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Validation of haemagglutination, haemagglutination inhibition and antibody neutralization assays used to determine the titre of CPV -2 virus and anti CVP – 2 egg yolk antibodies

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***Abstract*---**The safety and efficacy of a biological product is determined by the highly variable *in-vivo* or *in-vitro* assays. Hence the manufacturers have to minimize the test variabilities to an acceptable limit through optimization of test conditions and validation as per the regulatory guidelines. In this study the test variables were optimized and validated the methods HA, HI and antibody neutralisation assays. During optimisation of HA method, it was observed that no change in the HA titre either due to incubation temperatures (5 ± 3°C, 25 ± 2°C and 37 ± 2°C) or % of RBC used (1% and 0.5%). But the difference was observed for species of RBC used and time taken for HA reaction. CPV – 2 isolate 1 did not show HA activity with chicken RBC and showed

agglutination with porcine RBC, whereas isolate 2 was showing HA reaction with both chicken and porcine RBC. During validation studies of HA, HI, $CCID_{50}$ and antibody neutralization assays the % CV observed was 31, 31, 1.1 and 2.12 respectively which are less than the expected variability of > 50%.

***Keywords*---**validation, CPV - 2, HA, HI, $CCID_{50,}$ antibody neutralization assay.

Introduction

Canine Parvo viral enteritis is one of the common viral enteric infection of dogs caused by Canine Parvo virus – 2 (CPV - 2). This virus belongs to the family *Parvoviridae*, has a negative sense single strandard DNA and devoid of envelop. It is one of the smallest animal viruses and its size ranges from 20 to 26 nm in diameter. It has an icosahedral symmetry. This virus is causing 100% morbidity in dogs. In adult dogs it causes mortality up to 10%, where as in case of puppies the mortality rate is 91% (Nandi and Kumar, 2010). This viral infection occurs predominantly in young animals due to the declining of the maternal antibodies **(**Hoskins, 1998 and Larson and Schultz, 1997). Currently there is no specific antiviral treatment available for CPV – 2 infection and for other viral infections, only supportive therapy is given. Hence this necessitates to develop novel and alternate approaches to treat and manage any viral infections.

To fasten the recovery rate of the patients, as well as to prevent the patients from clinical infections, purified antibodies raised either in animals or obtained from donors or monoclonal antibodies or convalescent sera are administered by the clinicians. This treatment approach is called as Passive immunotherapy (Schade *et al.,* 1996). The major advantage of this immunotherapy is that the protection of a patient against a particular disease is immediate due to the usage of preformed antibodies, whereas the acquired immunity is a time-consuming process because the generation of antibodies takes time due to various complex mechanisms of immune system.

For routine passive immunotherapy the manufacturers have to produce large quantities of antibodies. One of the ways to produce bulk quantities of polyclonal antibodies is from the egg yolks of immunized chickens against a particular pathogen. The antibodies prepared from egg yolk is called as IgY antibodies. Administering egg yolk antibodies for passive immunotherapy is an accepted practice since 1996 onwards and in 1999 this practice was approved by the Veterinary Office of the Swiss Government (Office Vétérinaire Fédéral). These purified antibodies are being used successfully to treat clinical symptoms caused by venoms, toxins and certain viral infections especially Rabies, Hepatitis A and B, Varicella–zoster virus and Respiratory syncytial virus (Arturo Casadevall *et.al.,* 2004).

Experiments have been carried out to use chicken egg yolk antibodies as a therapeutic agent to treat the infected animals by different researchers. The egg yolk antibodies are given orally to the animals in the form of dried IgY powder

(Nguyen *et al.*, 2006) or intravenous (IV) infusion (Gusti *et al.,* 2014). Prior to release of any pharmaceutical and biological products to the end users their fitness to use is tested either based on quantitative or qualitative assays by the Quality Control department. The efficacy and safety of a product is evaluated either by *in-vivo* (animal based) or *in-vitro* (cell based) biological assays. These biological assays are highly variable in nature and the anticipated variation is > 50% (WHO/VSQ/97.02, 1997). Hence, to increase the reliability, repeatability and reproducibility of the test results the manufactures have to optimize the test variables and validate the assays (Darling *et al.,* 1998).

