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Original Article

Evaluation of live attenuated canine parvovirus vaccines using real-time PCR

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Canine parvovirus infections pose a significant global threat to canine health, necessitating effective vaccination strategies. This study evaluates the viral content in 20 batches of live attenuated canine parvovirus vaccines using real-time polymerase chain reaction and compares the results with the immunofluorescence test. The study involved canine parvovirus strain 39, adapted to Madin-Darby canine kidney cells, and vaccine batches sourced from various local and international suppliers. The immunofluorescence test results showed 18 batches met the permissible titer level of 3 $\log_{10}$ fluorescent antibody infective dose 50%/mL, while two batches (3 and 18) did not. Similarly, real-time polymerase chain reaction analysis confirmed the same 18 batches met the permissible titer level, with no significant difference between the methods as indicated by the 95% confidence interval for the difference in results (lower: 4.3949, upper: 5.3600). The findings support integrating advanced diagnostic technologies like real-time polymerase chain reaction into routine vaccine evaluation protocols, ensuring higher standards of veterinary biologics assessment; this transition aims to enhance the accuracy, efficiency, and overall quality of canine parvovirus vaccine evaluation, ultimately improving canine health protection.

Keywords: vaccine potency; canine parvovirus; vaccines; diagnostic testing; real-time polymerase chain reaction.

Introduction

Parvoviruses of the family *Parvoviridae* are non-enveloped, negative-sense single-stranded DNA viruses. Several parvoviruses infect different animal species worldwide, including domestic and wild mammals, crustaceans, and arthropods.(1) Canine parvovirus (CPV) infects domestic dogs and is transmitted via oral or nasal contact with excreta, fomites, or feces containing the virus.(2)

Canine parvovirus type 2 (CPV2) was recognized as a new virus in 1978 and is thought to have originated from feline panleukopenia virus (FPLV). There have been several outbreaks of this virus in raccoons in the southeastern United States. CPV2 is believed to be responsible for morbidity and mortality in wolf populations surrounding Yellowstone National Park, United States. There are three known strains of CPV2: CPV2a, CPV2b, and CPV2c. CPV2c has been found to infect captive Asian small-clawed otters. CPV1, another parvovirus that infects dogs, is of lesser concern to wildlife. Most CPV2 infections present with little or mild clinical signs.(3) Severe cases include anorexia, vomiting, bloody feces, and foul-smelling diarrhea. The disease can be more severe if there are co-infections with pathogens such as Salmonella spp. or Giardia spp. Rarely, young animals develop myocardial injury between 1 and 2 months of age. Raccoon dogs develop gastroenteritis.(4)

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Vaccination against parvovirus is recommended for some captive wildlife to prevent the spread of the virus and safeguard animal health. The vaccination with commercial modified live virus (MLV) CPV-2 vaccines induces immunity against all three CPV-2 strains. Immunity is mediated primarily by IgG neutralizing antibodies, which provide long-term protection. Secretory IgA and cell-mediated immunity play a role, but may be less critical in initiating immediate protection after vaccination.[5]

Evaluating the efficacy of CPV vaccines involves determining the antibody titers in the serum of vaccinated animals through several tests. These tests include enzyme-linked immunosorbent assay (ELISA), immunofluorescent techniques (IFT), hemagglutination inhibition (HI), and neutralization test (NT). Additionally, the titer of the vaccinal strain within the vaccine can be detected using IFT. These evaluations ensure that the vaccine induces a sufficient immune response to protect against the virus.[6]

In this study, we aimed to employ real-time polymerase chain reaction (qPCR) to detect and quantify the vaccinal strain within the vaccine. We compared the qPCR results with those obtained from the IFT to determine the relationship between the titers measured by IFT and qPCR. The use of qPCR for detection and quantification, not only enhances the efficiency of the evaluation process, but also reduces the reliance on experimental animals.

