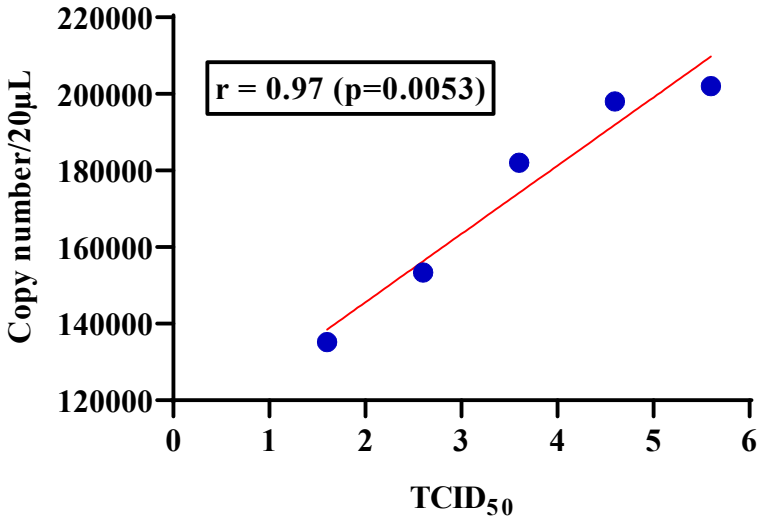


Fig. 12: Correlation between the ddPCR copy number and titrated live IBD virus

ddPCR, an excellent correlation (r =0.97) between virus titre ($TCID_{50}$ and the VP2 gene copy number/20 µL was observed (Fig 12).

4.2 Evaluation of ELISA based serological potency assay as an alternative potency test for inactivated rabies vaccine

In-house cELISA kit was evaluated for its suitability as an alternative to RFFIT (WHO recommended) for determination of rabies virus neutralizing antibodies (RVNA). Antibody titre in mice vaccinated with $1/5^{th}$ dose of rabies vaccine batches of 5 different manufacture (A-E) was conducted in RFFIT and cELISA. Subsequently, immunized mice of all groups were challenged with 39 LD_{50} CVS-11.

4.2.1 Determination of RABV-neutralizing antibodies by RFFIT

The methods for immunization, RFFIT, cELISA, and mouse challenge test are mentioned in the material and methods section 3.2.2.1 and 3.2.2.2. Rabies virus neutralizing antibodies were developed in the mice of all the groups vaccinated with 1/5th doses of the standard vaccine (0.2 IU) and vaccines from different manufacturers (A-E). The end point dilution of each serum that induced complete inhibition of fluorescence due to neutralization of virus was determined (Fig 13). The antibody titre in each serum in the term of IU was determined on basis of end point dilution of standard serum and test serum as per the formula mentioned in the section 3.2.2.2 of material and methods. The antibody titre (IU/mL) of serum collected from each mouse was calculated and mentioned in the table 17.

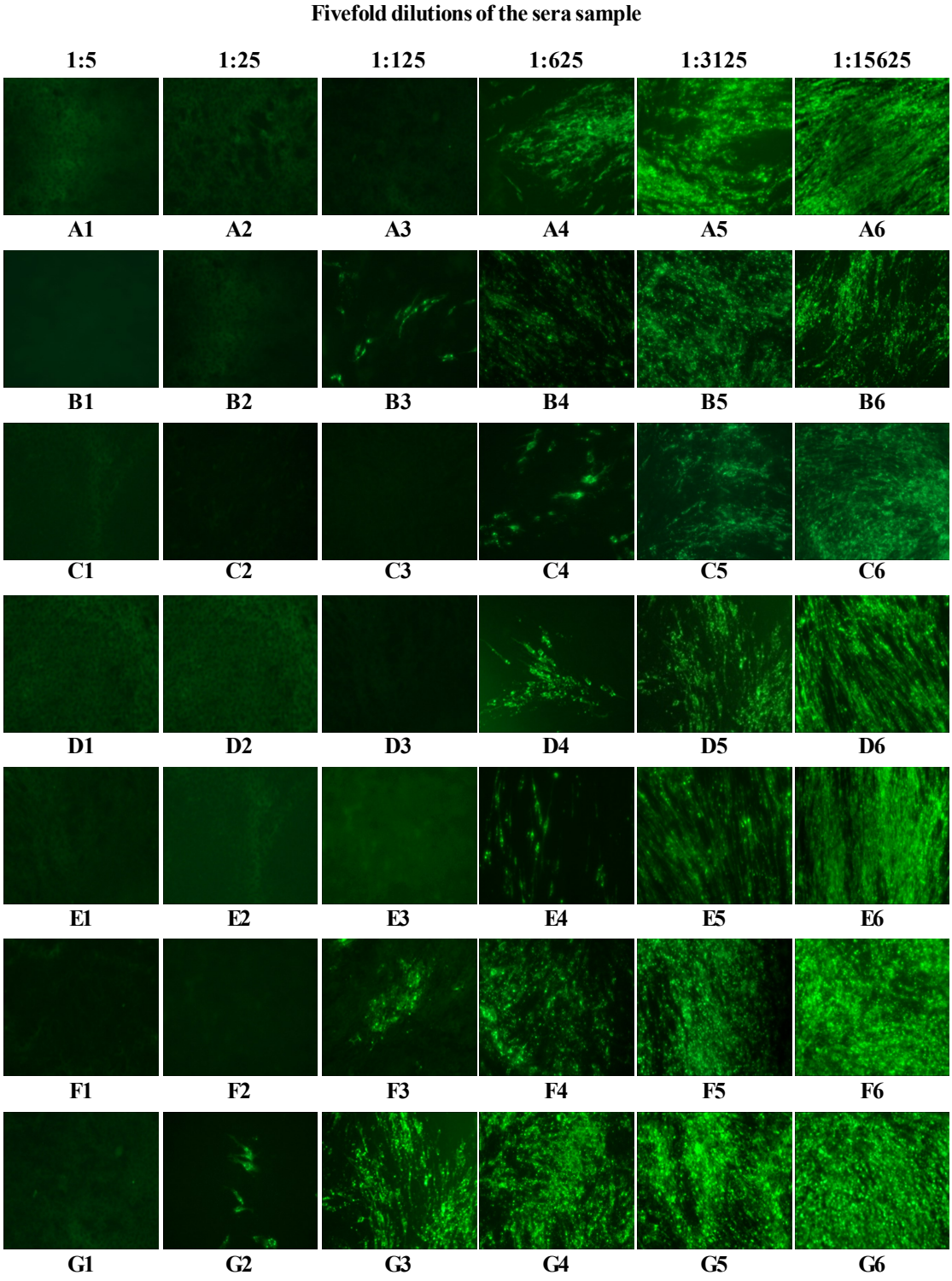


Fig. 13: Rapid fluorescent focus inhibition test (RFFIT) of five-fold diluted serum collected from mice immunized with reference and different test rabies vaccines (A1-A6: WHO Reference sera (2IU/mL); **B1-B6:** Reference Standard vaccine; **C1-C6:** Vaccine-A; **D1-D6:** Vaccine-B; **E1-E6:** Vaccine-C; **F1-F6:** Vaccine-D; **G1-G6:** Vaccine-E)

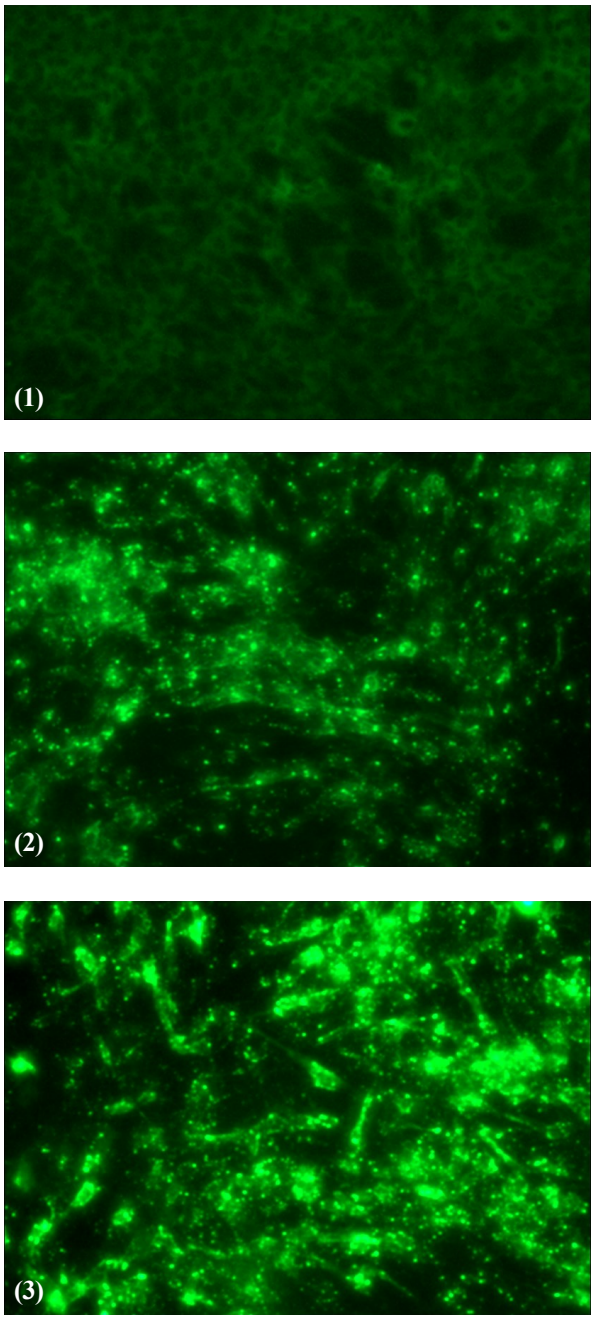


Fig. 14: RFFIT for cells control, negative serum control, and virus control {cells control (1), first well of negative serum control (2), and virus control (3)}

None of the control mice had RABV neutralizing antibodies, as checked by the RFFIT (Fig 14). Mice inoculated with 1/5 dose of commercial and standard rabies vaccines showed significant RVNA titres ranging from 0.31 to 5.28 mean IU/mL. Mice inoculated with commercial vaccines A, B, C, and D revealed significant protective antibody titres over 0.5 IU/mL, while vaccine E showed low VN antibody titres below 0.5 IU/mL 14th days after vaccination (Fig 15, table 17).

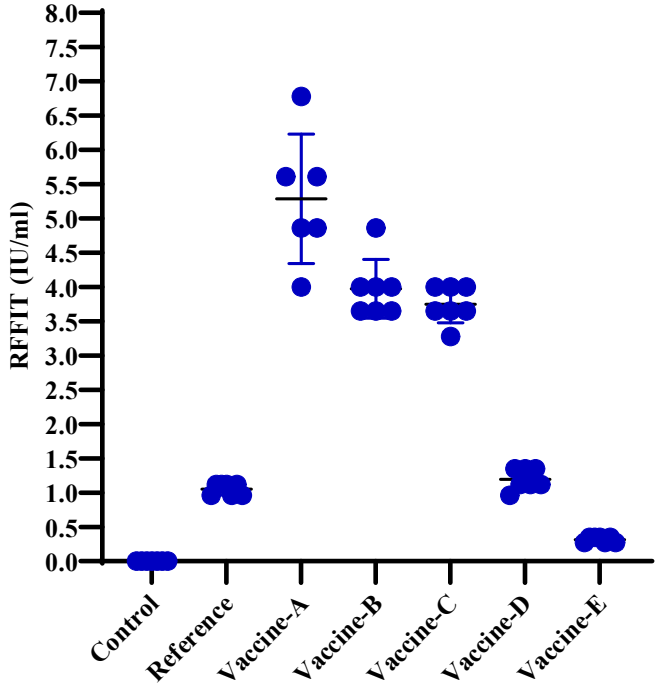


Fig. 15: Antibody responses in mice vaccinated with 1/5th dose of different vaccines and standard vaccine on 14th days post-vaccination by RFFIT

4.2.2 Determination of antibody response by in-house competitive ELISA (cELISA)

Serum samples tested by RFFIT were again tested by an in-house developed cELISA for determining RABV neutralizing antibody titre in term of percent inhibition (PI value). Based on our laboratory previous study on more than 150 serum samples, PI value less than 30 % is indicated antibody titre less than 0.5 IU/mL (data not shown). Mice inoculated with a 1/5th dose of commercial and standard rabies vaccines showed significant percent inhibition (PI), ranging from 21.37 to 80.61 mean PI. The PI value of serum collected from individual mice is mentioned in the table 17. Mice inoculated with commercial vaccines A, B, C, and D revealed more than 30% inhibition, while vaccine E showed less than 30% inhibition (Fig 16).