Validation of an analytical method is defined as, it is documented evidence to demonstrate that the test methods used to determine the quality parameters of an analyte are highly specific, accurate, precise, reliable, repeatable, consistent, reproducible and which could not be affected or influenced by external factors throughout its life cycle. Manufacturers have to validate their analytical methods as a part of licensing their product before supply to the end users as per the regulatory guidelines. Guidelines like ICH Q2(R1), USP (<1225>) and WHO (TRS 1019, 1999) *etc.,* are recommending the parameters to be validated based on the assay's nature and type of products (Table - 1). The parameters to be validated may vary case to case basis.

As a part of Quality control tests, to determine the titre of a virus HA and $CCID_{50}$ methods are being used. Similarly, to determine the titre of antibodies raised against a particular viral antigen, methods like HI and antibody neutralization assays are used. Since these methods are highly variable, this work was planned to optimize the test conditions to minimize the variability and validate these methods to demonstrate their repeatability in determining the titre of CPV - 2 virus and anti CPV - 2 IgY antibodies in the egg yolks of immunized chickens with CPV -2 vaccine strain.

Table 1: Parameters to be validated (ICH Q2R1)

S. No.	Characteristics	Type of analytical procedures			
		Identification test	Testing for impurities		Assay (Content/ potency)
			Quantitative	Limit	
1	Accuracy	-	+	-	+
2	Precision				
i	Repeatability	-	+	-	+
ii	Intermediate Precision	-	+ (1)	-	+ (1)
iii	Reproducibility	+	+	+	+
3	Specificity (2)	+	+	+	+
4	Detection Limit	-	- (3)	+	+
5	Quantitation Limit	-	+	-	-
6	Linearity	-	+	-	+
7	Range	-	+	-	+
8	Robustness*	+	+	+	+
9	Ruggedness **	+	+	+	+

Table – 1 is transcribed from ICH Q2 (R1).
Note:

- Signifies that parameter is not normally evaluated and + signifies that this parameter is normally evaluated
  (1) in case where reproducibility has been performed, intermediate precision is not needed
  (2) lack of specificity of one analytical procedure could be compensated by other supporting analytical procedure(s)
  (3) may be needed in some cases
    - This parameter is recommended by WHO TRS 1019 and USP (<1225>)
    - Performed by the manufacturers in case if they want to change the equipment or as the part of assay development and optimization with different equipment.

As per the recommendations of WHO/VSQ/97.02 (1997) the parameters *viz.*, Accuracy, Recovery and Range need not be considered since no pure material is available and then the inherent assay variability is > 50%.

Materials and Methods

Prior to validation, the assay variables which are having impact on test results were analysed and optimized for reliable and consistent test results.

Hemagglutination (HA) and Haemagglutination inhibition (HI) Tests

The variables (parameters) and their operational conditions which are having direct impact on HA and HI test results are presented in the table 2.

Table 2: Variables having direct impact on HA and HI test results

S. No.	Variables (Parameters)	Conditions employed	Remarks
1	Types of plates	V bottom	The equivalency of both the plates were demonstrated already. Hence the validation was done only with U bottom plate.
		U bottom	
2	Buffers	Normal saline	Only PBS was used in the assays performed in this study since its buffering capacity is more than Normal saline. The BSA was used in the buffer to nullify the non-specific reaction during the test especially with fecal materials. In this work only PBS without BSA was used since the test was carried out with known isolates of CPV - 2.
		PBS	
		Borate buffer	
		PBS + BSA	
3	Species of RBC	Porcine RBC	Helps in differentiating the strains of CPV - 2.
		Fowl (chicken)	
4	Percent of RBC	0.5 % v/v	It may have impact on the results of the titre or time taken for the complete HA
		1% v/v	

			reaction.
5	Incubation temperature	5 ± 3°C	These three conditions were employed to recommend a highly suitable incubation temperature for the test.
		25 ± 2°C	
		37 ± 2°C.	