Materials and Methods

Virus

CPV strain 39, which has been adapted on Madin-Darby canine kidney (MDCK) cells and has a titer of 6 $\log_{10}$ tissue culture infective dose 50% ($TCID_{50}$)/mL, was supplied by the Reference Strain Bank in Central Laboratory for Evaluation of Veterinary Biologics (CLEVB) in Abbassia, Cairo, Egypt. This virus was used as a positive control in the IFT and for generating the standard curve in the qPCR assay.[7]

Cell line

MDCK cells were provided by the Strain Bank at CLEVB. These cells were utilized in the IFT for vaccine titration and identification purposes.

Canine parvovirus vaccines

Twenty different batches of live attenuated CPV vaccines (n=20), including monovalent, bivalent, and polyvalent formulations sourced from both local manufacturers and international suppliers, were submitted to the CLEVB. These vaccines underwent rigorous evaluation for sterility, safety, and potency over the preceding two-year period.

Immunofluorescence technique

IFT was employed for titration and identification of the tested vaccinal strain of live attenuated CPV vaccines and the positive control parvovirus. This method utilized MDCK cell tissue culture in microtiter plates coated for immunofluorescence.[8] The fluorescent antibody infective dose 50% ($FAID_{50}$) of the test vaccines and positive control were calculated using the Spearman method.[9]

Real-time polymerase chain reaction for quantification of attenuated parvovirus vaccine

DNA extraction and dilution

First, DNA was extracted from both the reference parvovirus containing 10^6 $TCID_{50}$/mL and 20 batches of the vaccinal strain. This extraction was done using the Fast-Pure Viral DNA/RNA Mini Kit following the manufacturer's instructions (version 1). Next, the extracted DNA was serially diluted six times in a 10-fold dilution series.

qPCR

Each dilution of the extracted DNA, including those from the reference virus and all 20 vaccine batches, was then tested in triplicate using qPCR. The Taq PCR Master Mix kit (cat. nos. 201443 and 201445) was used for the PCR reaction itself. Primers and probe targeting the VP2 gene were designed according to Decaro, et al.[10] The specific sequences of these primers and probe are shown in Table 1.

Table 1. Primers and probe of qPCR for CPV.

Assay	Primer/ probe	Sequence 5′ to 3′	Polarity	Position	Amplicon size
TaqMan assay	CPV-For	AAACAGGAATTAACTATACTAATATATTTA	+	4104–4135	93 bp
	CPV-Rev	AAATTTGACCATTTGGATAAACT	-	4176–4198	
	Probe	FAM-TGGTCCTTTAACTGCATTAAATAATGTACC-TAMRA	+	4143–4172	

Thermal cycling conditions and Ct values

The qPCR reaction was conducted using a thermal cycling protocol that began with an initial activation of DNA polymerase at 95°C for 10 min. This was followed by 40 cycles consisting of denaturation at 95°C for 15 sec, primer annealing at 52°C for 30 sec, and extension at 60°C for 1 min. A cycle threshold (Ct) value of 37 or higher was considered negative, indicating no amplification was detected, whereas any reaction with a recorded Ct value was deemed positive, indicating amplification.

Standard curve, quantification, and limits

Standard curve was established using the Ct values obtained from the serially diluted reference virus, according to the guidelines of Abousenna et al.[11] The linear equation was then used to determine the quantity of viral particles in each vaccine batch based on their respective Ct values. Finally, the limit of detection (LOD) and limit of quantification (LOQ) for the qPCR assay were determined by analyzing each concentration only once for both the reference virus and vaccine batches.[12]

Statistical analysis

The data was analyzed using IBM SPSS Statistics version 21 for Windows. This software facilitated a comprehensive statistical analysis, including the calculation of confidence intervals for both the qPCR and the conventional IFT methods.

By employing confidence intervals, the analysis provided a nuanced understanding of the precision associated with the measurements from these two diagnostic methods. Confidence intervals essentially capture the range of values within which the true population mean is likely to lie, with a certain level of confidence (usually 95%).