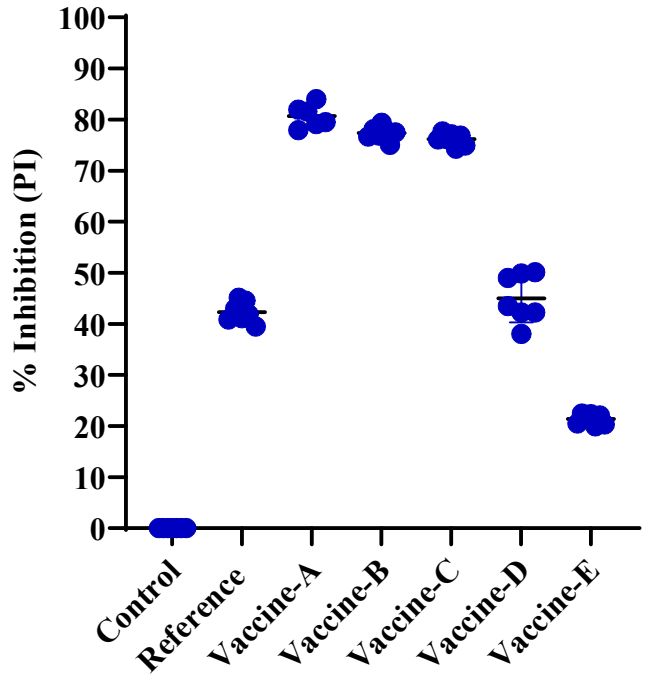


Fig. 16: Antibody responses in mice vaccinated with 1/5th dose of different vaccines and standard vaccine on 14th days post-vaccination by cELISA

4.2.3 Comparison of antibody response by RFFIT and cELISA

The comparison of two serological methods (RFFIT and cELISA) for determination of antibody titre in vaccinated mice is mentioned in the table 17.

The correlation study was indicated that both the tests are well correlated (r value = 0.9308) (P<0.0001) (Fig 17).

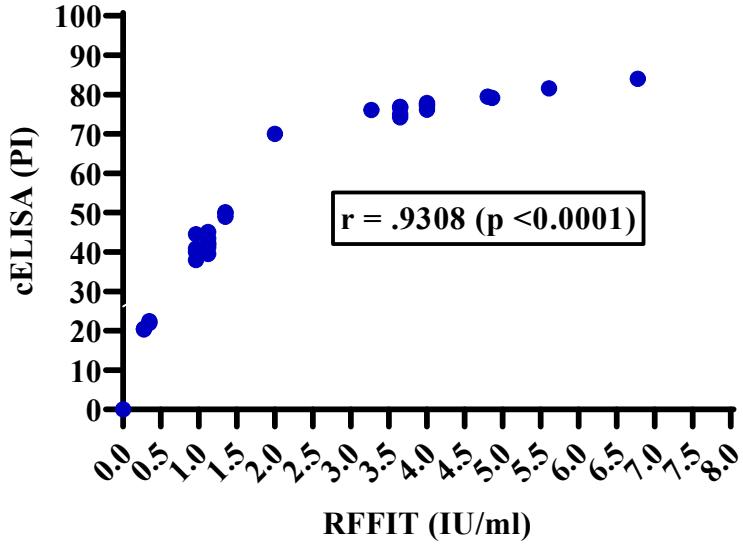


Fig. 17: Correlation between the rapid fluorescent focus inhibition test (RFFIT) and Competitive ELISA (cELISA)

Table 17: Results of RFFIT and cELISA for determination of antibody responses in immunized mice

Serum No.	Vaccine	RFFIT titre (IU/ml)	cELISA (% inhibition)
1	Reference Standard	0.96	40.87
2	Reference Standard	0.96	43.01
3	Reference Standard	1.12	39.48
4	Reference Standard	1.12	41.13
5	Reference Standard	1.12	44.54
6	Reference Standard	0.96	45.12
7	Reference Standard	1.12	41.85
8	Vaccine-A	4	77.89
9	Vaccine-A	5.61	81.58
10	Vaccine-A	5.61	81.95
11	Vaccine-A	4.8	79.51
12	Vaccine-A	6.78	83.99
13	Vaccine-A	4.8	79.13
14	Vaccine-B	3.65	76.89
15	Vaccine-B	4	78.09
16	Vaccine-B	3.65	76.68
17	Vaccine-B	4.86	79.38
18	Vaccine-B	4	77.50
19	Vaccine-B	4	78.09
20	Vaccine-B	3.65	75.01
21	Vaccine-C	3.27	76.10
22	Vaccine-C	3.65	74.26
23	Vaccine-C	4	77.67
24	Vaccine-C	4	77.12
25	Vaccine-C	4	76.15
26	Vaccine-C	3.65	76.87
27	Vaccine-C	3.65	74.89
28	Vaccine-D	1.35	49.87

29	Vaccine-D	1.35	48.98
30	Vaccine-D	0.96	38.01
31	Vaccine-D	1.12	43.46
32	Vaccine-D	1.35	50.10
33	Vaccine-D	1.12	42.24
34	Vaccine-D	1.12	42.21
35	Vaccine-E	0.34	21.98
36	Vaccine-E	0.27	20.34
37	Vaccine-E	0.34	22.45
38	Vaccine-E	0.34	22.07
39	Vaccine-E	0.27	19.94
40	Vaccine-E	0.34	22.31
41	Vaccine-E	0.27	20.50

4.2.4 Determination of protection level of mice vaccinated with 1/5th dose of commercial and standard vaccine

Mice inoculated with 1/5th doses of commercial and standard rabies vaccines showed CVS protection ranging from 0 to 100% mean survival rate. Mice inoculated with commercial vaccines A, B, C, and D revealed a greater than 80% mean survival rate, while vaccine E showed no protection against challenge. The results of the mouse protection test for determining RABV neutralizing antibodies (mean survival rate) in all individual mice immunized with commercial vaccines along with standard vaccines are presented in Table 18.

Table 18: The results of mouse protection tests for different rabies vaccine batches and the reference vaccine

Vaccine	Dilution	No. of mice used for immunization	No. of mice available at time of challenge	Non-specific deaths between 1-4th day of challenge	No. of survival mice from 5th to 14 days after challenge
Reference	1/5	10	7	1	4
Vaccine-A	1/5	9	6	0	6
Vaccine-B	1/5	10	7	0	6
Vaccine-C	1/5	10	7	1	5
Vaccine-D	1/5	10	7	2	4
Vaccine-E	1/5	10	7	1	0

4.2.5 Correlation between antibody titre and protection level

The antibody titres (IU/mL) obtained by RFFIT were marginally the same as those of percentage inhibition (PI) obtained by cELISA; there was a good correlation between the two (Fig 17; r = 0.9308). It can also be observed that there is a good correlation between RFFIT and cELISA with the mouse protection test. In regards to the correlation of serology results to survival from challenge, the relationship was mostly absolute: all mice with detectable rabies antibodies survived challenge, and all with no detectable antibody succumbed. None of the mice with levels below the cut-off (0.5 IU/mL) survived the challenge. However, in 5 out of 34 mice, the opposite trend occurred, where "positive" animals with antibody titres greater than 0.5 IU/mL were succumbed.

Table 19: Antibody response and mean survival rate of different groups of mice

S. No.	Parameter	Mean RVNA titres (IU/mL)	Mean % inhibition	Mean survival rate (%)
1	Control	0	0	0
2	Reference	1.05	42.28	67
3	Vaccine-A	5.28	80.67	100
4	Vaccine-B	3.97	77.38	86
5	Vaccine-C	3.74	76.15	83
6	Vaccine-D	1.19	44.98	71
7	Vaccine-E	0.31	21.37	0

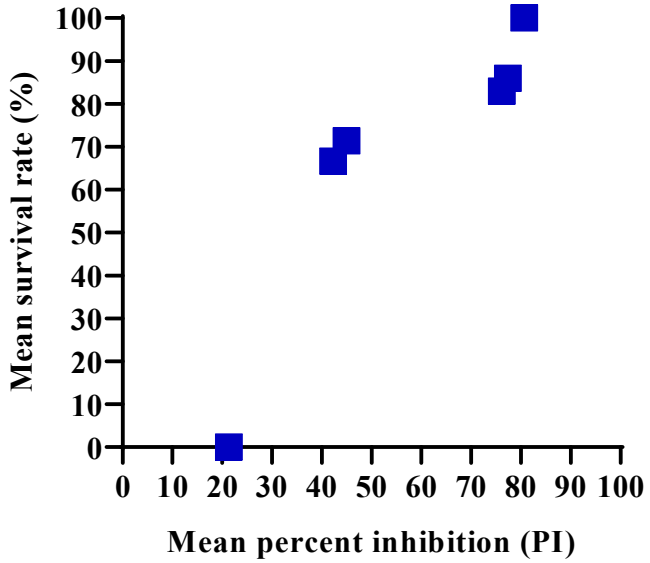


Fig. 18: Correlation of mean % inhibition (cELISA) and mean survival rate (mouse protection test) (r = 0.9092)

4.3 Evaluation of sandwich ELISA based antigen quantification assay for potency testing of inactivated rabies and IBD vaccine

In-house developed sELISA for quantification of rabies and IBDV antigen in inactivated vaccine was evaluated as per the protocol mentioned in the section 3.2.3 of material and methods. These ELISAs were performed in the IBD and rabies vaccine antigen extracted by IPM and extraction buffer, respectively. Five rabies (A to E) and five IBD vaccines (I-V) from different manufacturers were used in the present study.

4.3.1 Extraction of antigen from inactivated rabies and IBDV vaccine

Antigens from different doses of inactivated rabies and IBDV vaccine were extracted as per the methods mentioned in the material and methods. The volumes of extracted antigen from different doses are mentioned in the table 20, 21 and 22.