Note: The fowl and porcine RBCs were prepared and stored in Dulbecco's modified phosphate buffer saline.

Two tests were performed to optimize the HA test with the different variables presented in the table 2. The HA and HI tests were carried out with minor modifications as described by Carmichael *et al.*, (1980) and Kumar *et al.,* (2003). The plates were incubated at different temperatures, 5 ± 3°C, 25 ± 2°C and 37 ± 2°C. PBS was used as a diluent in these assays. The plates were observed every 5mins for complete HA reaction. The positive HA result was indicated by the absence of button formation. The HA titre was expressed as the reciprocal of highest dilution of virus showing agglutination. The HA test was carried out with both the isolates of CPV – 2. The mean HA titre of both the isolates was 256 (2^8). The HI test was carried out with 8HA units of CPV - 2 isolate 2 to determine the titre of the anti CPV -2 antibodies in the egg yolk purified by Sodium chloride (Hodek *et al.,* 2013). The HI plates were incubated at 5 ± 3°C. The HI titre was expressed as the reciprocal of highest purified egg yolk antibody dilution inhibiting the haemagglutination reaction.

The validation of test methods HA and HI were performed with optimized test variables like type of plate, species and % of RBC and incubation temperature and isolate 2 of CPV – 2. To validate both these assays, the parameter repeatability (intra assay precision) was considered. The repeatability expresses the precision under the same operating conditions over a short interval of time. To demonstrate the repeatability of the test method six tests were performed with 100% concentration of the analyte as per ICH guidelines.

Cell culture infective dose ($CCID_{50}$) and antibody neutralization assays

The important assay variables having impact on the test results of $CCID_{50}$ and antibody neutralization assays are suitable cell line, sensitivity of the cell line, cell culture medium, serum, passage level of the cell line, incubation temperature, % Co_2 level and incubation time. The following parameters were considered for test method optimization. These assays were performed under standard and optimized test conditions prescribed in standard cell culture protocols. A72 cell line was used in these assays.

1. Optimum cell concentration required
2. Edge effect

Optimum concentration of cells

For any cell-based assays, it is very important to use optimal cell concentration for reliable results since the duration of the assays ranges from 5 to 10 days based on the replication rate of the virus. In this study, to find out the optimum cell concentration required for the assay, the assays were performed with four different cell concentrations 6 x 10^4 cells / mL, 8 x 10^4 cell / mL, 1 x 10^5 cell / mL

and 1.2 x 10^5 cell/mL. Ten wells were seeded for each concentration and this procedure was repeated thrice to evaluate the repeatability. The optimum concentration of cells for the assay was concluded based on the time taken to form complete monolayer in the seeded wells.

Edge effect

To assess the edge effect (loss of medium due to evaporation during the incubation time), in one of the 96 well plate, the wells in the rows (A1 to A12 & H1 to H12) and in the column (B 1 to G1 and B12 to G12) an additional 50µL growth medium was added and, in another plate, the additional volume of medium was not added. $CCID_{50}$ and antibody neutralization assays were carried out as per the procedures described by Uma *et.al.,* 2022. The antibody neutralisation assay was carried out for the egg yolk antibody samples having peak HI titre. The parameter repeatability was considered to validate the $CCID_{50}$ and antibody neutralisation assays with the optimized test conditions *viz.,* optimal cell concentration and edge effect. Total 6 assays were carried out.

Results

HA results of CPV – 2

The HA results of CPV – 2 isolates 1 and 2 observed during the optimization of the test were presented in the tables 3 & 4.