Results

Immunofluorescence technique titration

The potency of each vaccine batch (n-20) was initially assessed using the IFT. Eighteen of the 20 batches achieved a protective level of no less than 3 $\log_{10}$ $FAID_{50}/mL$[13] Two batches recorded a titer below 3 $\log_{10}$ $FAID_{50}/mL$. The detailed results of the IFT titration, including the titer for each vaccine batch, are presented in Table 2.

Real-time polymerase chain reaction

Standard curve

Serial dilutions of a reference CPV stock were used to generate a standard curve, allowing for the correlation between Ct values and the actual amount of viral particles present (Fig. 1). The minimal concentration of CPV detectable by the assay was 10^1 $TCID_{50}/mL$. The linear equation relating Ct values to viral concentration, along with the mean Ct values for each dilution and the R^2 value, are presented in Figure 1 and Table 3. A high R^2 value indicates a strong correlation between Ct values and viral load.

Quantification of CPV in vaccine batches

Following the establishment of the standard curve, each of the 20 vaccine batches was analyzed using qPCR. The Ct values obtained were compared to the standard curve equation to determine the corresponding amount of CPV present in each batch. The detailed data on the quantified CPV load in each vaccine batch is shown in Table 4.

Comparison of qPCR and IFT

The viral load quantified by qPCR was compared to the titers obtained using IFT. Eighteen out of the 20 batches reached a permissible limit for viral load according to

Table 2. IFT titers of canine parvovirus vaccine batches.

No of batch	*IFT titer
1	4
2	5
3	2
4	5.5
5	5.5
6	5
7	5.5
8	5.5
9	4.5
10	5
11	4.9
12	5.5
13	5.5
14	5.5
15	5
16	4.9
17	5.3
18	2
19	5
20	5.5

IFT titer: titer determined by immunofluorescence technique; it is expressed as fluorescent antibody infective dose 50% ($FAID_{50}$)/mL. *: IFT titers ≥3 log $_{10}$ $FAID_{50}$/mL were considered for a protective level

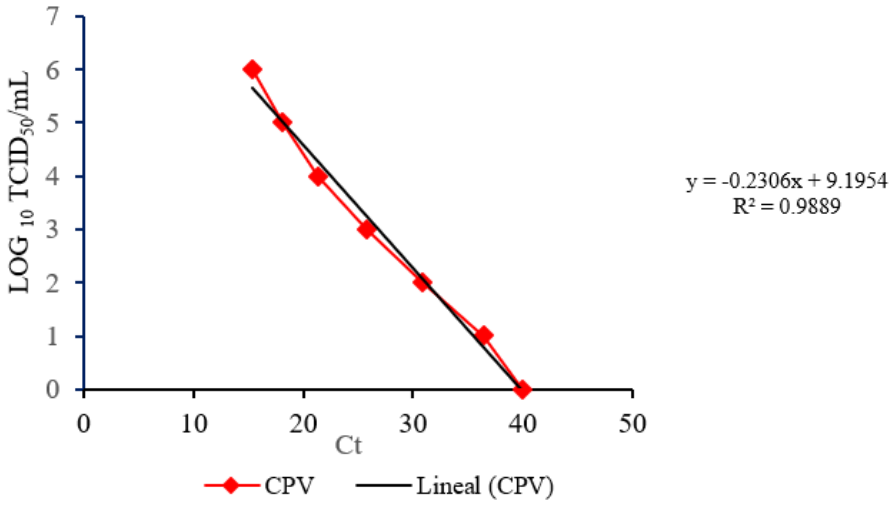

Fig. 1. Canine parvovirus qPCR standard curve with linear equation.

Table 3. Relationship between Ct values and log_{10} $TCID_{50}$/mL for CPV in qPCR analysis.

Ct values qPCR	15.4	18.1	21.4	25.8	30.9	36.5	40
Titer log_{10} $TCID_{50}$/mL	6	5	4	3	2	1	0

Table 4. Concordance between qPCR and IFT titers for canine parvovirus vaccines.

