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ISOLATION AND SELECTION OF POTENTIAL PARVOVIRUS STRAINS FOR VACCINE PRODUCTION AGAINST PORCINE MUMMIFICATION

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INTRODUCTION

1. Rationale of the Study

In Vietnam, no institution has yet produced or commercialized vaccines against reproductive failure in pigs caused by porcine parvovirus (PPV). All vaccines currently in use for the prevention of PPV-associated reproductive disorders are imported. These imported vaccines are often costly and may not be fully compatible with PPV strains circulating in Vietnam. Furthermore, reliance on imported vaccines places a financial burden on the national budget. For these reasons, the Ministry of Agriculture and Rural Development has identified the development of PPV vaccines for pigs as an urgent priority for the swine industry.

The dissertation entitled "*Isolation and Selection of Potential Parvovirus Strains for Vaccine Production against Porcine Mummification*" aims to isolate PPV strains with high virulence, genetic and biological stability, and strong immunogenicity. These strains will serve as the foundation for the development of PPV vaccines, thereby enabling proactive prevention and control of reproductive failure in pigs in line with the Government's strategic direction.

2. Research Objectives

2.1. General Objectives

To isolate, characterize, and establish a library of potential PPV strains for use in research and development of vaccines against porcine fetal mummification.

2.2. Specific Objectives

1) To isolate PPV strains from field samples.

2) To determine the viral type and analyze the VP2 gene of the isolated strains.

3) To characterize the biological properties, stability, and immunogenicity of the PPV strains.

3. Research Contents

1) Isolation of parvovirus strains causing fetal mummification from representative samples across various regions in Vietnam

2) Identification and classification of the isolated parvovirus strains

3) Genetic relationship analysis of the isolated strains

4) Characterization of the biological properties of the parvovirus strains

5) Evaluation of the stability, virulence, and immunogenicity of selected viral strains

4. Scientific and Practical Significance of the Study

4.1. Scientific Significance

This study represents the first successful isolation of PPV strains in Vietnam using the continuous PK-15 cell line. The research provides valuable insights into both the biological and genetic characteristics of PPV strains isolated in Vietnam, thereby contributing to a more comprehensive understanding of the PPV strains currently circulating in the country. Furthermore, two PPV strains with stable biological and genetic traits across multiple passages, high virulence, and strong immunogenicity were identified. These strains represent potential candidates for the development of vaccines against reproductive failure in pigs.

4.2. Practical Significance

The isolation and selection of PPV strains with high virulence, genetic and biological stability, and strong immunogenicity provide a foundation for the development of vaccines against reproductive failure in pigs. This will contribute to securing domestic vaccine supply, enhancing disease prevention, and supporting national strategies for epidemic control. The availability of locally produced vaccines, at lower cost and with more reliable supply compared to imported products, will empower farmers to implement effective preventive measures, improve livestock productivity, reduce production risks, and increase economic returns.

CHAPTER I
LITERATURE REVIEW

1.1. OVERVIEW OF PORCINE REPRODUCTIVE FAILURE CAUSED BY PARVOVIRUS

1.1.1. Historical Background

Porcine parvovirus (PPV) has been identified as a major causative agent of reproductive failure in swine, including embryonic death, mummification of fetuses, stillbirth, and infertility. The first strain of PPV identified was designated PPV1 to distinguish it from newly discovered variants. Among the known types, PPV1 remains the most widespread globally, as demonstrated in multiple studies. Importantly, PPV infections are restricted to pigs and do not affect other animal species. The virus specifically targets swine fetuses, and the severity of the outcome depends on the stage of gestation at which infection occurs. Clinical manifestations vary across fetal developmental stages. To date, seven PPV genotypes (PPV1 to PPV7) have been described.

1.1.2. Pathogenesis

PPV infects fetuses primarily through transplacental transmission. Initially, the virus may affect only one or a few fetuses, but it rapidly spreads to other fetuses within the uterus. The infection disrupts the circulatory system, leading to edema, hemorrhage, and accumulation of blood-tinged fluid in body cavities. These severe lesions result in the complete destruction of the fetal circulatory system. Transmission of PPV from the dam to the fetus via the placenta is well established.

The pathological outcome of PPV infection is dependent on the stage of gestation at which the fetus becomes infected. Infection occurring between 10 and 30 days of gestation usually results in embryonic death or resorption due to the small size and underdeveloped immune system of the fetus. Infections between 30 and 70 days of gestation often lead to fetal dehydration, mummification, and the so-called “wooden fetus” phenomenon. When infection occurs after day 70 of gestation, fetuses are capable of mounting an immune response against PPV, thereby increasing their chances of survival.

1.1.3. Clinical Signs

Following infection with PPV, the virus replicates rapidly within host tissues, with a particular tropism for lymphoid organs. In pigs, a transient leukopenia commonly occurs

5–10 days post-infection, irrespective of age or sex, though this manifestation is typically mild. The predominant clinical outcome of PPV infection is reproductive failure. Infected sows may present with anestrus, return to estrus, reduced litter size, mummified fetuses, abortions, stillbirths, and decreased neonatal survival rates.

1.2. OVERVIEW OF PARVOVIRUS

1.2.1. Taxonomy

Family: Parvoviridae

Subfamily: Parvovirinae

Genus: *Protoparvovirus*

Species: *Ungulate protoparvovirus*

Subspecies: Porcine parvovirus

1.2.2. Viral Structure

The PPV genome consists of linear single-stranded DNA with an approximate size of 5 kb. PPV is a small, non-enveloped, spherical virus with a diameter ranging from 18 to 26 nm, characterized by icosahedral symmetry.

1.3. PREVALENCE OF PPV

1.3.1. Global Prevalence of PPV

Table 1.1. Distribution and prevalence of PPV worldwide

PPV Classification	Europe		America		Asia		Africa	
	Infection rate (%)	Ref.	Infection rate (%)	Ref.	Infection rate (%)	Ref.	Infection rate (%)	Ref.
PPV1	0.5-7.5	Csagola et al., 2012; Milek et al., 2019; Milek et al., 2020	14.7-25.9	Serena et al., 2019; Opriessnig et al., 2014; Garcia -Camacho et al., 2020	4.5-53	Zhang et al., 2011; Xiao et al., 2013 and 2013b; Ni et al., 2014; Kim et al., 2022; Parthiban et al., 2022; Saekhow et al., 2015	29.1	Afolabi et al., 2019; Bisimwa et al., 2021
PPV2	6.4-48.7		72-90		6.4-83		21.8	
PPV3	9.7-25.2		19.2-58.8		9.7-73		17.5	
PPV4	8.2-94		5.9-25.9		1.5-44		43.6	
PPV5	22.6-41.3		7-32.4		2.6-14.3			
PPV6	37.1-38		74.7		21.5			
PPV7	18.2				14.2-18			

The prevalence of PPV across different types is summarized in Table 1.1, ranging from 0.5% to 94%. These findings indicate that the prevalence of PPV is not dependent on geographical region, tissue origin of samples, or pig breeds.