Table 3: HA titre of CPV -2 isolate 1

Variables (Parameters)	Plate - 1	Plate - 2	Plate - 3	Plate - 4	Plate - 5	Plate - 6	Plate - 1	Plate - 2	Plate - 3	Plate - 4	Plate - 5	Plate - 6
Isolate	CPV Isolate - 1											
Type of plate	U	U	U	U	U	U	U	U	U	U	U	U
Buffer	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS
Species of RBC	Chicken						Porcine					
Percent of RBC	1%	1%	1%	0.5%	0.5%	0.5%	1%	1%	1%	0.5%	0.5%	0.5%
Incubation temperature	5 ± 3°C	25 ± 2°C	37 ± 2°C	5 ± 3°C	25 ± 2°C	37 ± 2°C	5 ± 3°C	25 ± 2°C	37 ± 2°C	5 ± 3°C	25 ± 2°C	37 ± 2°C
HA result - Test 1	Nil	Nil	Nil	Nil	Nil	Nil	256 (45 min)	256 (20min)	256 (25 min)	256 (50 min)	256 (35min)	256 (25 min)
HA result - Test 2	Nil	Nil	Nil	Nil	Nil	Nil	256 (50 min)	256 (25 min)	256 (20 min)	256 (55min)	256 (35 min)	256 (30 min)
Mean	Nil	Nil	Nil	Nil	Nil	Nil	256	256	256	256	256	256

Table 4: HA titre of CPV -2 isolate 2

Variables (Parameters)	Plate - 1	Plate - 2	Plate - 3	Plate - 4	Plate - 5	Plate - 6	Plate - 1	Plate - 2	Plate - 3	Plate - 4	Plate - 5	Plate - 6
Isolate	CPV Isolate – 2											
Type of plate	U	U	U	U	U	U	U	U	U	U	U	U
Buffer	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS
Species of RBC	Chicken						Porcine					
Percent of RBC	1%	1%	1%	0.5%	0.5%	0.5%	1%	1%	1%	0.5%	0.5%	0.5%
Incubation temperature	5 ± 3°C	25 ± 2°C	37 ± 2°C	5 ± 3°C	25 ± 2°C	37 ± 2°C	5 ± 3°C	25 ± 2°C	37 ± 2°C	5 ± 3°C	25 ± 2°C	37 ± 2°C
HA result - Test 1	256 (40 min)	256 (25 min)	256 (15min)	256 (50 min)	256 (30 min)	256 (25 min)	256 (45min)	256 (35 min)	256 (25 min)	256 (60 min)	256 (35min)	256 (30 min)
HA result - Test 2	256 (45 min)	256 (20 min)	256 (15 min)	256 (55 min)	256 (35 min)	256 (30min)	256 (40min)	256 (30 min)	256 (25 min)	256 (55 min)	256 (35 min)	256 (30 min)
Mean	256	256	256	256	256	256	256	256	256	256	256	256

Note: The values presented in the parenthesis of tables 3 & 4 denotes time taken for complete haemagglutination reaction.

From the data presented in the tables 3 & 4, it was observed that CPV – 2 isolate 1 can react only with porcine RBC. Whereas the isolate 2 can react with the RBC of both the species. It was also observed that there was no change in the HA titre of both the virus isolates when the HA plates were incubated at different temperature and % of RBC used in the tests. The only difference observed was time taken for the complete haemagglutination (HA) reaction. The HA reaction was faster in the plates incubated at 37 ± 2°C, followed by the plates incubated at 25 ± 2°C. The HA reaction was much slower in the plates incubated at 5 ± 3°C. Similarly, the time taken for complete HA reaction was more in case of tests performed with 0.5% RBC when compared with the tests carried out with 1% RBC and the chicken RBC gave quicker results when compared with swine RBC.

The validation studies for HA tests were carried out with the optimised test variables based on the results observed during optimisation studies *viz.,* U bottom plate, 1% chicken RBC, CPV – 2 isolate 2 and incubation temperature 5 ± 3°C. The results observed in validation studies are presented in the table 5. Total 6 assays were carried out with 100% analyte concentration. The observed mean HA titre of the CPV - 2 isolate 2 was 213 ± 66 and the percentage of CV observed among the six assays was 31.

HI results

The HI tests for the purified anti CPV – 2 IgY antibodies were performed with the optimized test variables of HA test *viz.,* U bottom plate, 8 HA units CPV -2 isolate 2,1% of Chicken RBC and incubation temperature at 5 ± 3°C. Total 6 assays were performed with 100% analyte concentration to demonstrate the repeatability of the test method. The results are presented in table – 5. The mean HI titre of the anti CPV – 2 IgY antibodies was 853 ± 264 and the percentage of CV was 31.