Batch no	**Ct**	**Equation**	**qPCR titer (log_{10} $TCID_{50}$/mL)**	**IFT titer (log_{10} $FAID_{50}$/mL)**
1	22.5	Y=-0.230x+9.195	4.02	4
2	17.3		5.216	5
3	30.5		2.18	2
4	16.4		5.423	5.5
5	15.6		5.60	5.5
6	17.5		5.17	5
7	16.9		5.30	5.5
8	15.2		5.69	5.5
9	19.8		4.641	4.5
10	18.5		4.94	5
11	19.2		4.77	4.9
12	15.5		5.63	5.5
13	16.5		5.4	5.5
14	15.7		5.58	5.5
15	17.8		5.1	5
16	18.8		4.87	4.9
17	16.8		5.33	5.3
18	31.2		2.019	2
19	17.6		5.14	5
20	15.9		5.53	5.5
95% Confidence interval of the difference				
Lower			Upper	
4.39			5.36	

the qPCR analysis. This result is comparable to the findings from the IFT assay, which also identified 18 batches exceeding the minimum potency requirement. Two batches showed CPV levels below the permissible limit in both qPCR and IFT analyses, as shown in Table 4.

When the statistical analysis was performed to assess the agreement between the two methods, a 95% CI for the difference between the qPCR and IFT measurements was calculated. The CI ranged from -4.3949 to 5.3600. Since the CI includes zero, there is no statistically significant difference between the qPCR and IFT results, as shown in Table 4.

Discussion

Parvovirus infections in dogs are a significant global challenge due to their contagious nature and severe health impacts, particularly on puppies and unvaccinated dogs. Symptoms include severe vomiting, bloody diarrhea, lethargy, and fever, and the disease can be fatal without prompt treatment.[14] Vaccination is the recommended medical prophylaxis against CPV, providing effective protection and significantly reducing infection rates. Puppies typically start their vaccination schedule at 6 to 8 weeks old, with boosters every 3 to 4 weeks until 16 to 20 weeks old. Adult dogs require regular booster shots to maintain immunity.[15]

The evaluation of live attenuated CPV vaccines at the CLEVB traditionally relies on IFT using tissue culture for qualitative and quantitative detection of viral content in the vaccines.[11] While effective, this method can be time-consuming and labor-intensive.

Recent advancements in diagnostic technologies aim to provide more rapid, specific, and accurate methods for vaccine evaluation. For instance, rapid ELISA tests, such as the SNAP Parvo test, have been investigated for their sensitivity compared to IFT, offering a quick preliminary assessment of live attenuated CPV vaccines.[16] Similarly, the lateral flow assay for CPV (LFA-CPV) antigen test, developed in this study, emerges as another promising alternative. Positioned as a point-of-care diagnostic tool for CPV, this assay presents a cost-effective, user-friendly, and rapid on-site solution for both preliminary evaluation of CPV vaccines. While demonstrating potential in semi-quantitative analysis, particularly in detecting CPV titers exceeding 10^3 $TCID_{50}$.[11] Furthermore, the World Organization for Animal Health (WOAH) recommends using qPCR for CPV identification due to its precision and efficiency.[17]

The use of qPCR has proven effective in evaluating live virus vaccines, as demonstrated by its application in assessing live attenuated sheep pox virus vaccines.[18] In this case, qPCR was found to surpass traditional tissue culture titration in identifying and rapidly evaluating the vaccinal strain. Similarly, qPCR has been established for the differential detection of wild-type and vaccine strains of canine distemper virus in China, showcasing its versatility and reliability in virological assessments.[19]

In this study, we employed qPCR to evaluate the viral content in live attenuated CPV vaccines across 20 batches. These batches had previously undergone assessments for sterility, safety, and potency. The qPCR results were then compared with those obtained from the IFT.