1.3.2. Prevalence of PPV in Vietnam

In Vietnam, a serological survey conducted by Ho Dinh Chuc reported that 50% to 60% of sows had been infected with PPV (Ho Dinh Chuc, 1993). In another study, the same author employed serological methods to determine the prevalence of PPV in the central region, revealing an infection rate of approximately 21.5% (Pham Hung, 1999). A survey carried out in Long An Province found that 69.5% of 4,166 serum samples collected from sow farms tested positive for PPV (Nguyen Huynh et al., 2000). More recently, a study on the circulation of PPV1 in Hanoi and surrounding areas demonstrated that 88.2% (60/68) of samples contained antibodies against PPV1 (Huynh Thi My Le et al., 2020).

Collectively, these findings indicate that the prevalence of PPV in Vietnam has been reported to range from 10% to 88%.

1.4. CURRENT STATUS OF VACCINE PRODUCTION AGAINST PORCINE REPRODUCTIVE FAILURE CAUSED BY PARVOVIRUS IN THE WORLD AND IN VIETNAM

1.4.1. Global status of parvovirus vaccine production for porcine reproductive failure

Worldwide, numerous vaccines have been licensed for the prevention of porcine parvovirus (PPV)-induced reproductive disorders. However, these imported vaccines are not only costly but may also be inadequately matched with circulating strains in Vietnam. Moreover, several studies have documented the emergence of novel PPV strains that differ genetically and antigenically from previously isolated ones. For instance, Serena et al. (2019) reported amino acid substitutions at positions 436 (S–P) and 565 (R–K) in studied PPV strains. Similarly, Chung et al. (2020) identified mutations in PPV7 strains, including deletions within the VP2 gene. In this study, amino acid variations in the VP2 protein were observed in 6 out of 12 strains, which were predicted to result in substantial differences in antigenic indices compared to PPV1 strains.

These findings highlight the urgent need to isolate and select PPV strains circulating in Vietnam that possess suitable characteristics for the development of effective vaccines against reproductive failure in pigs.

1.4.2. Status of parvovirus vaccine production for porcine reproductive failure in Vietnam

At present, no studies in Vietnam have focused on the isolation and selection of PPV strains for vaccine development. Existing research has mainly concentrated on determining prevalence using PCR-based methods. Globally, no published work has reported the isolation of PPV strains originating from Vietnam. Consequently, research on viral isolation and vaccine production in the country remains limited.

Developing vaccines based on PPV strains circulating in Vietnam would improve disease prevention efficacy. Domestic vaccine production would not only ensure a stable supply but also reduce costs compared to imported vaccines, thereby enhancing accessibility for farmers. Such efforts would strengthen disease prevention measures and contribute significantly to the sustainable development of Vietnam's swine industry.

CHAPTER II
MATERIALS AND METHODS

2.1. RESEARCH SUBJECTS

The research subjects were Parvovirus isolated from six provinces of Vietnam: Thai Binh, Thanh Hoa, Binh Dinh, Dak Lak, Dong Nai, and Binh Duong.

2.2. RESEARCH METHODS

2.2.1. Method for isolating porcine parvovirus causing reproductive failure from representative clinical samples collected in different localities

*** Sample collection**

The sampling procedure was conducted in compliance with the national technical regulation QCVN 01-83:2011/BNNPTNT: Animal diseases – General requirements for sampling, preservation, and transportation of clinical specimens.

*** Sample processing**

Serum samples: Serum samples were centrifuged at 3,000 rpm for 10 minutes, and the supernatant was collected and stored at –20 °C (Tuck et al., 2009).

Embryonic tissue samples: Liver and lung tissues from embryos were collected and prepared as a 10% suspension in PBS (Nguyen et al., 2022).

*** Identification of PPV-positive samples**

Samples were screened for PPV by PCR, following the method described by Lyoo et al., 2001.

*** Determination of samples eligible for virus isolation**

Samples were tested for the presence of other viruses, including PRRSV (according to TCVN 8400-21:2014), PCV (TCVN 8400-36:2015), CSFV (TCVN 8400-47:2019), and ASFV (TCVN 8400-41:2019). Only samples negative for all the above viruses were considered eligible for PPV isolation using the PK-15 continuous cell line.

*** PPV isolation method**

The isolation of PPV was carried out by culturing on PK-15 cells, as described by Kim et al., 2022.

2.2.2. Determination of parvovirus types

The isolated viral strains were typed using the PCR method of Kim Seung-Chai et al., 2022.

2.2.3. Method for determining the genetic relationships of parvovirus strains

The NS1 gene fragment was amplified using the primer pair PPVF and PPVR, as described by Lyoo et al., 2001. The VP2 gene fragment was amplified using PCR according to the method of Xu et al., 2013. The amplified DNA was sequenced at First BASE Laboratories (Singapore). Sequence data were analyzed using BLAST against the NCBI GenBank database. Phylogenetic trees were constructed based on nucleotide sequences of the NS1 gene from the isolated PPV strains, referenced with selected sequences from GenBank, using MEGA6 software with the Neighbor-Joining method (Saitou and Nei, 1987). Bootstrap analysis with 1,000 replicates was performed to assess the reliability of the phylogenetic tree (Felsenstein, 1985).

2.2.4. Methods for determining selected viral characteristics

*** Determination of $TCID_{50}$ titer**

The TCID50 was determined using the method of Reed and Muench (1938).

*** Determination of optimal culture conditions**

The culture conditions evaluated included temperatures of 33°C, 37°C, and 39°C, and fetal bovine serum (FBS) concentrations of 2%, 3%, and 5%. Parvovirus was inoculated onto PK-15 cells at a multiplicity of infection (MOI) of 0.01. The virus was then cultured under the respective conditions for 7 days. Every 24 hours, 200 µl of viral suspension was collected, followed by DNA extraction and quantification of viral load using real-time PCR.