Table 20: Extracted antigen volumes from different doses of inactivated rabies vaccine

Vaccine	Doses	Volume of extracted antigen after conc. (µL)	Volume of PBS used (µL)	Total volume added per well (µL)
A	2	100	0	100
	1	80	20	100
	$1/5^{th}$	50	50	100
	$1/25^{th}$	20	80	100
	$1/125^{th}$	16	84	100
B	2	100	0	100
	1	50	50	100
	$1/5^{th}$	25	75	100
	$1/25^{th}$	20	80	100
	$1/125^{th}$	16	84	100
C	2	100	0	100
	1	55	45	100
	$1/5^{th}$	30	70	100
	$1/25^{th}$	25	75	100
	$1/125^{th}$	16	84	100
D	2	100	0	100
	1	60	40	100
	$1/5^{th}$	50	50	100
	$1/25^{th}$	30	70	100
	$1/125^{th}$	16	84	100
E	2	100	0	100
	1	55	45	100
	$1/5^{th}$	40	60	100
	$1/25^{th}$	32	68	100
	$1/125^{th}$	16	84	100

Table 21: Extracted antigen volumes from different doses of inactivated IBDV vaccine

Vaccine	Doses	Extracted amount (µL)	Extracted amount divided by 1/3rd	Volume of PBS used (µL) to make 100 µL
I	2	270	90	10
	1	130	43.33	56.67
	1/2nd	60	20	80
	1/4th	32	10.66	89.34
	1/8th	16	5.33	94.67
II	2	350	116.66	0
	1	177	59	41
	1/2nd	88	29.33	70.67
	1/4th	40	13.33	86.67
	1/8th	20	6.66	93.34
III	2	320	106.66	0
	1	162	54	46
	1/2nd	81	27	73
	1/4th	40	13.33	86.67
	1/8th	20	6.66	93.34
IV	2	280	93.33	6.67
	1	140	46.66	53.34
	1/2nd	70	23.33	76.67
	1/4th	30	10	90
	1/8th	15	5	95
V	2	235	78.33	21.67
	1	117	39	61
	1/2nd	58	19.33	80.67
	1/4th	30	10	90
	1/8th	14	4.66	95.34

Table 22: Extracted antigen volumes from different doses of new batch of vaccine III inactivated IBDV vaccine

Doses	Extracted amount (µL)	Volume of PBS used (µL)	Total volume added per well (µL)
1	170	0	100
	Concentrate to 100 µL		
1/2.5th	70	30	100
1/5th	35	65	100
1/10th	17	83	100
1/20th	9	91	100
1/40th	4.5	95.5	100
1/50th	3.6	96.5	100
1/100th	1.8	98.2	100

4.3.2: Quantification of antigen extracted from different doses of inactivated rabies vaccines by sELISA

In sELISA of rabies, antigens extracted from the 5 different doses (2 dose, 1 dose, 1/ 5th, 1/25th, and 1/125th) of rabies vaccine (A-E) and standard vaccine at different antigen mass (2-0.0016 IU) were showed a significant OD value at 450 nm (Table 23 and Fig 19). The OD_{450} values are directly correlated with the antigenic mass in different doses of the vaccines. The potency ranking of each vaccine based on the antigenic mass were as follow B >C >A >D >E.

Table 23: Quantification of rabies viral antigen content at different doses of reference and test vaccine by using in-house sELISA

S.No.	Vaccine	Rabies sELISA mean OD450 nm				
		2 dose	1 dose	1/5th dose	1/25th dose	1/125th dose
1	Reference	1.80	1.710	1.489	1.241	0.9
2	Vaccine-A	1.121	1.012	0.854	0.663	0.580
3	Vaccine-B	1.2	1.1	0.931	0.789	0.678
4	Vaccine-C	1.171	1.069	0.90	0.722	0.668
5	Vaccine-D	0.910	0.689	0.568	0.399	0.288
6	Vaccine-E	0.621	0.532	0.415	0.325	0.2
7	DHPPi	-	0.4	-	-	-

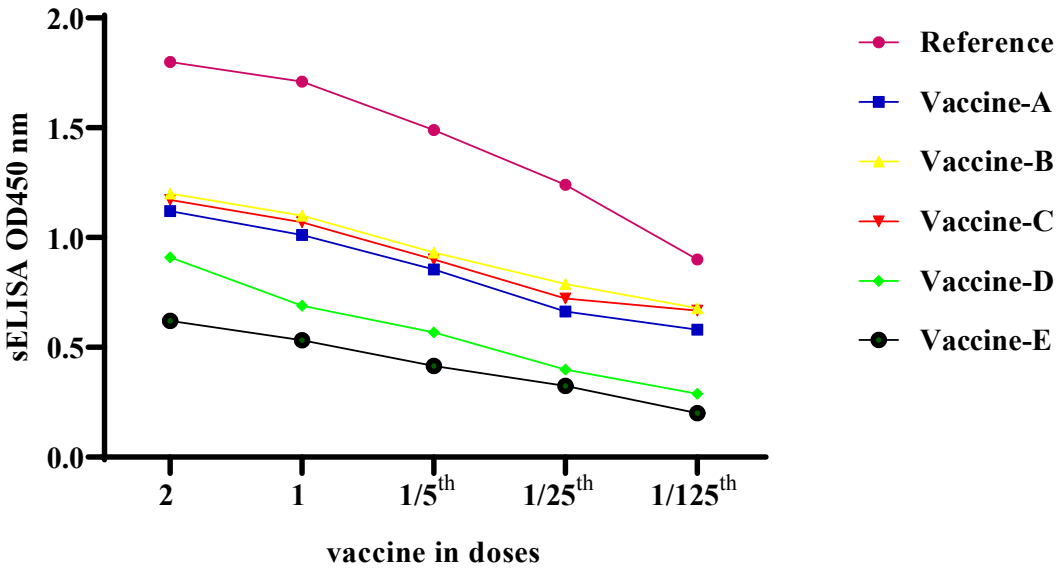


Fig. 19: Standard graph of Sandwich ELISA of reference and test rabies vaccine (X axis-the antigenic mass of different doses, Y axis-OD value of different doses)

High OD_{450} values were observed at different antigenic mass (2 -0.0016 IU equivalent to 2- 1/125th dose) of freeze-dried standard rabies vaccine compared to the antigen recovered from the different doses (2- 1/125th dose) of aluminum hydroxide or phosphate binds adjuvanted vaccines. These results indicated that antigen recovery was not complete from the adjuvanted vaccine as aluminum hydroxide or phosphate binds to a large portion of the antigenic mass. When, results of RFFIT and mouse protection test of standard and test vaccines at 1/5th dose was compared with the OD_{450} values at different doses of adjuvanted vaccines doses and reference vaccine, we assumed that antigen from all the inactivated vaccines was not recovered completely. The OD_{450} values of all the vaccines are mentioned in the table 23.

4.3.3: Quantification of antigen extracted from different doses of inactivated IBDV vaccines

In sELISA of IBD, antigens extracted from the 5 different doses (2 dose, 1 dose, 1/ 2nd, 1/4th, and 1/8th) of IBD vaccine (I-V) were showed a significant high OD_{450} value, whereas the value of vaccines other than IBD was negligible even at a single dose (Table 24 and Fig 20). The OD_{450} values are directly correlated with the antigenic mass in different doses of the vaccines. The potency ranking of each vaccine based on the antigenic mass were as follow V >IV>III >I >II.

Table 24: Quantification of IBD viral antigen content at different doses of test vaccine by using in-house sELISA

S. No.	Vaccine	IBD sELISA mean OD450 nm				
		2 dose	1 dose	1/2nd dose	1/4th dose	1/8th dose
1	Vaccine- I	2.301	2.2555	1.9475	1.722	1.41
2	Vaccine- II	1.999	1.906	1.7335	1.516	0.946
3	Vaccine- III	2.3125	2.204	1.904	1.6495	1.445
4	Vaccine- IV	2.399	2.289	2	1.689	1.354
5	Vaccine- V	2.401	2.342	2.197	2.098	1.6
6	Reo vaccine	-	0.30	-	-	-

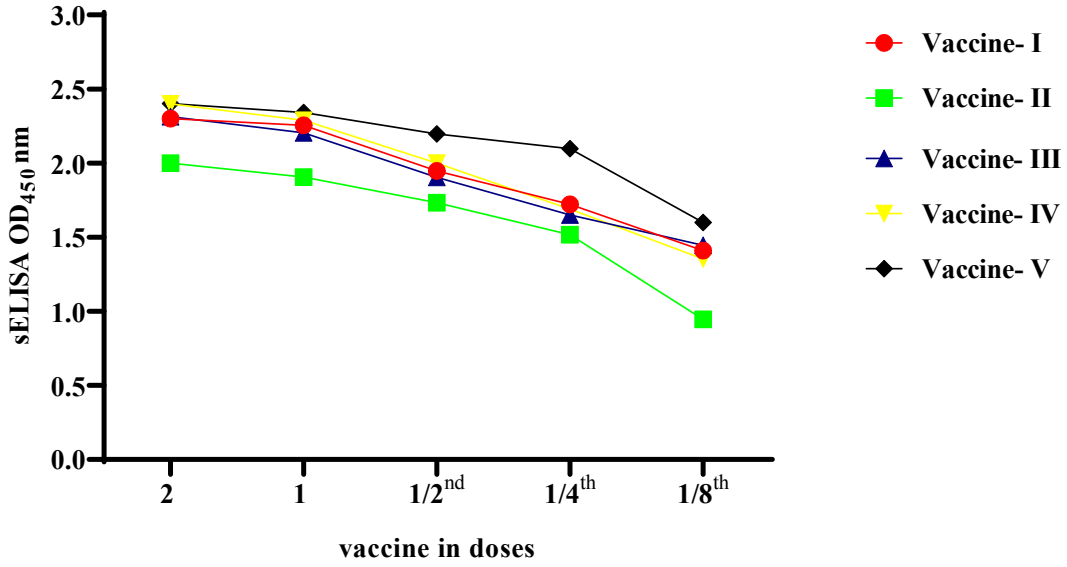


Fig. 20: Standard graph of Sandwich ELISA of different test IBD vaccine (X axis-the antigenic mass of different doses, Y axis-OD value of different doses)

Out of 5 vaccines, vaccine V was found highest amount of antigenic mass. The antigen from the new batch of vaccine III was extracted with 8 different doses ranging from 1 to 1/100th dose (1 dose, 1/2.5th, 1/5th, 1/10th, 1/20th, 1/40, 1/50th, and 1/100th) and tested by sELISA. The OD_{450} value showed a significant fall as reduction in the antigenic mass at different doses (Fig 21).

S.No.	IBD sELISA mean OD450 nm							
1	1 dose	1/2.5th dose	1/5th dose	1/10th dose	1/20th dose	1/40th dose	1/50th dose	1/100th dose
2	2.3905	2.2115	2.054	1.858	1.5555	1.199	1.014	0.7855

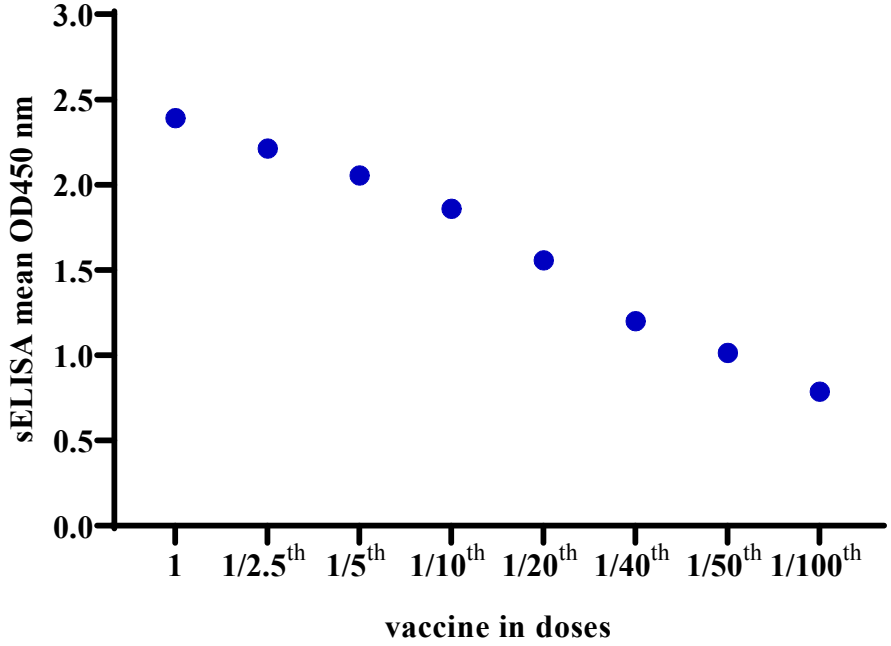


Fig. 21: Antigen quantification assay of extracts from different doses of new IBD vaccine III by using Sandwich ELISA

4.4 Correlation of standard serological assay of rabies and IBD vaccine with copy number and antigen mass of respective vaccine

4.4.1 Correlation of standard serological assay of IBD vaccine with copy number and antigen mass

4.4.1.1 IDEXX ELISA

Serological test (IDEXX ELISA) of serum collected from the different groups of chicks vaccinated with 1 dose, 1/2nd, and 1/4th dose of inactivated vaccine was conducted as per the protocol mentioned in the section 3.2.4.1 of material and methods. Chicks immunized with three different dose volumes of each inactivated IBD vaccine (1 dose, 1/2nd, and 1/4th dose) showed significant protective antibody titres in the serum by IDEXX ELISA. The results of the IDEXX ELISA for IBDV-neutralizing antibody titre in the sera of all individual chicks immunized with five commercially available inactivated vaccines along with their respective