The IFT results for the 20 batches indicated that 18 batches achieved the permissible titer level of no less than 3 $\log_{10}$ $FAID_{50}$/mL, indicated that these batches contain a sufficient viral load to be effective.[13] Only two batches, specifically batch numbers 3 and 18, recorded titers below this permissible limit, this suggests that these batches may not meet the minimum potency requirement for the vaccine. Similarly, the qPCR results, calculated using Ct values and corresponding equations for the 20 batches, confirmed that the same 18 batches met the permissible titer level, while batches 3 and 18 did not. The statistical analysis of the results, using a 95% confidence interval of the difference with lower and upper bounds of 4.3949 and 5.3600, respectively, indicated a strong concordance between the two methods for potency evaluation of the CPV vaccine, showing no significant difference between them. This reflects that both qPCR and IFT are compatible in terms of assessing the titer of the vaccinal strain, leading to consistent final decisions regarding the vaccine batches.

Our findings align with previous studies,[11,16] highlighting that while traditional methods like IFT remain valuable, modern techniques such as qPCR, rapid ELISA and LFA-CPV offer enhanced efficiency and accuracy for evaluating live attenuated vaccines. This supports the ongoing transition towards integrating these advanced methods into routine vaccine evaluation protocols, ensuring higher standards of veterinary biologics assessment.

Conclusion

The qPCR method proved to be an accurate, simple, and rapid tool for evaluating the CPV vaccinal strain, aligning with traditional IFT results. By adopting qPCR as a standard supervisory method and incorporating tools, veterinary biologics assessment can become more precise and efficient.

Conflict of interest

The authors declare that there is no conflict of interest.

Author's contributions

Nermeen Gouda Shafik: conceptualization, validation, and investigation.

Sara El Sawy Ahmed: methodology, validation, investigation.

Mohamed Samy Abousenna: conceptualization, methodology, formal analysis, investigation, data curation, writing-original draft preparation, writing-review and editing.

Fady Abd El Mohsen Shasha: methodology, formal analysis, investigation.

Darwish Mahmoud Darwish: methodology, formal analysis, and investigation

Heba A. Khafagy: methodology, formal analysis, and data curation

Amal Abd El Moneim Mohamed: methodology, validation, formal analysis, writing-original draft preparation, writing-review and editing.

All authors read and approved the final manuscript.

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Evaluación de vacunas vivas atenuadas contra el parvovirus canino mediante PCR

Resumen

Las infecciones por parvovirus canino suponen una importante amenaza mundial para la salud canina, lo que exige estrategias de vacunación eficaces. En el presente estudio se evalúa el contenido viral en 20 lotes de vacunas vivas atenuadas contra el parvovirus canino mediante la reacción en cadena de la polimerasa en tiempo real y se comparan los resultados con la prueba de inmunofluorescencia. En el estudio se utilizó la cepa 39 del parvovirus canino, adaptada a células de riñón canino Madin-Darby y lotes de vacunas procedentes de diversos proveedores locales e internacionales. Los resultados de la prueba de inmunofluorescencia mostraron que 18 lotes cumplieron el nivel de título permitido de 3 $\log_{10}$ dosis infecciosa determinada por inmunofluorescencia 50%/mL, mientras que dos lotes (3 y 18) no. Del mismo modo, el análisis de la reacción en cadena de la polimerasa en tiempo real confirmó que los 18 lotes cumplieron el nivel de título permitido, sin diferencias significativas entre los métodos, como indica el intervalo de confianza del 95% (inferior: 4,3949, superior: 5,3600). Los resultados apoyan la integración de tecnologías avanzadas de diagnóstico como la reacción en cadena de la polimerasa en tiempo real en los protocolos rutinarios de evaluación de vacunas, garantizando estándares más altos en la evaluación de biológicos veterinarios; esta transición pretende mejorar la precisión, la eficiencia y la calidad general de la evaluación de vacunas contra el parvovirus canino y, en última instancia, mejorar la protección de la salud canina.

Palabras clave: potencia de la vacuna; parvovirus canino; vacunas; pruebas de diagnóstico; reacción en cadena en tiempo real de la polimerasa.

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