*** Determination of viral growth curve**

Viral strains were inoculated onto PK-15 cells at an MOI of 0.01. The viruses were cultured in E-MEM supplemented with 5% FBS and 1% penicillin–streptomycin at 37°C with 5% CO_2. Samples were collected every 24 hours for 7 days post-inoculation. The viral load at each time point was determined by real-time PCR, as described by Miao et al., 2009.

2.2.5. Determination of stability, virulence, and immunogenicity

*** Determination of stability in selected biological and genetic characteristics**

Viral samples were serially passaged for 15 generations. At passages 1, 5, 10, and 15, evaluations were conducted on growth kinetics, $TCID_{50}$ titers, cytopathic effects, and sequence analysis of the NS1 and VP2 genes.

*** Assessment of virulence**

The virulence of parvovirus strains was determined following the description of Zeeuw et al., 2007.

*** Assessment of immunogenicity**

Evaluation of immunogenicity in alternative animal models: The immunogenicity in guinea pigs was assessed according to the method described by Ma et al., 2011.

Evaluation of immunogenicity in pigs: The immunogenicity of parvovirus strains was evaluated in pregnant sows following the protocol described by Foerster Tessa et al., 2016.

2.3. DATA PROCESSING METHODS

All experiments were repeated three times. Data obtained from the experiments on optimal culture conditions, $TCID_{50}$, HI titers, and ELISA titers were analyzed using the General Linear Model (GLM) combined with Tukey's test in Minitab18 software to evaluate statistically significant differences. The prevalence of PPV infection was analyzed using the Chi-square test.

2.4. ANIMAL ETHICS

All animal experiments conducted in this study were approved by the Animal Ethics Committee of Nong Lam University, Ho Chi Minh City, under certificate number NLU-230313, issued on May 24, 2023.

CHAPTER III
RESULTS AND DISCUSSION

3.1. ISOLATION OF PARVOVIRUS CAUSING REPRODUCTIVE FAILURE FROM REPRESENTATIVE CLINICAL SPECIMENS COLLECTED IN DIFFERENT REGIONS

3.1.1. Detection of Parvovirus-Positive Samples

A 330 bp fragment of the NS1 gene, located between positions 1,453 and 1,782, represents a highly conserved region among PPV strains and was used to identify PPV-positive samples (Lyoo et al., 2001). Based on PCR results, 32 out of 392 samples were confirmed positive for PPV, accounting for 8.2% (Table 3.1). The infection rate varied within a narrow range, from 4.8% in Thanh Hoa to 10.0% in Dong Nai and Binh Duong. Although absolute differences in prevalence were observed among provinces, a *p-value* > 0.05 indicated that these differences were not statistically significant.

Table 3.1. PPV infection rates by location

Location	Total number of samples	PPV positive sample	Infection rate (%)
Thai Binh	60	5	8.3
Thanh Hoa	63	3	4.8
Binh Dinh	60	4	6.7
Dak Lak	69	6	8.7
Dong Nai	70	7	10
Binh Duong	70	7	10
Total	392	32	8.2

The seropositive rate for PPV was 9.1% in serum samples and 9.4% in embryonic tissue samples. The infection rate in multiparous sows was 9.4%, whereas that in primiparous sows was 4.6%. A *p-value* > 0.05 indicated that these differences were not statistically significant. In this study, the number of samples examined was limited compared with the minimum sample size required to detect small differences at a statistically significant level. To further clarify this issue, differences between multiparous and primiparous sows should be investigated through epidemiological studies with a sufficiently large sample size and multivariate analyses to better control potential confounding factors.

3.1.2. Isolation of Parvovirus on PK-15 Cell Line

3.1.2.1. Screening of samples eligible for isolation

All 32 PPV-positive samples tested negative for CSFV, PRRSV, PCV2, and ASFV, thereby meeting the criteria for subsequent PPV isolation procedures

3.1.2.2. Observation of cytopathic effects (CPE)

The observed CPE revealed that within 24 hours post-infection, the virus induced cellular alterations, including irregular cell morphology, nuclear condensation, and granulation (Figure 3.1). The virus subsequently replicated and caused progressive cell destruction. By day 5 post-infection, extensive cellular damage was evident, resulting in the formation of clear areas on the surface of the culture flask (Figure 3.2)

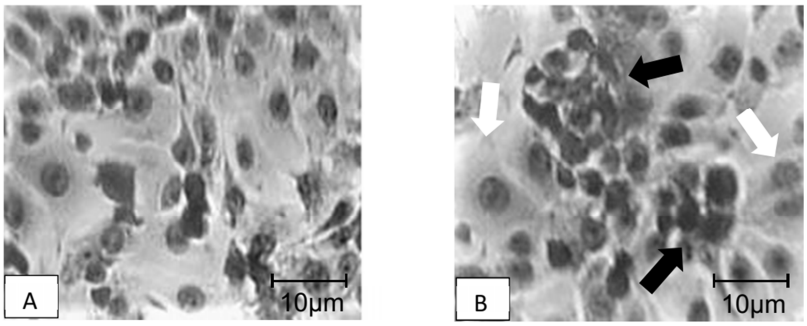

Figure 3.1. PK-15 cells 24 hours post-infection with PPV

Magnification: 100X under inverted microscope

(A) Uninfected PK-15 cells, serving as the negative control.

(B) PK-15 cells 24 hours post-infection with parvovirus. The white arrow indicates normal cells, the black arrow highlights virus-infected cells exhibiting nuclear clumping and morphological alterations.

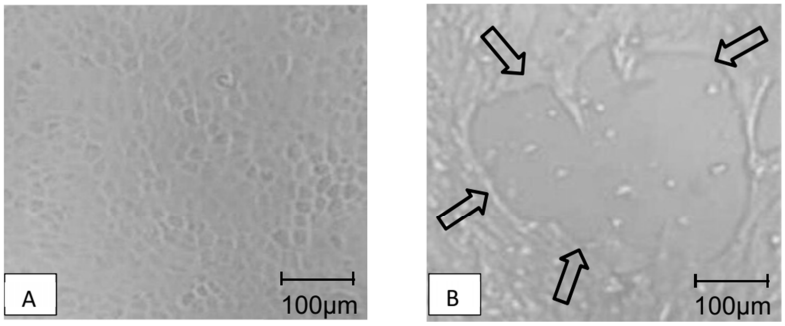

Figure 3.2. Cytopathic effects on PK-15 cells 5 days post-infection with PPV

Magnification: 10X under inverted microscope

(A) PK-15 cells remained uninfected after five days, serving as the negative control.