Table 25: IDEXX IBD-XR Ab ELISA titre in chicks on day of immunization and 21 days post-immunization with different doses of the IBD vaccine

		21 days post-immunization			Pre-immune serum		
		OD650	S/P	Titres	OD650	S/P	Titres
	C-	0.052	0	0			
	C+	0.4685	1	2291			
Chick 1	I-1 dose	1.701	3.95	10266	0.101	0.11	222
Chick 2	I-1 dose	1.301	2.99	7584	0.121	0.16	323
Chick 3	I-1 dose	1.632	3.79	9798	0.049	-0.00	0
Chick 4	I-1 dose	1.714	3.99	10354	0.051	-0.00	0
Chick 5	I-1 dose	1.959	4.57	12028	0.053	0.00	3
Chick 6	I-1/2 dose	1.357	3.13	7955	0.154	0.24	494
Chick 7	I-1/2 dose	1.602	3.72	9596	0.118	0.15	308
Chick 8	I-1/2 dose	1.636	3.80	9825	0.053	0.00	3
Chick 9	I-1/2 dose	1.284	2.95	7471	0.069	0.04	70
Chick 10	I-1/2 dose	1.335	3.08	7809	0.058	0.01	23
Chick 11	I-1/4 dose	1.304	3.00	7603	0.066	0.03	57
Chick 12	I-1/4 dose	1.363	3.14	7995	0.049	-0.00	0
Chick 13	I-1/4 dose	1.456	3.37	8615	0.084	0.07	140
Chick 14	I-1/4 dose	0.89	2.01	4909	0.048	-0.00	0
Chick 15	I-1/4 dose	1.072	2.44	6081	0.088	0.08	159
Chick 16	II-1 dose	1.458	3.37	8628	0.06	0.01	31
Chick 17	II-1 dose	1.611	3.74	9657	0.069	0.04	70
Chick 18	II-1 dose	1.158	2.65	6642	0.062	0.02	39
Chick 19	II-1 dose	1.693	3.93	10211	0.112	0.14	277
Chick 20	II-1 dose	1.096	2.50	6237	0.064	0.02	48
Chick 21	II-1/2 dose	1.514	3.51	9004	0.108	0.13	257
Chick 22	II-1/2 dose	0.842	1.89	4603	0.057	0.01	18

Chick 23	II-1/2 dose	1.431	3.31	8448	0.091	0.09	173
Chick 24	II-1/2 dose	1.75	4.07	10599	0.108	0.13	257
Chick 25	II-1/2 dose	0.482	1.03	2372	0.077	0.06	107
Chick 26	II-1/4 dose	1.342	3.09	7855	0.051	-0.00	0
Chick 27	II-1/4 dose	0.29	0.57	1245	0.087	0.08	154
Chick 28	II-1/4 dose	1.55	3.59	9245	0.059	0.01	27
Chick 29	II-1/4 dose	1.278	2.94	7431	0.07	0.04	75
Chick 30	II-1/4 dose	1.106	2.53	6303	0.051	-0.00	0
Chick 31	III-1 dose	1.432	3.31	8454	0.091	0.09	173
Chick 32	III-1 dose	1.718	4.00	10381	0.063	0.02	44
Chick 33	III-1 dose	1.886	4.40	11527	0.055	0.00	11
Chick 34	III-1 dose	1.656	3.85	9961	0.063	0.02	44
Chick 35	III-1 dose	1.689	3.93	10184	0.058	0.01	23
Chick 36	III-1/2 dose	1.21	2.78	6983	0.054	0.00	7
Chick 37	III-1/2 dose	1.424	3.29	8401	0.047	-0.01	0
Chick 38	III-1/2 dose	0.908	2.05	5024	0.125	0.17	343
Chick 39	III-1/2 dose	1.097	2.50	6244	0.049	-0.00	0
Chick 40	III-1/2 dose	0.836	1.88	4565	0.053	0.00	3
Chick 41	III-1/4 dose	0.736	1.64	3934	0.058	0.01	23
Chick 42	III-1/4 dose	1.247	2.86	7227	0.048	-0.00	0
Chick 43	III-1/4 dose	1.06	2.42	6003	0.081	0.06	125

Chick 44	III-1/4 dose	0.865	1.95	4749	0.058	0.01	23
Chick 45	III-1/4 dose	1.142	2.61	6538	0.05	-0.00	0
		21 days post-immunization			Pre-immune serum		
		OD650	S/P	Titres	OD650	S/P	Titres
	C-	0.0465	0	0			
	C+	0.4195	1	2291			
Chick 46	IV-1 dose	2.055	5.38	14354	0.052	0.01	23
Chick 47	IV-1 dose	2.008	5.25	13988	0.043	-0.00	0
Chick 48	IV-1 dose	1.991	5.21	13856	0.044	-0.00	0
Chick 49	IV-1 dose	1.97	5.15	13693	0.05	0.00	14
Chick 50	IV-1 dose	1.769	4.61	12141	0.045	-0.00	0
Chick 51	IV-1/2 dose	1.495	3.88	10052	0.113	0.17	350
Chick 52	IV-1/2 dose	1.415	3.66	9448	0.056	0.02	42
Chick 53	IV-1/2 dose	1.726	4.50	11811	0.095	0.13	248
Chick 54	IV-1/2 dose	1.584	4.12	10727	0.051	0.01	19
Chick 55	IV-1/2 dose	1.589	4.13	10765	0.045	-0.00	0
Chick 56	IV-1/4 dose	1.045	2.67	6701	0.062	0.04	71
Chick 57	IV-1/4 dose	1.139	2.92	7391	0.074	0.07	134
Chick 58	IV-1/4 dose	0.799	2.01	4923	0.045	-0.00	0
Chick 59	IV-1/4 dose	0.828	2.09	5130	0.068	0.05	102
Chick 60	IV-1/4 dose	1.415	3.66	9448	0.043	-0.00	0
Chick 61	V-1 dose	2.482	6.52	17710	0.056	0.02	42
Chick 62	V-1 dose	1.4	3.62	9335	0.046	-0.00	0

Chick 63	V-1 dose	1.969	5.15	13685	0.047	0.00	2
Chick 64	V-1 dose	2.017	5.28	14058	0.065	0.04	87
Chick 65	V-1 dose	1.998	5.23	13910	0.047	0.00	2
Chick 66	V-1/2 dose	1.626	4.23	11046	0.045	-0.00	0
Chick 67	V-1/2 dose	1.874	4.89	12950	0.043	-0.00	0
Chick 68	V-1/2 dose	1.693	4.41	11558	0.076	0.07	144
Chick 69	V-1/2 dose	1.213	3.12	7939	0.047	0.00	2
Chick 70	V-1/2 dose	2.744	7.23	19796	0.118	0.19	378
Chick 71	V-1/4 dose	1.722	4.49	11780	0.052	0.01	23
Chick 72	V-1/4 dose	1.731	4.51	11849	0.043	-0.00	0
Chick 73	V-1/4 dose	1.975	5.17	13732	0.061	0.03	66
Chick 74	V-1/4 dose	2.003	5.24	13949	0.094	0.12	242
Chick 75	V-1/4 dose	1.505	3.91	10127	0.049	0.00	10

pre-immune sera titres are presented in the table 25. Out of 100 pre-immune sera, only one serum sample had an IBDV neutralizing antibody titre greater than 396 at the day of IBD vaccine immunization. Chicks immunized with 1 dose, 1/2nd dose, and 1/4th dose of commercial IBD vaccines showed significant neutralizing antibody titres ranging from high to medium. Chicks immunized with commercial vaccine V revealed a high protective antibody titre, while vaccine II showed the lowest VN antibody titre among all selected vaccines. The potency ranking of each vaccine based on the antibody titre was as follow V >IV>I >III >II.

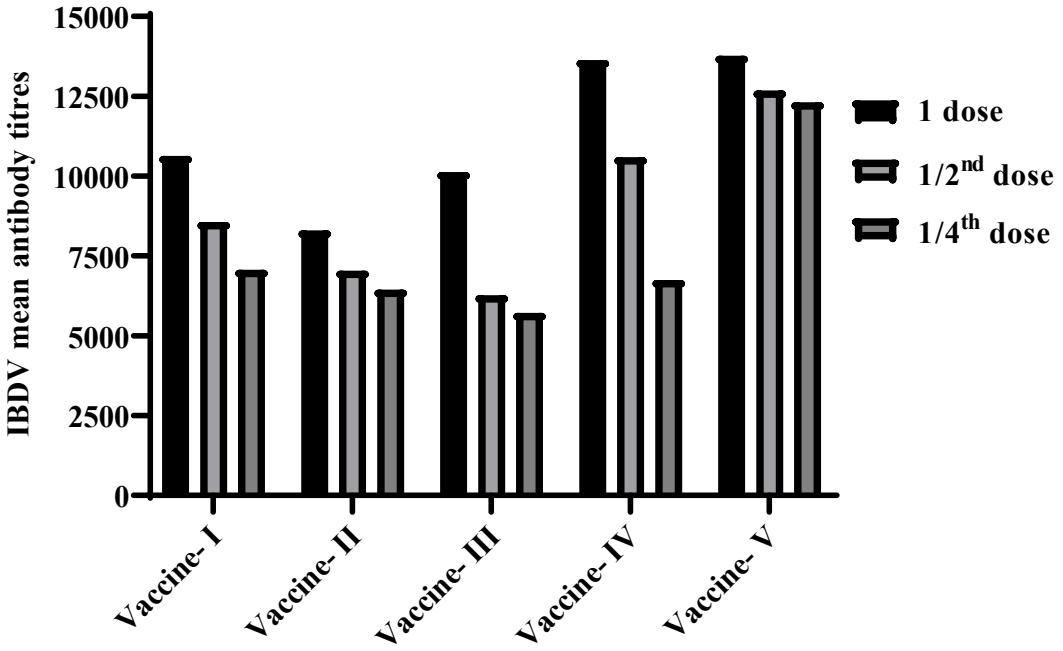


Fig. 22: Serum IBDV antibody titres of the immunized chicks at 21 days post-immunization with three different doses of each IBD vaccine

In a separate study, chicks immunized with 1 dose, 1/2.5th dose, 1/5th dose, 1/10th dose, 1/20th dose, 1/40th dose, 1/50th dose, and 1/100th dose showed a significant decrease in protective antibody titre ranging from high, medium, low, to negative as per the IDEXX ELISA kit interpretation. The result of the serological response of chicks immunized with different dose volumes (1 dose, 1/2.5th dose, 1/5th dose, 1/10th dose, 1/20th dose, 1/40th dose, 1/50th dose, and 1/100th dose) showed a gradual fall in the antibody titre (Table 26 and Fig. 23). The result of the IBDV neutralizing antibody titre showed that out of all dose volumes, only 1/100th of the dose volume was considered negative in accordance with the IDEXX ELISA test.