Table 5: Validation study results

Assay No.	HA titre of CPV -2 isolate 2	HI titre of anti CPV – 2 Purified IgY antibodies	$CCID_{50}$ titre of CPV – 2 isolate 2	PD_{50} titre of purified IgY
1	128	1024	-6.50	- 4.50
2	256	1024	-6.66	- 4.41
3	256	512	-6.60	- 4.65
4	128	1024	-6.63	- 4.53
5	256	512	-6.55	- 4.43
6	256	1024	-6.49	- 4.4
Mean	213	853	-6.57	-4.49
SD	66	264	0.07	0.10
% CV	31	31	1.1	2.12

Determination of titre of CPV - 2 by $CCID_{50}$ method

Three experiments were carrier out to determine the optimum cell concentration required for reliable test results. The results observed in these tests was indicating that the optimum concentration required for the assay was 6000 to 8000 cells / well. In this study it was observed that a healthy monolayer was formed in the wells seeded with 6000 to 8000 cells / well after 72 hrs of incubation and the cells were healthy till 5^{th} day with less number of dead cells. Whereas in case of wells seeded with 10,000 and 12, 000 cells / well a monolayer was formed within 24 hrs of incubation and more number of dead cells were observed on day 5. Hence the validation of $CCID_{50}$ test was performed with 6000 cells / well. The results of validation studies are presented in table – 5. The titre of CPV - 2 isolate 2 was Log $^{-6.57 \pm 0.07}$/ 100µL of virus and the percentage of CV was 1.1. The study results of edge effect indicating that evaporation of medium was observed in the wells A1 to A12, B1 to H1, A12 to G12 and H2 to H12 and the monolayers in the wells were not either healthy or dried.

Determination of Protective dose 50 (PD_{50}) of specific anti CPV -2 purified IgY antibodies (Antibody neutralisation assay)

The validation study results of antibody neutralisation assay for the purified IgY antibodies were presented in the table 5. The PD_{50} of purified anti CPV – 2 egg yolk antibodies of white Leghorn chicken was log -4.49 ± 0.1 / 50µL and the percentage of CV was 2.12.

Discussions

Canine Parvo viral enteritis is one of the most common viral enteric infection of dogs and caused by Canine Parvo virus – 2. This virus is causing 100% morbidity and 10% mortality in case of adult dogs, where as in case of puppies the mortality rate is 91% (Nandi and Kumar, 2010). This viral infection occurs predominantly in young animals due to the declining of maternal antibodies to a lower level **(**Hoskins, 1998 and Larson and Schultz, 1997). Currently no specific antiviral treatment is available for CPV – 2 infection and other viral infections. Only supportive therapy is given. Hence this necessitates to develop alternate

approaches to treat and manage CPV -2 infections. An alternate approach for the treatment is passive immunotherapy. In this therapy the infected animals will be treated with purified antibodies derived from the animal sera or hyper immune serum or convalescent sera. Due to the awareness and implementations of "3 Rs.", the manufacturers are looking for alternate approaches for the production of larger quantities of antibodies and one of the identified options is using avian eggs. The antibodies processed from the egg yolk is called as IgY antibodies. Administering egg yolk antibodies for passive immunotherapy is an accepted practice since 1996 onwards and in 1999 this practice was approved by the Veterinary Office of the Swiss Government (Office Vétérinaire Fédéral). In case of CPV -2 experimental infection, the dogs were fed orally with dried IgY powder (Nguyen *et al.*, 2006) and given intravenous (IV) infusion with purified IgY antibodies (Gusti *et al.,* 2014). The results were encouraging.