(B) PK-15 cells exhibiting cytopathic effects following five days of parvovirus infection. Arrows indicate virus-infected cells that have undergone lysis, leading to the formation of gaps on the surface of the culture flask.

All 32 PPV strains in this study exhibited cytopathic effects on PK-15 cells following infection.

3.1.2.3. Results of viral replication dynamics during cell culture

Table 3.2. Quantification of viral load after three serial passages

Location	**Sample**	**Mean logarithmic viral copy number ± SD (n = 3)**			**Tukey's mean comparison group (95% CI, n = 9)**
		P1	P2	P3	
Thai Binh	DH2	3.36 ± 0.04	4.86 ± 0.05	5.16 ± 0.18	B, C, D
	DH4	2.82 ± 0.04	4.32 ± 0.03	4.62 ± 0.16	B, C, D
	DH11	3.78 ± 0.02	5.28 ± 0.13	5.58 ± 0.10	A, B, C, D
	DH15	2.81 ± 0.02	4.31 ± 0.11	4.61 ± 0.07	C, D
Thanh Hoa	KX24	4.23 ± 0.02	5.73 ± 0.12	6.03 ± 0.16	A, B, C, D
	TS8	2.79 ± 0.01	4.29 ± 0.09	4.59 ± 0.09	D
	TX7	**5.13 ± 0.05**	**6.63 ± 0.12**	**7 ± 0.11**	**A**
	TX13	2.78 ± 0.03	4.28 ± 0.09	4.58 ± 0.10	D
Binh Dinh	PM21	3.29 ± 0.04	4.79 ± 0.06	5.09 ± 0.18	B, C, D
	PM27	2.77 ± 0.20	4.27 ± 0.08	4.57 ± 0.10	D
	PC7	3.76 ± 0.12	5.26 ± 0.07	5.56 ± 0.14	A, B, C, D
	PC15	2.75 ± 0.15	4.25 ± 0.09	4.55 ± 0.07	D
Dak Lak	EK18	4.31 ± 0.07	5.81 ± 0.13	6.11 ± 0.09	A, B, C
	EK20	2.74 ± 0.12	4.24 ± 0.08	4.54 ± 0.09	D
	EK27	3.78 ± 0.11	5.28 ± 0.05	5.58 ± 0.10	A, B, C, D
	EK31	2.79 ± 0.12	4.29 ± 0.11	4.59 ± 0.16	B, C, D
	MD22	2.82 ± 0.06	4.32 ± 0.05	4.62 ± 0.10	B, C, D
	MD28	2.83 ± 0.04	4.33 ± 0.09	4.63 ± 0.08	B, C, D
Dong Nai	**VC5**	**5.02 ± 0.13**	**6.52 ± 0.04**	**6.92 ± 0.08**	**A**
	VC10	4.23 ± 0.07	5.73 ± 0.06	6.03 ± 0.07	A, B, C, D
	VC15	2.82 ± 0.06	4.32 ± 0.04	4.62 ± 0.08	B, C, D
	VC32	3.33 ± 0.12	4.83 ± 0.06	5.13 ± 0.03	B, C, D
	LT23	2.79 ± 0.06	4.29 ± 0.05	4.59 ± 0.05	D
	LT32	2.73 ± 0.12	4.23 ± 0.07	4.53 ± 0.07	D
	LT36	3.21 ± 0.09	4.71 ± 0.05	5.01 ± 0.03	B, C, D
Binh Duong	DA6	2.86 ± 0.06	4.36 ± 0.10	4.66 ± 0.06	B, C, D
	DA12	3.73 ± 0.16	5.23 ± 0.10	5.53 ± 0.08	A, B, C, D
	DA27	2.82 ± 0.10	4.32 ± 0.08	4.62 ± 0.12	B, C, D
	BC2	3.37 ± 0.11	4.87 ± 0.10	5.17 ± 0.18	B, C, D
	BC4	2.76 ± 0.08	4.26 ± 0.07	4.56 ± 0.04	D
	BC11	4.32 ± 0.12	5.82 ± 0.07	6.12 ± 0.08	A, B
	BC15	2.87 ± 0.09	4.37 ± 0.07	4.67 ± 0.10	B, C, D

Note: P1 – First passage; P2 – Second passage; P3 – Third passage
** p-value* < 0.001

From the results presented in Table 3.2, all 32 PPV isolates demonstrated an increase in viral copy number during cell culture. Tukey's pairwise comparison indicated that samples VC5 and TX7 exhibited the highest viral copy numbers, with statistically significant differences. Taken together with the observation of CPE and the confirmed increase in viral replication during culture, it was evident that all 32 PPV-positive samples consistently exhibited CPE and viral proliferation. These findings confirm the successful isolation of 32 PPV strains.

3.2. PARVOVIRUS TYPING

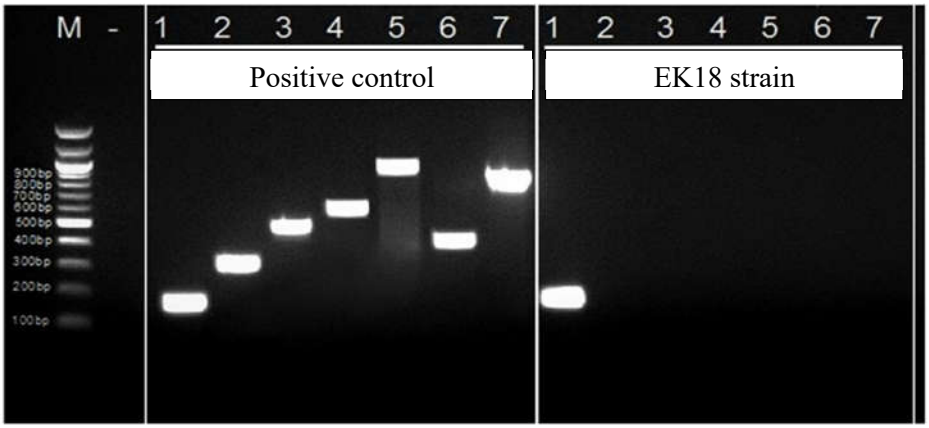

Figure 3.3. Typing of EK18 virus strain by PCR

M: 100 bp plus DNA ladder (Gold Biotechnology, USA); – : Negative control (PCR sample without template DNA); Positive controls 1–7: PCR samples with positive DNA from type 1 to type 7 (Parvovirus DNA provided by Kangwon University, Republic of Korea); EK18 virus strain 1–7: PCR samples with DNA of EK18 virus strain tested for type 1 to type 7.