Table 26: IDEXX IBD-XR Ab ELISA titre in chicks on day of immunization and 21 days post-immunization with different doses of the new IBD vaccine III

		21 days post-immunization				Pre-immune serum		
		OD650	S/P	Titres		OD650	S/P	Titres
	C-	0.0445	0	0				
	C+	0.366	1	2291				
Chick 76	1 dose	1.201	3.59	9247		0.047	0.00	12
Chick 77	1 dose	1.493	4.50	11819		0.05	0.00	27
Chick 78	1 dose	1.171	3.50	8986		0.052	0.02	38
Chick 79	1/2.5 dose	0.748	2.18	5379		0.046	0.00	7
Chick 80	1/2.5 dose	0.955	2.83	7125		0.045	0.00	2
Chick 81	1/2.5 dose	0.874	2.58	6437		0.045	0.00	2
Chick 82	1/5 dose	0.67	1.94	4732		0.047	0.00	12
Chick 83	1/5 dose	0.671	1.94	4740		0.049	0.01	22
Chick 84	1/5 dose	0.68	1.97	4815		0.047	0.00	12
Chick 85	1/10 dose	0.614	1.77	4272		0.052	0.02	38
Chick 86	1/10 dose	0.603	1.73	4182		0.047	0.00	12
Chick 87	1/10 dose	0.62	1.79	4321		0.047	0.00	12
Chick 88	1/20 dose	0.503	1.42	3373		0.044	-0.00	0
Chick 89	1/20 dose	0.52	1.47	3510		0.047	0.00	12
Chick 90	1/20 dose	0.512	1.45	3445		0.048	0.01	17
Chick 91	1/40 dose	0.197	0.47	1016		0.048	0.01	17
Chick 92	1/40 dose	0.202	0.48	1052		0.051	0.02	33
Chick 93	1/40 dose	0.199	0.48	1031		0.049	0.01	22
Chick 94	1/50 dose	0.133	0.27	561		0.055	0.03	55
Chick 95	1/50 dose	0.135	0.28	575		0.049	0.01	22
Chick 96	1/50 dose	0.116	0.22	445		0.046	0.00	7
Chick 97	1/100 dose	0.102	0.17	351		0.046	0.00	7
Chick 98	1/100 dose	0.105	0.18	371		0.046	0.00	7
Chick 99	1/100 dose	0.107	0.19	384		0.053	0.02	44

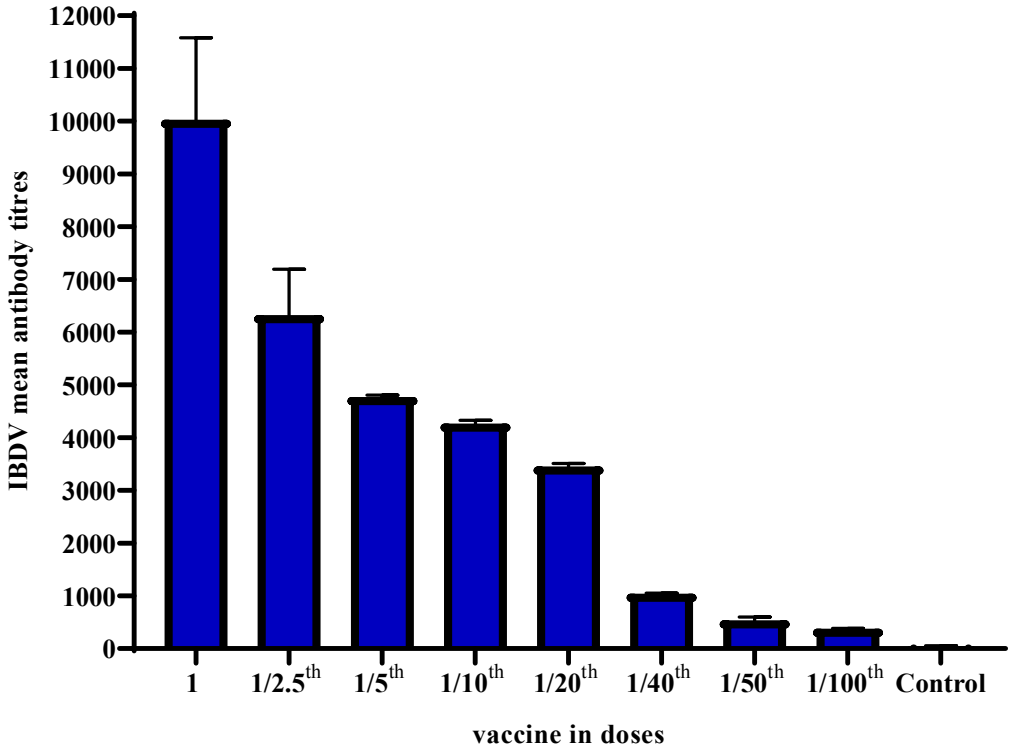


Fig. 23: Serum IBDV antibody titres of the immunized chicks at 21 days post-immunization with eight different doses of IBD vaccine

4.4.1.2: Correlation of results of IDEXX ELISA, sELISA, and ddPCR for inactivated IBDV vaccine

Serological test (IDEXX ELISA) of serum collected from the different groups of chicks vaccinated with full, 1/2nd, and 1/4th dose of inactivated vaccine, antigen quantification by sELISA from 2, 1, 1/2nd, 1/4th, and 1/8th dose of inactivated vaccine and copy number in same doses were established. Serological test and antigen quantification assay was found well correlated as r value was found to be 0.9029 (Fig 24). The correlation was found to be even better when we increased the dose regimen from 1 to 1/100th dose (r = 0.9418) (Fig 25).

Ranks of the vaccines based on the antibody titre, amount of antigen (proportional to the OD_{450} value), and copy numbers of the VP2 gene were calculated and mentioned in the table 27.

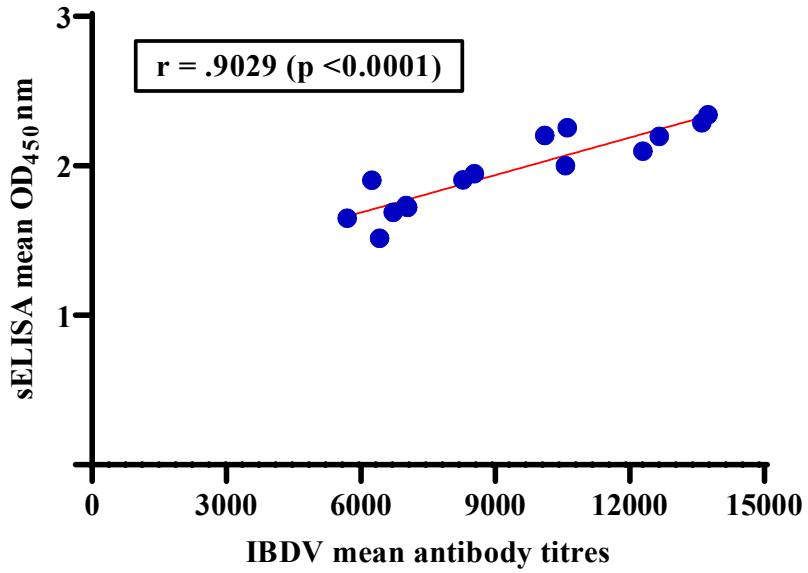


Fig. 24: Correlation between antigen content at three different doses of IBD vaccines by using Sandwich ELISA and serological response after immunization by using IDEXX ELISA

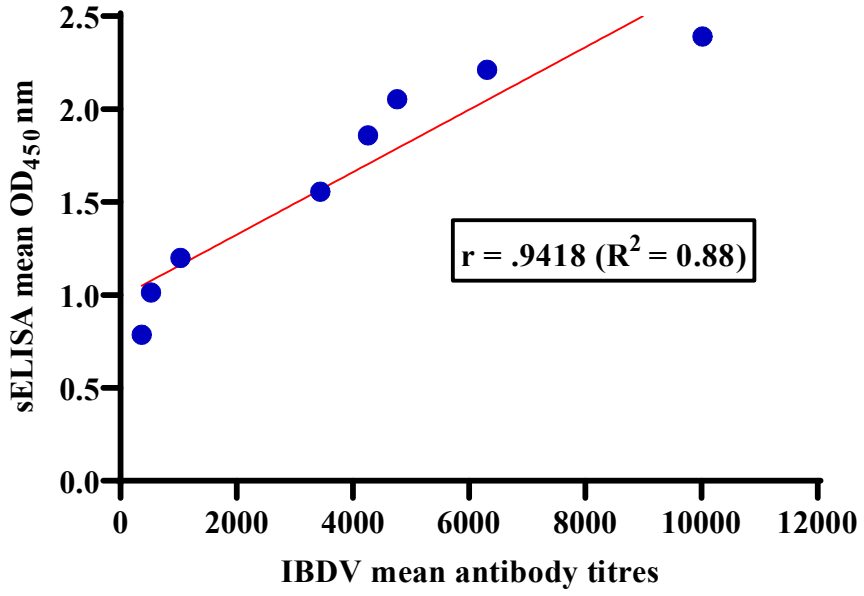


Fig. 25: Correlation between antigen content at eight different doses of IBD vaccines by using Sandwich ELISA and serological response after immunization by using IDEXX ELISA

Table 27: Rank of the IBD vaccines by standard and alternative methods

Vaccine	Dose	Serology (Standard test rank)	Antigen quantification	Copy number
V	Full	1	1	1
	1/2nd	1	1	1
	1/4th	1	1	1
IV	Full	2	2	3
	1/2nd	2	2	3
	1/4th	3	3	3
I	Full	3	3	2
	1/2nd	3	3	2
	1/4th	2	2	2
III	Full	4	4	4
	1/2nd	5	4	4
	1/4th	5	4	4
II	Full	5	5	5
	1/2nd	4	5	5
	1/4th	4	5	5

4.4.2 Correlation of rabies virus neutralizing antibody titre (RFFIT titre), antigenic mass, and copy number

RFFIT titre in serum collected from the mice vaccinated with 1/5th dose of different vaccines and quantity of antigen were very well correlated as r-value was found to be 0.94.

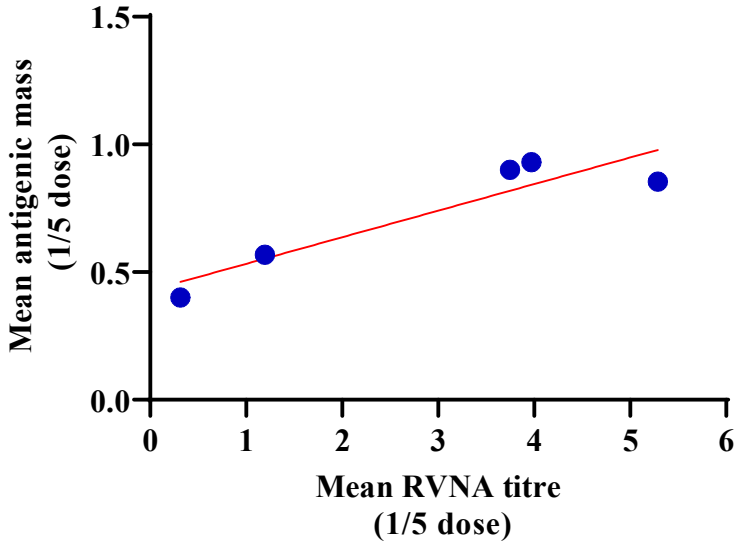


Fig. 26: Correlation between antigen content of different rabies vaccines at 1/5th dose (Sandwich ELISA) and serological response after immunization with 1/5th dose (RFFIT)

Ranks of the vaccines based on the antibody titre, amount of antigen (proportional to the OD_{450} value), and copy numbers of the glycoprotein gene were given and mentioned in the table 28

Table 28: Rank of the rabies vaccines by standard and alternative methods

Vaccine	Dose	Serology (Standard test rank)	Antigen quantification	Copy number
A	1/5th	1	3	3
B	1/5th	2	1	1
C	1/5th	3	2	2
D	1/5th	4	4	-
E	1/5th	5	5	5

Discussion

5 DISCUSSION

Potency testing of veterinary vaccines is generally conducted in animals by post-vaccination challenge or serological methods. Attempts are currently being made all over the world to follow the 4Rs concept, such as reducing the number of animals (reduction), using humane endpoints (refinement), using serology instead of immunization-challenge tests (a combination of reduction and refinement), using antigen quantification or molecular methods for quantification of antigen/nucleic acid per dose in the vaccine (replacement), and reusing animals after experimentation (rehabilitation) (Hendriksen, 2007; Jennings *et al.,* 2010; Woodlands, 2011; Hans and Drayer, 2011; and Romberg *et al.,* 2012). For live vaccines, virus titration or live bacterial count/dose has been considered as an alternative test in the recent years and mentioned in many Pharmacopoeias for batch release. In case of inactivated vaccine, quantification of antigen /dose of the vaccine by antigen quantification assays particularly sandwich ELISA has been considered as an alternative test for few vaccines (NDV, EHV-1, EHV-4, canine coronavirus etc.) and mentioned in the latest addition of European Pharmacopoeia and accepted by Animal and Plant Health Inspection Service (APHIS), USDA. The present study was designed in the similar direction and focused on alternative tests for inactivated rabies and IBD vaccines using molecular and immunological approaches (serology and antigen quantification).