In biopharmaceutical industries as a part of quality control of a product either quantitative or qualitative assays are being used. To determine the efficacy and safety of a product either *in-vivo* (animal-based) or *in-vitro* methods (cell based) are being used. These biological assays are highly variable in nature and the anticipated variability is > 50% (WHO/VSQ/97.02,1997). Hence, to increase the reliability, repeatability and reproducibility of the test results manufacturers have to minimize the test variabilities to an acceptable limit by optimising the test conditions and validating the assays. Manufacturers have to validate their analytical methods as a part of licensing their product before supply to the end users as per the regulatory guidelines.

In the Biopharmaceutical industries to determine the titre of a virus or to determine the LD_{50} of the virus and the protective dose 50 (PD_{50}) of a vaccine or antibodies, the methods $CCID_{50}$ or antibody neutralisation assays are used. Hence in this study it was planned to validate the HA, HI, $CCID_{50}$ and antibody neutralisation assays since these methods were critical for the titration of CPV – 2 and anti CPV - 2 IgY antibodies.

During the optimization of HA test, it was observed that the CPV -2 isolate 1 gave positive HA reactions only with Swine RBC not with Chicken RBC. Whereas the isolate 2 gave positive HA reaction with Swine and Chicken RBCs. This result concurs with the observation made by (Bayati *et al.,* 2010). No difference in the HA titre was observed for both the CPV -2 isolates when the plates incubated at 37 ± 2°C, 25 ± 2°C and 5 ± 3°C and % of RBC (1% and 0.5%) used. Whereas Kumar *et al.,* (2003) reported that plates incubated at 2°C gave better result when compared with the plates incubated at 37°C and 23°C to 26°C. This could be due to lab-to-lab variability and strain of virus tested. In this study the only difference observed was time taken for complete hemagglutination reaction and the chicken RBC gave quicker results when compared with the Porcine RBC. This could be due to the chicken RBCs are nucleated and bigger in size. The HA with chicken RBC was also reported in earlier studies (Khan *et al.,* 2006).

In validation studies it was observed that the mean HA titre of CPV - 2 isolate 2 was 213 ± 66 and the percentage of CV was 31 which was less than the expected variability of > 50% (WHO/VSQ/97.02,1997). This indicating that the optimized

test conditions followed in this work for HA method were adequate to give reliable and consistent results.

In validation studies of HI method, it was observed that the mean HI titre of anti CPV – 2 egg yolk antibodies was 853 ± 264 and the percentage of CV was 31 which was less than the expected variability of > 50 % (WHO/VSQ/97.02,1997). This indicating that the optimized test conditions followed in this method were adequate to give reliable and consistent results. During standardisation of $CCID_{50}$ method it was observed that the cell concentration 6000 to 8000 cells / well was adequate to produce CPE with less number of normal dead cells. In the validation studies of $CCID_{50}$ method it was observed that the mean titre of CPV - 2 isolate 2 was Log $^{-6.57 \pm 0.07}$ with percentage of CV 1.1 which is less than the acceptable limit of the assay variability 0.5 log (WHO, TRS 840, Annexure 3, 1994). Hence the optimized test conditions followed in this study were adequate to give reliable and consistent results.

In the validation studies of antibody neutralisation assay the mean PD_{50} value of the anti CPV -2 egg yolk antibodies Log $^{-4.49 \pm 0.1}$ with percentage of CV 2.12 which is less than the acceptable limit of the assay variability 0.5 log (WHO, TRS 840, Annexure 3, 1994). Hence the optimized test conditions followed in this study were adequate to give repeatable, reliable and consistent results.

Conclusions

In this study it was observed that the incubation temperatures and % of RBC have no impact on titre of the CPV -2 isolates 1 & 2. The only difference observed was the time taken for complete HA reactions. The lab can use either porcine or chicken RBC for HA and HI methods based on the sensitivity of the CPV -2 strain. For antibody neutralisation assay the lab can standardize the cell seeding density based on their lab conditions. From this study it is concluded that the manufacturers have to optimise the test variables which can impact the test results and validate their assays in-order to ensure their reliability, repeatability and consistency of their test methods used to determine the quality parameters of their products. They can set appropriate validity criteria for the test methods to ensure that the methods are performed correctly by the analysts and thereby ensuring the quality of the products before release to the end users.

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