All 32 parvovirus isolates obtained in this study were identified as type 1 (Figure 3.3). In Vietnam, a 2020 study reported that 88.2% (60/68) of serum samples collected from pig farms in six provinces, including Hanoi, Vinh Phuc, Bac Ninh, Hung Yen, Hoa Binh, and Hai Duong, contained antibodies against PPV1. Genetic analysis of 10 samples, based on the NS1 gene, confirmed that these strains all belonged to PPV1 (Huynh Thi My Le et al., 2020). More recent studies in Vietnam have documented the circulation of PPV1–PPV4 (Nguyen et al., 2022; Tran Van Trung, 2024). However, those prevalence rates were derived from slaughterhouse samples (Tran Van Trung, 2024) or samples that had already tested positive for other viruses (Nguyen et al., 2022). Because slaughterhouses mainly process finishing pigs or sows that are no longer reproductively active, and the slaughtering conditions are not sterile (Dang-Nguyen et al., 2010), the

reported PPV prevalence rates were considerably higher than those observed in the present study. In contrast, the samples in this study were collected from breeding herds maintained at farms or household production units, which explains the lower prevalence rate (8.2%) and the differences in type distribution. Only PPV1 was detected in this study.

These findings are consistent with the global predominance of PPV1. Currently, PPV1 is the most prevalent genotype in most countries worldwide (Streck and Truyen, 2020). Furthermore, most commercially available vaccines are developed based on PPV1 strains. Porcilis® Parvo (inactivated vaccine, Netherlands), Eryseng® Parvo (inactivated vaccine, Spain), and ReproCyc® ParvoFLEX (subunit vaccine, Germany) all employ PPV1-derived antigens (Vereecke et al., 2022). Numerous studies on PPV subunit vaccines have also been conducted using PPV1 strains.

The PPV1 isolates obtained in this study may therefore represent valuable seed strains for further research on parvovirus vaccine development in Vietnam.

3.3. GENETIC RELATIONSHIP ANALYSIS OF PARVOVIRUS

3.3.1. Sequence analysis of the NS1 gene

Phylogenetic analysis revealed that both VC5 and TX7 strains belong to PPV1 (Figure 3.4). PPV1 is the most prevalent genotype, accounting for approximately 80% of PPV strains worldwide (Streck et al., 2015). The use of a highly prevalent genotype as a vaccine seed strain is expected to provide enhanced protective efficacy. Therefore, the PPV strains isolated in this study are well-suited for vaccine development and hold promise for achieving effective disease prevention.

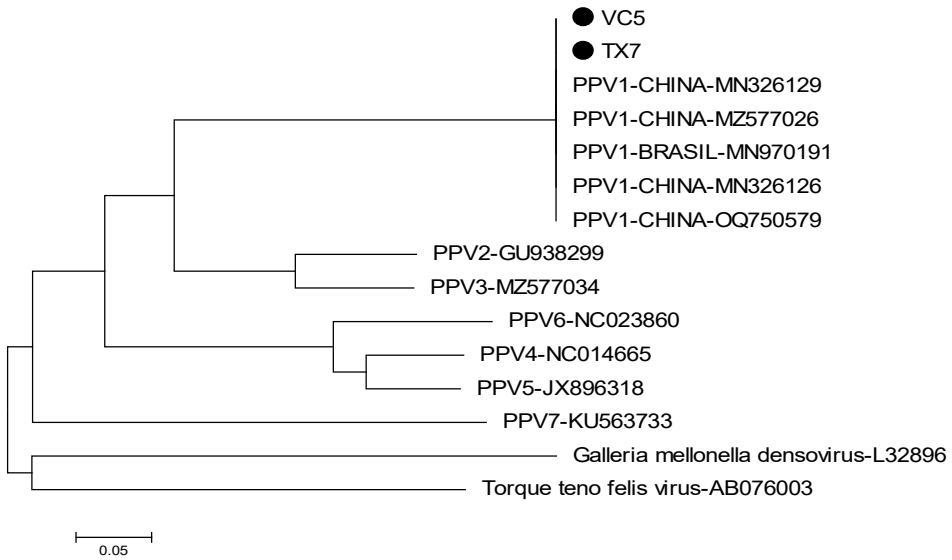

Figure 3.4. Phylogenetic tree based on NS1 gene sequence

3.3.2. Sequence analysis of the VP2 gene

Sequence analysis of the VP2 gene from 32 PPV isolates in this study revealed 100% identity. Since no sequence variation was detected among the isolates, six sequences representing six strains from different provinces were selected for submission to the NCBI GenBank database under accession numbers OR263486–OR263491. Phylogenetic analysis demonstrated that the PPV isolates were most closely related to the Kresse strain (isolated in Canada in 1996) and the PPV1-0225-L-SD strain (isolated in China in 2022) (Figure 3.5).

In a recent study comparing the protective efficacy of several commercial PPV vaccines, a vaccine derived from the K22 PPV "Kresse-like" strain conferred superior protection when challenged with the PPV-27a strain, compared with commercial vaccines developed from NADL-2- and NADL-like strains (Kiss et al., 2020). Based on the phylogenetic results of the present study, the isolates exhibited the closest relationship to the Kresse strain. This genetic similarity further supports the potential of these isolates as candidate strains for vaccine development.