In the recent years, research related to absolute quatification of copy number of viral genes in the live vaccines and their relationship with virus titre have been conducted to find out the possibilities of quantitative real-time PCR as an alternative to virus titration for potency testing of several vaccines such as Camelpox and Buffalopox vaccine (Prabhu *et al*., 2012),

Goatpox vaccine (Kallesh *et al*., 2009), Measles vaccine (Schalk *et al*., 2004), Rotavirus vaccine (Ranheim, 2006), Multivalent MMR vaccine (Schalk *et al*., 2005). The quantification of copy number by real-time PCR needs a highly precise standard curve. Keeping the above possibilities in mind, one of the objective of present study was focused on the quantification of virus genes in inactivated vaccines using digital droplet PCR (ddPCR). The quantification of gene copy number without the need for a standard curve makes this technique more reliable than the quantitative real-time PCR. It was previously reported that ddPCR was used to quantify viruses during influenza vaccine preparation in cell culture and subsequent downstream purifying steps (Veach *et al*., 2015).

In the present study, the copy number of the glycoprotein gene of rabies in the antigen extracted from different doses (2 dose, 1 dose, $1/5^{th}$, $1/25^{th}$, and $1/125^{th}$) of rabies vaccine and the copy number of the VP2 gene of IBDV in the antigen extracted from different doses (2 dose, 1 dose, $1/2^{nd}$, $1/4^{th}$, and $1/8^{th}$) of IBD vaccine were determined by ddPCR. Glycoprotein and VP2 genes were selected for ddPCR, as proteins coded by these genes are known to induce neutralizing antibodies against rabies and IBDV viruses, respectively (Prehaud *et al.,* 1989; Macreadie *et al.,* 1990). Globally, a variety of RABV strains (CVS, Pasteur RIVM, Louis Pasteur's original 1882, PV-11, VP12) and IBDV strains (D78, NEV39, 228E, Georgia, Delaware variant) have been used for the preparation of these vaccines. Therefore, IBDV and rabies primers were designed to amplify all strains currently used in the respective vaccines.

Worldwide, inactivated rabies vaccines are prepared by inactivation of rabies vaccine strains by beta-propiolactone (BPL), or acetylethylamine, or binary ethylenimine (BEI). These agents act on the nucleic acids (RNA/ DNA) leading to inactivation of virus (Groseil *et al*., 1995). In the present study, five different vaccine batches, in which rabies virus was inactivated by BEI (A and E) or BPL (B and C) or undisclosed chemical (D) were used for quantification of nucleic acid. The copy number glycoprotein gene in the $1/5^{th}$ dose of BPL inactivated vaccines (10304000 in B, 9600000 in C) were found more than the BEI inactivated vaccines (27760 in A). Contrary to this, by RFFIT (WHO recommended test), BEI inactivated vaccine A induced more RVNA titre (5.28 IU/ml) in the immunized mice than BPL inactivated vaccine B (3.97 IU/ml) and C (3.74 IU/ml) at $1/5^{th}$ dose. These findings indicated that damage of

nucleic acid by BEI was more as compared to BPL and was supported by the earlier observation (Groseil *et al*., 1995). Hence, quantification of nucleic acid in BPL or BEI inactivated vaccines had given erroneous results (low/incorrect copy number) due to damage of targeted nucleic acid. Therefore care should be taken for determination of copy number in inactivated rabies vaccines and types of inactivating agents should also be considered for determination for copy number. However, for determination of copy number in live rabies virus, ddPCR was found most suitable and good correlation (r = 0.999) was established between virus titre ($FFID_{50}$) and the copy number/reaction of the immunogenic gene of the rabies virus. The correlation was also calculated between virus titre ($FFID_{50}$/mL) and the copy number/mL of glycoprotein gene (r = 0.999). Same observations have been reported by other workers, where copy number of viral gene showed good correlation with virus titre of African rabies and rabies-related lyssaviruses by using quantitative real-time PCR assay (Coertse *et al*., 2010).

On the other hand, inactivated IBD vaccines were prepared by inactivation of IBD virus by denaturing of the viral protein by formalin without any deteriorating effects on nucleic acid (Habib *et al.,* 2006). Therefore, copy number of each dose of vaccine (2 dose, 1 dose, $1/2^{nd}$, $1/4^{th}$, and $1/8^{th}$) determined by ddPCR was found correlated with the amount of the antigen and antibodies response induced by the antigen present in the same doses of IBD vaccine of different manufacturers (I-V). All the vaccines got the same rank in almost all doses based on the copies number of VP2 gene by ddPCR, antigen quantification by sELISA and antibody titre induced by different doses of inactivated IBD vaccine. Based on the present study, ddPCR may be applied for copy number determination of formalin inactivated IBD vaccine and may be used to determined comparative efficacy of different IBD vaccines.

In order to determine the potency of inactivated rabies vaccines for humans and animals, rapid fluorescent focal inhibition test (RFFIT) is a widely accepted alternative to the mouse challenge test (Smith *et al.,* 1996; Moore and Hanlon, 2010; World Health Organization/ Department of Control of Neglected Tropical Disease, 2018, European Pharmacopoeia, 2018). The present study was also aimed to replace RFFIT with the in-house developed competitive ELISA (cELISA) which leads to avoid the handling of rabies challenge virus standard (CVS) in the laboratory to conduct RFFIT. This in-house cELISA was already optimized and validated

in three different institutes in the earlier study. Based on the 130 pre-immune and post-immune human samples tested by cELISA and commercial ELISA (Platelia Rabies II kit, Bio-Rad Laboratories Inc.) in the earlier study, the relative sensitivity and specificity of in-house cELISA was claimed to be 100 %. In the present study, we evaluated comparative performance of cELISA and RFFIT for determination of RVNA in mice immunized with 1/5th dose five commercial rabies vaccines and a reference standard. A single dose (1/5th dose) was used in the present study to minimize the number of mice as per the guideline of European Pharmacopoeia 2021. A comparison of mean RVNA titres (RFFIT), mean percent inhibition (cELISA), and mean survival rate (mouse protection test) in six groups of mice immunised with five different vaccines along with standard vaccine, and one group of control mice was performed for this purpose. RVNA titre of RFFIT and PI value of cELISA in serum samples taken from mice were showed excellent correlation as 0.9308 r-value was obtained using two-tailed Spearman correlation analysis (Zar 1972). The comparative efficacy of all the five different vaccines (A-E) was also found to be same by both RFFIT and cELISA. The good correlation was observed between PI value and percentage protection after challenge as r value 0.9092 was obtained. All mice with detectable rabies antibodies survived challenge, and all with no detectable antibody succumbed. None of the mice with levels below the cut-off (0.5 IU/mL) survived the challenge. However, in 5 out of 34 mice, the opposite trend occurred, where "positive" animals with antibody titres greater than 0.5 IU/mL succumbed. As mouse-challenge test involved a living system, it suffered from frequent invalid results and high variability as reported in the earlier studies (Bruckner *et al*., 1986, Barth *et al*., 1988, Perrin *et al*., 1990, Wilber *et al*., 1997). In conclusion, in-house cELISA can be used as an alternative to RFFIT for determination of RVNA in the mice and would be a promising test for mice serology based potency testing of rabies vaccine.

In the recent years, antigen quantification based ELISAs for potency testing of several vaccines has been accepted by regulatory authorities of many countries. Theses ELISAs based alternative methods have been included in EP and 9CFR for potency testing of several inactivated vaccines such as inactivated polio vaccines, Hepatitis A, Hepatitis B, NDV vaccine (Ph. Eur. Monograph. 870; Hendriksen, 2007; Claassen *et al.,* 2004), canine coronavirus vaccine

(USDASAM 322. 2007), bovine respiratory disease vaccine (9CFR113.216 BRV; 9CFR113.115 BVD), Leptospira vaccine (USDA), EHV-1 and EHV-3 (USDA-APHIS) etc. The present study was aimed at the same direction, therefore suitability of in-house developed sELISA for quantification of extracted antigen from 1/5th dose of five inactivated vaccines was compared with RFFIT titre induced by 1/5th dose of the same vaccine batches. In the present study, antigen extracted from the five different doses of inactivated adjuvanted rabies vaccines were tested with different units antigen in freeze dried standard vaccine by sELISA. The higher OD_{450} values of different doses [2 IU (double), 1 IU (full), 0.2 IU (1/5th), 0.04 IU (1/125th) , 0.008 IU (1/625th), and 0.0016 IU (1/3125th)] of standard vaccine (1 IU/ml) were obtained as compare to same doses of test vaccines (A, B, C, D, and E), even standard vaccine induced low mean RFFIT titre (1.05 IU/mL) in mice immunized with at 1/5th dose (0.2 IU). The low OD values in sELISA were due to partial recovery of antigen from the adjuvanted vaccines and high RFFIT titres were due to effects of adjuvant to enhance antibody mediate response of test vaccines as compared to freeze-dired standard vaccine (Pulendran *et al*., 2021). Therefore, pre-qualified inactivated and adjuvanted vaccines should be quantified along with the inactivated test vaccine by sELISA to determine the relative potency of test vaccine. Freeze-dried standard vaccine was not found suitable and should be avoided for determination of relative potency of liquid vaccine as complete recovery of antigen from the liquid vaccine is not possible by any methods. The OD_{450} values of commercial rabies vaccines A, B, and C revealed high antigenic mass, while vaccine E showed lesser antigenic mass in all doses. Vaccine D had an antigenic mass in between. The antigen content of different rabies vaccines at 1/5th dose (Sandwich ELISA) and the serological response after immunization with 1/5th dose (RFFIT) had an excellent correlation. The calculated r-value was 0.94.