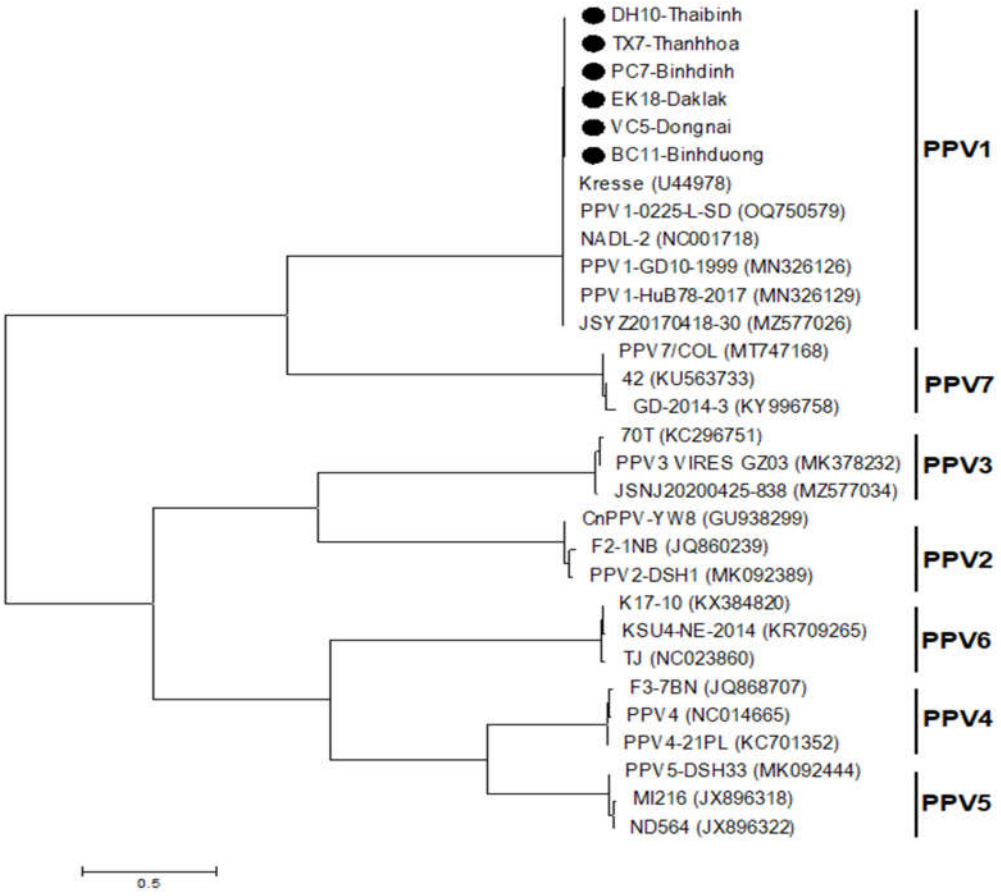

Figure 3.5. Phylogenetic tree based on VP2 gene sequence

3.4. CHARACTERIZATION OF PARVOVIRUS ISOLATES

3.4.1. Determination of PPV growth curves on PK-15 cell

After investigation, the growth curves of the PPV strains in this study were determined as follows: the virus replicated rapidly from day 1 to day 4, reached its peak growth on day 5, and remained stable from day 5 to day 7. Based on the established growth curves, it was observed that when PPV was inoculated at an MOI of 0.01, the viral load reached its maximum after 5 days of culture. This time point represents the optimal stage for virus harvest.

3.4.2. Determination of $TCID_{50}$

The TCID50 titers of all 32 successfully isolated PPV strains were determined. The results of TCID50 determination for the 32 isolates are presented in Table 3.3.

Table 3.3. Determination of $TCID_{50}$ of PPV isolates

Location	Sample	Mean TCID50/1ml (log10) ± SD (n = 3)	Tukey's mean comparison group (95% CI, n = 3)
Thai Binh	DH2	5.1 ± 0.1	D
	DH4	4.6 ± 0.1	E
	DH11	5.4 ± 0.1	C
	DH15	4.5 ± 0.0	E
Thanh Hoa	KX24	6.1 ± 0.1	A, B
	TS8	4.5 ± 0.0	E
	TX7	**6.3** ± 0.0	**A**
	TX13	4.6 ± 0.1	E
Binh Dinh	PM21	5.1 ± 0.1	D
	PM27	4.5 ± 0.0	E
	PC7	5.5 ± 0.1	C
	PC15	4.5 ± 0.0	E
Dak Lak	EK18	4.5 ± 0.0	E
	EK20	4.5 ± 0.0	E
	EK27	5.5 ± 0.1	C
	EK31	4.6 ± 0.1	E
	MD22	4.6 ± 0.1	E
	MD28	4.6 ± 0.1	E
Dong Nai	**VC5**	**6.3** ± 0.0	**A**
	VC10	6 ± 0.1	B
	VC15	4.5 ± 0.0	E

Location	Sample	Mean TCID50/1ml (log10) ± SD (n = 3)	Tukey's mean comparison group (95% CI, n = 3)
	VC32	5.1 ± 0.1	D
	LT23	4.5 ± 0.0	E
	LT32	4.5 ± 0.0	E
	LT36	5 ± 0.0	D
Binh Duong	DA6	4.6 ± 0.1	E
	DA12	5.5 ± 0.1	C
	DA27	4.5 ± 0.0	E
	BC2	5 ± 0.0	D
	BC4	4.5 ± 0.0	E
	BC11	6 ± 0.1	B
	BC15	4.5 ± 0.0	E

* *p-value* < 0,001

The $TCID_{50}$/1ml titers of the isolated PPV strains ranged from 4.5 to 6.3 log $TCID_{50}$/1ml. Among them, two strains, VC5 and TX7, exhibited the highest average titers (6.3 log $TCID_{50}$/1ml), which were categorized into group A and showed a statistically significant difference compared to most of the other strains. These findings indicate that VC5 and TX7 possess strong replication capacity in PK-15 cells, comparable to the titers of several PPV strains that have been utilized in vaccine development studies (Zeew et al., 2007, Van den Born et al., 2020). Therefore, VC5 and TX7 can be considered potential candidates for further investigations on virulence and immunogenicity in sows, thereby contributing to the development of PPV vaccines in Vietnam.

3.4.3. Determination of the optimal culture conditions

Based on statistical analyses and growth curves (Figure 3.6), the optimal conditions for PPV cultivation were determined to be 37°C with 5% FBS. Under these conditions, the virus exhibited stable growth over time, with a marked increase after day 3, reaching the peak of replication on day 5, and remaining stable through days 6–7. Significant statistical interactions were observed between Temperature–Time and FBS concentration–Temperature (*p-value* < 0.05). In contrast, the interactions between FBS concentration–Time and the three-way interaction of Temperature–FBS concentration–Time were not statistically significant (*p-value* > 0.05)

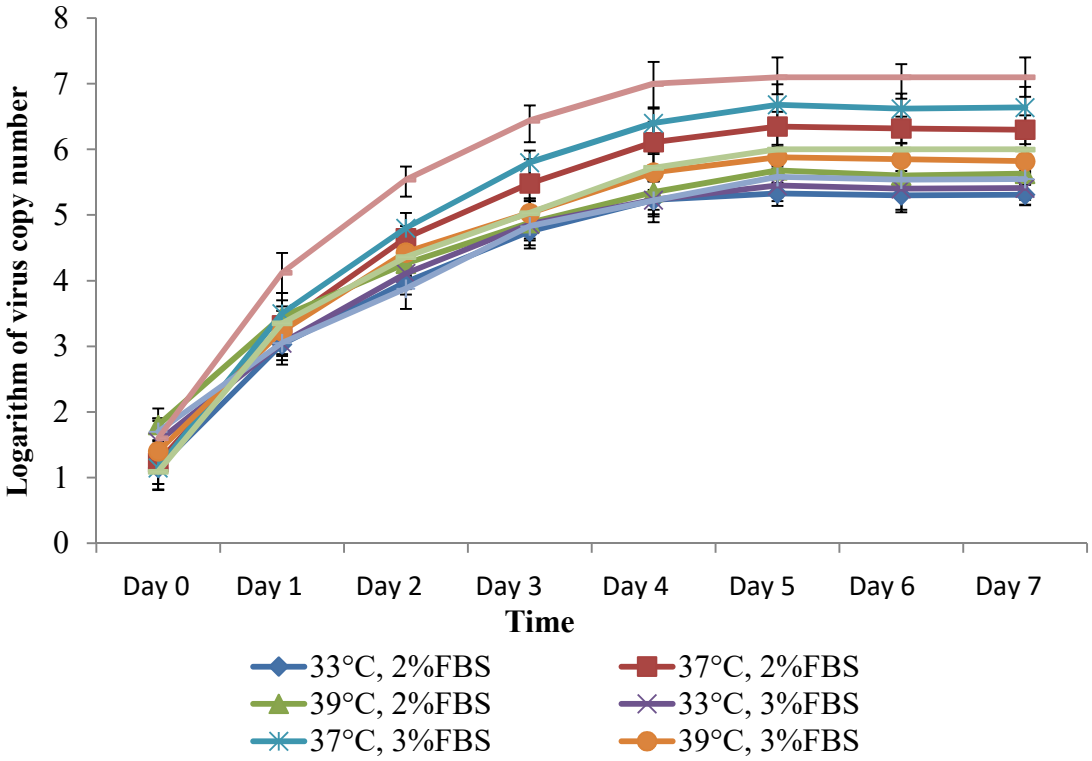

Figure 3.6. Growth curve analysis for determining optimal culture conditions

3.5. EVALUATION OF STABILITY, VIRULENCE, AND IMMUNOGENICITY OF PARVOVIRUS

3.5.1. Assessment of Parvovirus Stability

Genetic analysis revealed no differences in the genotypes of the isolates included in this study. Results of virus propagation and $TCID_{50}$ determination demonstrated that two isolates, VC5 and TX7, exhibited robust replication capacity with high viral titers. Therefore, these two isolates were selected for further evaluation of biological and genetic stability.

3.5.1.1. Evaluation of Biological Stability

*** Culture Characteristics Based on Cytopathic Effects (CPE)**

Observation of CPE on PK-15 cells indicated that after 15 serial passages, both VC5 and TX7 consistently induced stable cytopathic effects. These included granulation, morphological alterations of the cells, and extensive cell destruction leading to the formation of large clear areas on the surface of the culture flasks.

*** Growth Curves across passages**

The growth kinetics of isolates TX7 and VC5 were highly consistent across passages. Both isolates exhibited rapid viral replication from day 1 to day 4, reached peak titers at day 5, and remained stable from days 5 to 7. No significant differences in viral

copy numbers were observed among the passages, indicating stable replication profiles of both isolates after 15 passages.

*** $TCID_{50}$ titers across passages**

Determination of infectious titers showed that viral concentrations remained stable over 15 serial passages. $TCID_{50}$ values consistently ranged from 6.3 to 6.5 log_{10} $TCID_{50}$/1ml, confirming the biological stability of isolates VC5 and TX7 during extended passaging.

3.5.1.2. Evaluation of Genetic Stability

*** Stability of the NS1 Gene**

Comparison of NS1 gene sequences across 15 serial passages revealed no alterations, indicating that the NS1 gene remained stable during extended propagation.

*** Stability of the VP2 Gene**

Similarly, no sequence variation was detected in the VP2 gene of isolates VC5 and TX7 after 15 passages.

These findings demonstrate that both VC5 and TX7 are genetically stable in addition to maintaining biological stability across multiple passages. Therefore, these isolates may serve as suitable candidates for further research on vaccine development against porcine parvovirus.

3.5.2. Evaluation of Parvovirus Virulence

The hemagglutination inhibition (HI) titers in pregnant sows following viral challenge showed marked variation over time and significant differences among the experimental groups. The effects of "group," "time," and the interaction "group × time" were all highly significant (*p-value* < 0.001). By day 7 post-infection, specific antibodies were already detectable, with mean titers of 4.67 log_2 in the VC5 group and 5.33 log_2 in the TX7 group, whereas the control group remained at 0. The sharp increase during the first week indicated that both isolates replicated efficiently and elicited an early humoral immune response.

Antibody titers continued to rise, reaching their peak on day 21 with 10.67 log_2 in the VC5 group and 11.33 log_2 in the TX7 group, representing the highest values recorded during the entire experiment. Tukey pairwise analysis indicated that at day 21, TX7 achieved the highest mean titer (11.33 log_2, group A), followed by VC5 (10.67 log_2, group A–B), while the control group showed no detectable antibody response (0 log_2, group F). By day 49, HI titers in both experimental groups declined slightly but remained high (9.67 log_2 for VC5 and 10.0 log_2 for TX7).

Necropsy performed at day 90 of gestation revealed that all fetuses in the control group were healthy, whereas 100% of fetuses in the virus-infected groups were dead. Among them, 71% of fetuses from sows infected with isolate TX7 and 80% from those infected with isolate VC5 exhibited typical mummification. Mummified fetuses were characterized by dark brown coloration, desiccation, and dehydration. Furthermore, 88–90% of fetuses in the infected groups tested positive for PPV, while no evidence of PPV was detected in fetuses from the control group.