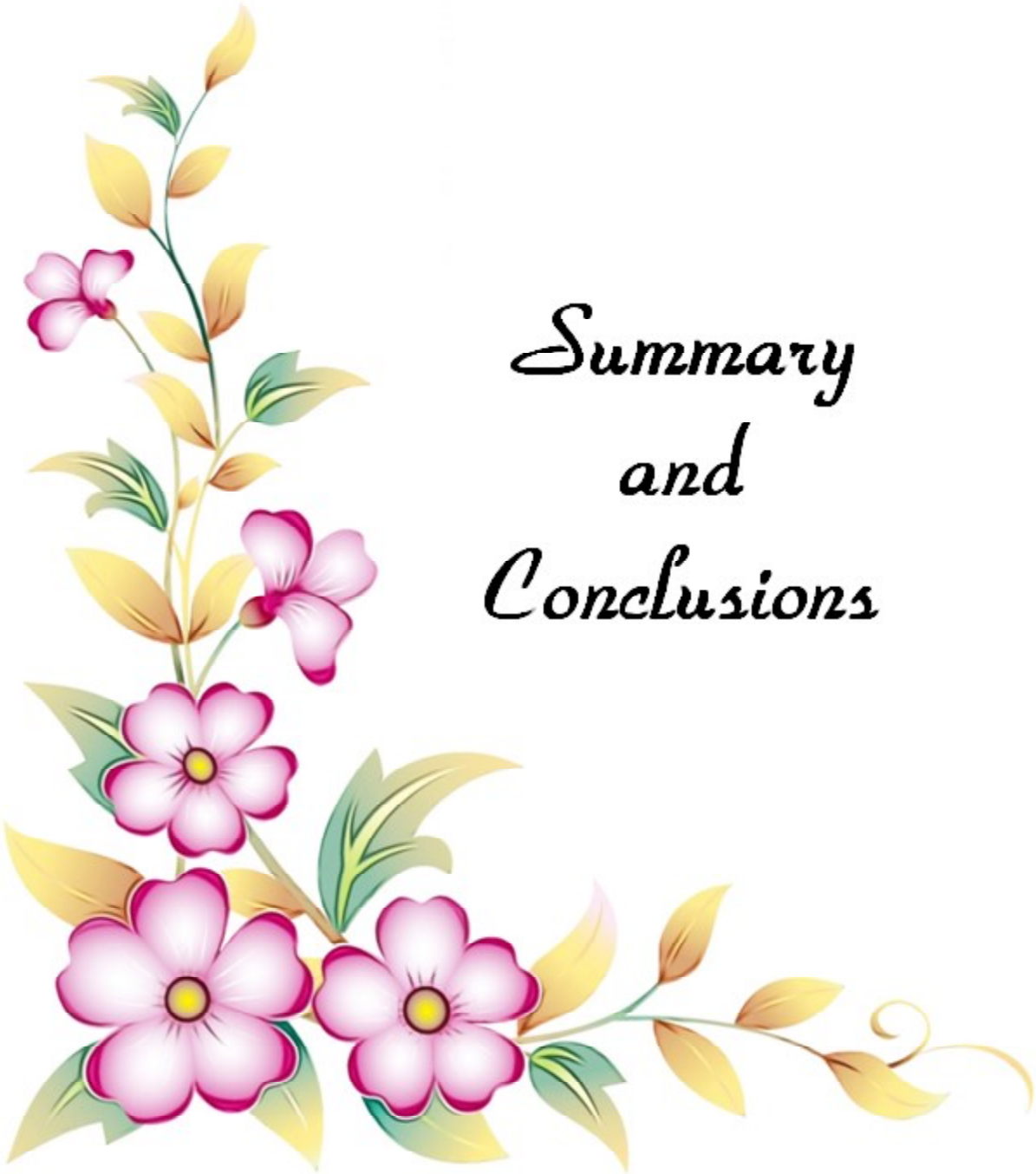
Serological methods are commonly used for potency testing of several inactivated poultry viral vaccines including Newcastle disease, infectious bronchitis, EDS, reovirus, CIAV etc. (Goddard *et al*., 1988; Horvath *et al*., 1999; Indian Pharmacopoeia, 2018). The serological method is also used for potency testing of inactivated IBD vaccine for determination of antibody titre in the immunized chicks (Indian pharmacopeia, 2018). In the present study, antibody titre induced by three different doses (full, 1/2nd, 1/4th) of five commercial batches (I-V) of IBD

vaccine in chicks was monitored using the IDEXX IBD-XR ELISA kit. Serological analysis revealed that immunization of chicks produced protective mean antibody titres ranging from high 13740 to medium 5690, whereas all the 100 pre-immune sera samples except 1 were found negative in IDEXX ELISA. Earlier studies of IBDV antibody immune response in vaccinated chicks have been indicated three range of antibody titre from high (>10000), medium (5000-9000), and low (<4000) (IDEXX Poultry Diagnostic). In the present study, Chicks immunized with commercial vaccine V revealed highest antibody titre in all the doses, while vaccine II showed the lowest antibody titre in all the doses among all selected vaccines. All the serum samples collected from different groups of chicks vaccinated with $1/4^{th}$ dose of vaccine batches were also showed significant antibody titre ranging from 12288 to 5690. Therefore, to determine the end point titre as per IDEXX kit (396), another experiment was conducted by immunizing the chicks with different doses (full, $1/2.5^{th}$, $1/5^{th}$, $1/10^{th}$, $1/20^{th}$, $1/40^{th}$, $1/50^{th}$, $1/100^{th}$) to get end point titre (396). The result of the IBDV antibody titre in the serum of different groups chicks, serum collected from $1/100^{th}$ dose immunized chicks was considered negative in accordance with the IDEXX ELISA test.

In the subsequent study, antibody response induced by different doses of inactivated IBD vaccine with quantity of antigen present in these doses was determined by in-house sELISA. The OD_{450} values of commercial IBD vaccines I, III, IV, and V revealed high antigenic mass, while vaccine II showed less antigenic mass in all doses. In this ELISA, we have found that the vaccine V contains the highest antigenic mass (proportional to the OD_{450} value). A good correlation was demonstrated between the antigen content of inactivated IBD vaccines, and the serological response after immunization with 1, $1/2^{nd}$, and $1/4^{th}$ dose of IBD vaccine ($r = 0.9029$). The correlation was found to be even better between serological test and sELISA, when we increased the dose regimen from 1 to $1/100^{th}$ ($r = 0.9418$). Same ranking of all five IBD vaccines (I-VI) was obtained by both serological test and sELISA. Similar type of method for ranking of inactivated vaccine was followed for evaluation of sELISA based alternative potency test of inactivated NDV vaccine (Claassen *et al*., 2004).

Based on the above results, the serological potency assay and the *in vitro* antigen

quantification methods complemented each other in their efficiency. Out of these tests, cELISA of rabies and sELISA of IBDV were found as promising alternative methods for potency testing of these vaccines. These tests should further be used along with standard methods to generate sufficient data for at least twenty batches of each vaccine. Such studies are crucial for the acceptance of *in vitro* tests by regulatory authorities and inclusion of these *in vitro* methods in Indian Pharmacopoeia.



Summary and Conclusions

6 SUMMARY AND CONCLUSIONS

The purpose of this study was to establish a reliable alternative test for inactivated rabies and IBD vaccines to completely replace or minimize the use of animals for potency testing. One of the aims of present work was to develop the ddPCR assay for copy number determination of inactivated rabies and IBD vaccine viruses. Furthermore, ddPCR was also carried out to determine the absolute copy number of the immunogenic gene in the live rabies and IBD viruses. Initially, annealing temperature and template concentration in RT-PCR for glycoprotein gene of rabies and VP2 gene of IBDV were optimized. The distinction in signals between the fluorescent channels were found maximum at 55°C annealing temperature for rabies virus and at 58.8°C annealing temperature for IBD virus. In the present study, the copy number of the glycoprotein gene of rabies in the antigen extracted from different doses (2 dose, 1 dose, $1/5^{th}$, $1/25^{th}$, and $1/125^{th}$) of rabies vaccine and the copy number of the VP2 gene of IBDV in the antigen extracted from different doses (2 dose, 1 dose, $1/2^{nd}$, $1/4^{th}$, and $1/8^{th}$) of IBD vaccine were determined by ddPCR.

The different batches of inactivated rabies vaccines selected in the present studies from different manufacturers (A, B, C, D, and E). As rabies virus inactivated by BEI (A and E) or BPL (B and C) or undisclosed chemical (D) were used for quantification of nucleic acid. These agents act on the nucleic acids (RNA/ DNA), leading to the inactivation of viruses. At $1/5^{th}$ dose of rabies vaccine, highest copy number was detected in vaccine B (10304000), followed by vaccine C (9600000), A (27760), and the lowest in vaccine E (17160). The ddPCR of vaccine D was not conducted as amplification was not observed in the RT-PCR from the cDNA generated from vaccine D. In contrast, the BEI inactivated vaccine A induced

a higher RVNA titre (5.28 IU/mL) in immunised mice than the BPL inactivated vaccines B (3.97 IU/mL) and C (3.74 IU/mL) using the WHO-recommended RFFIT test at 1/5th dose. These findings indicated that BEI caused more damage to nucleic acid than BPL and were supported by several earlier observations. Therefore, quantification of nucleic acid in BPL or BEI inactivated vaccines by ddPCR had given erroneous results (low or incorrect copy number) due to damage to the targeted nucleic acid. Therefore, care should be taken for the determination of copy number in inactivated rabies vaccines, and types of inactivating agents should also be considered for the determination of copy number. Contrary to inactivated rabies vaccine, for determination of copy number in live rabies virus, ddPCR was found most suitable, and a good correlation was established with virus titration. In ddPCR, an excellent correlation (r = 0.999) was found between virus titre ($FFID_{50}$/mL) and the copy number/ml of the immunogenic gene of the rabies virus.

The different batches of inactivated IBD vaccines were selected from different manufacturers (I, II, III, IV, and V). The IBDV virus in these vaccines was inactivated by formalin. In a single dose, the highest copy number was detected in vaccine V (3960000), followed by vaccine I (3520000), vaccine IV (3480000), vaccine III (3280000), and the lowest in vaccine II (2800000). As formalin inactivates viruses through the cross-linking of virus surface proteins, BEI/BPL inactivates viruses mainly via the acetylation or alkylation of virus DNA or RNA. According to these findings, the damage to nucleic acid caused by formalin was negligible when compared to that caused by BPL. Therefore, quantification of nucleic acid in formalin-inactivated IBD vaccines gave accurate results (correct copy number) as there was no damage to targeted nucleic acid. Therefore, ddPCR may be used for absolute quantification of immunogenic VP2 gene in inactivated IBD vaccine.

Potency testing of inactivated rabies vaccines mostly carried out using the gold standard RFFIT test and mouse protection test to determine the antibody titre developed after immunizing mice. With the objective of adapting a new alternative method for the batch release of inactivated rabies vaccine, we evaluated the suitability of the use of in-house cELISA for the alternative potency test instead of the *in vivo* mouse challenge test and gold standard RFFIT test. We evaluated its performance on mice immunized with 1/5th dose of five commercial rabies vaccines

and a reference standard (1 IU/mL). RFFIT and cELISA were used to determine antibody titre in mice vaccinated with 1/5th doses of rabies vaccine batches from five different manufacturers (A–E). Following that, all groups of immunized mice were challenged with 50 LD_{50} CVS-11. The antibody titres (IU/mL) obtained by RFFIT were marginally the same as those of percentage inhibition (PI) obtained by cELISA; there was a good correlation between the two (r = 0.9308). It can also be observed that there is a good correlation between RFFIT and cELISA with the mouse protection test. In regards to the correlation of serology results to survival from challenge, the relationship was mostly absolute: all mice with detectable rabies antibodies survived challenge, and all with no detectable antibody succumbed. None of the mice with levels below the cut-off (0.5 IU/mL) survived the challenge. However, in 5 out of 34 mice, the opposite trend occurred, where "positive" animals with antibody titres greater than 0.5 IU/mL succumbed. As the mouse challenge test involved a living system, it suffered from frequent invalid results and high variability, even though the mean % inhibition (cELISA) was found to be well correlated with the mean survival rate (r = 0.9092).

The RFFIT titre induced by 1/5th dose of inactivated vaccine and amount of extracted antigen quantified by in-house developed sELISA were also compared in the present study. All the vaccines selected for potency testing were in liquid form and contained adjuvant. Antigen extraction was done from the different doses of inactivated rabies vaccine by using chemical methods of extraction. The OD_{450} value of different doses of vaccines was determined. In sELISA for rabies, antigens extracted from the 5 different doses (2 doses, 1 dose, 1/5th, 1/25th, and 1/125th) of rabies vaccine (A-E) and standard vaccine at different antigen masses (2-0.0016 IU) showed a significant OD value at 450 nm. The potency rankings of each vaccine based on the antigenic mass were as follows: B >C >A >D >E. There was a strong correlation between the antigen content of different rabies vaccines at 1/5th dose (Sandwich ELISA) and the serological response following immunization with 1/5th dose (RFFIT). R-value was calculated to be 0.94.

The serological assay for inactivated IBD vaccine was evaluated as per the IDEXX IBD-XR ELISA kit protocol to know the protective titre developed after immunizing chicks with 3 different doses (full dose, half and 1/4th) of inactivated IBD vaccine (I-V) as well as

eight different doses (full, 1/2.5th, 1/5th, 1/10th, 1/20th, 1/40th, 1/50th, and 1/100th) of a new batch of IBD vaccine (III). Chicks immunized with full dose, half, and 1/4th dose of commercial IBD vaccines showed significant neutralizing antibody titres ranging from high to medium. Chicks immunized with commercial vaccine V revealed a high protective antibody titre, while vaccine II showed the lowest VN antibody titre among all selected vaccines. The potency ranking of each vaccine based on the antibody titre was V >IV >I >III >II. The result of the serological response of chicks immunized with different dose volumes (full, 1/2.5th , 1/5th, 1/10th , 1/20th, 1/40th, 1/50th, and 1/100th) showed a gradual fall in the antibody titre ranging from high, medium, low, to negative as per the IDEXX ELISA kit interpretation. The result of the IBDV neutralizing antibody titre showed that out of all dose volumes, only 1/100th of the dose volume was considered negative in accordance with the IDEXX ELISA test.

The current study further established the relationship between the amount of antigen present in the different doses of the inactivated IBD vaccine and the antibody response that was induced by these doses. In sELISA for IBD, antigens extracted from the 5 different doses (2 doses, 1 dose, 1/2nd, 1/4th, and 1/8th) of the IBD vaccine (I-V) showed a significant high OD_{450} value. In this ELISA, we have found that the vaccine V contains the highest antigenic mass. The potency ranking of each vaccine based on the antigenic mass was V >IV >III >I >II. The serological test (IDEXX ELISA) and antigen quantification assay (sELISA) were found to be well correlated (r = 0.9029). Same ranking of all five IBD vaccines (I-V) was obtained by both serological test and sELISA at full dose. Results of Sandwich ELISA of a IBD vaccine batch at different doses ranging from 1 dose, 1/2.5th dose, 1/5th dose, 1/10th dose, 1/20th dose, 1/40th dose, 1/50th dose, and 1/100th dose showed a significant fall in antigenic mass at different doses. The correlation was found to be even better when we increased the dose regimen from 1 to 1/100th dose (r = 0.9418).

Based on the results of the present study, cELISA of rabies and sELISA of IBDV were found as promising alternative methods for potency testing of these vaccines. These tests should further be used along with standard methods to generate sufficient data for at least twenty batches of each vaccine. Such studies are crucial for the acceptance of in vitro tests by regulatory authorities. Following things should also be considered for using alternative *in vitro* test for potency testing of inactivated rabies and IBD vaccines.

- Pre-qualified inactivated and adjuvanted vaccines should be quantified along with the inactivated test vaccine by sELISA to determine the relative potency of test vaccine.
- Freeze-dried standard vaccine was not found suitable and should be avoided for determination of relative potency of liquid vaccine as complete recovery of antigen from the liquid vaccine is not possible by any methods.


Mini Abstract

7 MINI ABSTRACT

In the present study, two ddPCR were standardized for quantification of immunogenic genes of IBD and rabies virus; cELISA was evaluated as an alternative to RFFIT; and two sELISA were assessed for quantification of antigens in inactivated rabies and IBD vaccines. Droplet digital PCR for the glycoprotein gene of rabies and the VP2 gene of IBDV was optimized at 55°C and 58.8°C, respectively. Copy number of glycoprotein gene in inactivated rabies vaccines of different manufactures by ddPCR gave erroneous results (low/incorrect copy number) even induced high RFFIT titre in mice. This specious result was due to damage of nucleic acid by BPL or BEI at different levels. However, copy number of the VP2 gene in different doses of formalin-inactivated IBD vaccine was found to be correlated with the amount of the antigen and antibodies induced by the different doses. The results of the present study also indicated the suitability of in-house cELISA as a potential alternative to RFFIT. Rabies virus neutralizing antibody titre (IU/mL) by RFFIT in mice induced by $1/5^{th}$ dose of five different vaccines and standard vaccine were showed excellent correlation (r = 0.9308) with the PI value of cELISA in the same mice serum**.** The comparative efficacy of all five different vaccines (A-E) was also found same by both RFFIT and cELISA. The good correlation between PI value and percentage protection after challenge as r value 0.9092 was obtained. In the subsequent study, the RFFIT titre induced by $1/5^{th}$ dose of the inactivated vaccine and amount of extracted antigen quantified by in-house developed sELISA was showed excellent correlation (r = 0.94). The correlation of antibody response (IDEXX ELISA) induced by three different doses (1, $1/2^{nd}$, and $1/4^{th}$ dose) of inactivated IBD vaccine with amount of antigen present in these doses by in-house sELISA also showed a good correlation (r = 0.9029). The correlation was found to be even better when we increased the dose regimen from 1 to $1/100^{th}$ dose (r = 0.9418). The potency ranking of the IBD vaccines based on the antigenic mass and serological response was found almost similar. The present study was conducted in five IBD and rabies vaccine. Based on the results of present study, cELISA of rabies and sELISA of IBDV were found as promising alternative methods for potency testing of these vaccines. These tests should be used along with standard methods to generate sufficient data for at least twenty batches of each vaccine. Such studies are crucial for the acceptance of *in vitro* tests by regulatory authorities.

लघु सारांश

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मौजूदा अध्ययन में, दो ddPCR को IBD और रेबीज वायरस के इम्युनोजेनिक जीन के परिमाणीकरण के लिए मानकीकृत किया गया था, cELISA का RFFIT के विकल्प के रूप में मूल्यांकन किया गया था, और निष्क्रिय रेबीज और IBD टीकों में एंटिजनों की मात्रा का पता करने लिए दो sELISA का मूल्यांकन किया गया था। रेबीज के ग्लाइकोप्रोटीन जीन और IBD के VP2 जीन के लिए ड्रॉपलेट डिजिटल पीसीआर को क्रमशः 55 डिग्री सेल्सियस और 58.8 डिग्री सेल्सियल पर अनुकूलित किया गया था। ddPCR द्वारा अलग–अलग निर्माता कंपनी के निष्क्रिय रेबीज टोकों में ग्लाइकोप्रोटीन जीन की कॉपी संख्या को गलत परिणाम (कम/गलत कॉपी नंबर) आया था, यहाँ तक कि वो चूहों में उच्च REEIT अनुमाप प्रेरित किया था। यह दिखावटी परिणाम विभिन्न स्तरों पर BPL या BEI द्वारा न्यूक्लिक अम्ल को नुकसान पहुँचाने के कारण हुआ। हालांकि, फॉर्मेलिन निष्क्रिय IBD टीका की अलग–अलग खुराक में VP2 जीन की कॉपी संख्या को अलग–अलग खुराक में उपस्थित एंटीजन की मात्रा और प्रेरित एंटीबॉडी की मात्रा के साथ सहसंबंध पाया गया।

मौजूदा अध्ययन के परिणामों ने RFFIT के संभावित विकल्प के रूप में इन हाउस cELISA की उपयुक्तता का भी संकेत दिया। चूहो में पाँच अलग–अलग टीकों और एक मानक टीका की 1/5 वीं खुराक से प्रेरित RFFIT द्वारा रेनीज वायरस को बेअसर करने वाले ऍंडीबॉंडी टाइटर (IU/mL) को उसी चूहों के सीरम के cELISA के PI मान के साथ उत्कृष्ट सहसंबंध स्थापित (r=0.9308) किया गया।

सभी पाँच अलग–अलग टीकों (A-E) की तुलनात्मक प्रभावकारिता भी RFFIT और cELISA दोनों द्वारा समान पाई गई। चैलेंज के बाद PI मान और प्रतिशत सुरक्षा के बीच अच्छा संबंध (r=0.9092) प्राप्त किया गया था। आगामी अध्ययन में, निष्क्रिय टीके की 1/5 वीं खुराक से प्रेरित RFFIT अनुमाप और इन हाउस विकसित SELISA द्वारा परिमाणित निकाले गए एंटिजन की मात्रा से उत्कृष्ट सहसंबंध (r=0.94) पाया गया।

निष्क्रिय IBD टीकों की तीन अलग–अलग खुराक (1, 1/2 और 1/4 खुराक) से प्रेरित ऍंटीबाडी प्रतिक्रिया (IDEXX ELISA) का इन–हाउस विकसित sELISA द्वारा इन खुराकों में मौजूद एंटीजन की मात्रा के साथ एक अच्छा सहसंबंध पाया गया (r=0.9029)। सहसंबंध तब और भी बेहतर पाया गया जब हमने खुराक आहार को 1 खुराक से लेकर 1/100 वीं खुराक तक कर दिया (r=0.9418)। एंटीजेनिक द्रव्यमान और सीरोलॉजिकल प्रतिक्रिया के आधार पर IBD टीकों की क्षमता रैकिंग लगभग समान पाई गई।

मौजूदा अध्ययन पाँच IBD और रेबीज टीकों में आयोजित किया गया था। मौजूदा अध्ययन के परिणामों के आधार पर, रेबीज के cELISA और IBD के sELISA को इन टीकों के शक्ति परीक्षण के लिए आशाजनक वैकल्पिक तरीकों के रूप में पाया गया। प्रत्येक टीके के कम से कम बीस बैचों के लिए प्रर्याप्त डेटा उत्पन्न करने के लिए मानक विधियों के साथ इन परीक्षणों का उपयोग किया जाना चाहिए। नियामक अधिकारियों द्वारा इन–विट्रो परीक्षणों की स्वीकृति के लिए ऐसे अध्ययन महत्वूपर्ण हैं।

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Appendix

APPENDIX

A) Reagents for Agarose Gel Electrophoresis

1. Tris-acetate- EDTA (TAE) buffer 50X

Tris base	242 g
Glacial acetic acid (Central Drug Huse)	57.1 ml
0.5 M EDTA (pH 8.0)	100 ml

Distilled water was added to make the final volume up to 1000 ml. Aworking solution of 1X was used.

2. Ethidium bromide stock solution (10mg/ml)

Ethidium bromide	100 mg
Distilled water	10 ml

The solution was mixed and stored at 4ºC. A concentration of 0.5 ìg/ml was used in preparing agarose gel.

3. 1% Agarose Gel

Agarose (GCC Biotech)	0.8gm
TAE (1x)	80 ml
Ethidium Bromide	4µl

B) Reagents and solutions for ELISA

1. Phosphate buffer saline (PBS) 10X

$Na_2HPO_4.2H_2O$ or NaH_2PO_2	14.4 gm
KH_2PO_2	2 gm
KCl	2 gm
NaCl	80 gm
DW	1000 ml

2. Coating Buffer

Na_2CO_3	0.159 g
$NaHCO_3$	0.293 g
DW	100 ml

pH was adjusted to 9.6, sterilized by filtration and stored at -20ºC as aliquots.

3. Phosphate buffer saline Tween 20 (PBST)

Phosphate buffer saline (1X)	1000 ml
Tween-20 (VWR life Science)	1 ml

4. Washing buffer

10x Phosphate buffer saline	100 ml
Tween-20	500 µl
DW	900 ml

5. Blocking Buffer (5%SMP)

Skim milk powder	5 gm
PBST	100 ml

6. Blocking Buffer (5% SMP +1% Horse Serum)

Skim milk powder	0.25 gm
Horse Serum	50 µl
PBST	5 ml

7. Blocking Buffer (2% BSA +1% Horse Serum)

Bovine SerumAlbumin	0.1 gm
Horse Serum	50µl
PBST	5 ml

8. Blocking Buffer (5% SMP +2% BSA)

Skim milk powder	0.25 gm
Bovine SerumAlbumin	0.1 gm
PBST	5 ml

9. Blocking Buffer (2% SMP)

Bovine SerumAlbumin	0.1 gm
PBST	5 ml

10. Serum and conjugate dilution buffer

Skim milk powder	5 gm
PBST	100 ml

C) MISCELLANEOUS BUFFER AND SOLUTION

1. Extraction Buffer

Disodium EDTA.$2H_2O$	100 mM
Sodium citrate dihydrate	0.60 M
SDS	30 mM

2. CVS diluent

Heat-inactivated, rabies antibody free horse serum	2ml
Penicillin	50, 000 units
Streptomycin	0.1 gram
De-ionized water to make	100 ml

Adjust pH to 7.6 with 7.5% sodium bicarbonate and aliquot 2-ml, 5-ml, 10-ml and 25-ml then store at 4°C.