3.5.3. Evaluation of Immunogenicity of Parvovirus Isolates

3.5.3.1. Assessment of the Immunogenicity of VC5 and TX7 in a Substitute Animal Model

To identify potential PPV isolates for vaccine development, it is essential to evaluate their immunogenicity in substitute animal models. This approach provides a scientific foundation for establishing quality control assays without relying exclusively on the target host. The use of substitute animals offers several advantages, including reduced cost, ease of availability, and simplified husbandry, and has been widely applied in the testing of various vaccines.

Ma et al. (2011) previously assessed the immunogenicity of PPV in guinea pigs, with antibody titers determined by the HI assay. Accordingly, in this study, the immunogenic potential of PPV isolates VC5 and TX7 was evaluated in guinea pigs.

Five guinea pigs per experimental group were inoculated with one-fourth of the dose used in pigs. Results demonstrated that HI titers varied significantly by group, number of immunizations, and sampling time, with highly significant differences (*p-value* < 0.001). Following the first immunization, HI titers increased only slightly, ranging from 0.47 to 1.47 $\log_2$ for both TX7 and VC5 between days 7 and 14. Tukey pairwise analysis indicated that animals receiving a single dose reached a mean HI titer of only 0.91 $\log_2$ (group B), which was significantly lower than that of the two-dose group (3.53 $\log_2$, group A).

A marked increase in HI titers was observed after the booster immunization. At day 0 before the booster, titers had already reached 3.6 $\log_2$ (TX7) and 3.2 $\log_2$ (VC5). Titers then continued to rise rapidly, reaching 6.2–7.6 $\log_2$ (TX7) and 5.6–7.4 $\log_2$ (VC5) between days 14 and 21. The highest antibody levels were recorded at day 21 post-booster.

Importantly, throughout the experiment, no abnormal clinical signs or local adverse reactions at the injection sites were observed in guinea pigs, indicating that both isolates

were safe in this substitute animal model. Demonstrating safety in guinea pigs provides critical scientific and practical evidence to support subsequent immunogenicity assessments in the target host species, pigs.

3.5.3.2. Evaluation of the Immunogenicity of VC5 and TX7 in Sow

The results demonstrated a progressive increase in mean HI titers in immunized pigs. After the first immunization, mean HI titers reached only 1.56 and 1.44 $\log_2$ in the TX7 and VC5 groups, respectively. A sharp increase was observed following the booster dose, with mean titers at day 21 post-booster reaching 7.33 $\log_2$ in the VC5 group and 7.67 $\log_2$ in the TX7 group. In contrast, no PPV-specific antibodies were detected in the negative control group throughout the experiment (HI = 0). Statistical analysis confirmed that treatment group, number of immunizations, and time significantly influenced HI titers (*p-value* < 0.001).

Following virulent challenge, 18 of 22 fetuses (81.82%) in the VC5-immunized group and 18 of 21 fetuses (82.35%) in the TX7-immunized group remained viable, whereas 100% of fetuses in the control group died and exhibited typical pathological lesions. Approximately 75% of fetuses in the control group showed signs of mummification, characterized by dehydration, darkened coloration, firmness, and reduced size compared with healthy fetuses.

PPV was detected in 90% of fetuses in the control group, while only 2 of 22 (9.09%) and 2 of 21 (9.52%) fetuses were positive in the VC5- and TX7-immunized groups, respectively. These findings are consistent with previous studies evaluating inactivated PPV vaccines.

ELISA analysis of PPV-specific antibody responses in pigs revealed a clear increase in PI values in both immunized groups (TX7 and VC5). After the first immunization, PI values increased but did not exceed the positivity threshold. Following the booster dose, PI values rose sharply in both vaccine groups, surpassing 50% and peaking at day 21 (TX7: 72.48%; VC5: 71.51%). Statistical analysis confirmed that treatment group, number of immunizations, and time significantly influenced ELISA values (*p-value* < 0.001).

Tukey pairwise comparisons showed that both TX7 and VC5 induced significantly higher mean PI values compared with the control group (*p-value* < 0.05), while no significant difference was observed between TX7 and VC5. Regarding the number of immunizations, the booster dose induced significantly higher PI values compared with the primary dose (36.74 vs. 17.62; *p-value* < 0.05). With respect to time, PI values increased

progressively post-immunization and reached the highest level at day 21 (41.94), which was statistically different from all other time points.

These results are comparable to international studies on PPV vaccines. For instance, an evaluation of Parvoruvax (based on strain K22, “Kresse-like”), Vaccine B (based on strain NADL-2), and Vaccines C and D (based on strain 014, “NADL-2-like”) showed that antibody titers measured by ELISA remained low after immunization, with only 15% of vaccinated animals exceeding the positive cutoff of the assay (Kiss et al., 2020).

In summary, isolates VC5 and TX7 demonstrated strong virulence and robust immunogenicity. These two isolates represent promising candidates for future PPV vaccine development.

- **EVALUATION OF THE POTENTIAL USE OF PPV STRAINS IN VACCINE PRODUCTION**

The two strains selected in this study, VC5 and TX7, belong to PPV1. PPV1 is the most widely distributed group worldwide (Streck and Truyen, 2020). Based on VP2 gene analysis, these two strains showed the closest relationship to the Kresse strain (isolated in Canada in 1996). According to recent studies, vaccines derived from the Kresse strain provide superior protection compared with commercial vaccines based on NADL-like strains (Kiss et al., 2020).

Moreover, VC5 and TX7 exhibited strong replication capacity in PK-15 cells, with $TCID_{50}$ values consistently above 6.0 log_{10} across multiple passages. In vaccine research, PPV strains are commonly used for vaccine production when $TCID_{50}$ values exceed 5.0 log_{10}, and for challenge experiments when $TCID_{50}$ values are above 6.0 log_{10} (Van den Born et al., 2020; Zeeuw et al., 2007; Foerster et al., 2016).

Both strains also demonstrated high stability in terms of biological and genetic characteristics. Their virulence and immunogenicity were comparable to findings reported for several commercial PPV vaccines (Van den Born et al., 2020; Zeeuw et al., 2017; Foerster et al., 2016; Kiss et al., 2020; Jóźwik et al., 2009; Noguera et al., 2021; Garcia-Morante et al., 2020; Ma et al., 2011).

Taken together, these results indicate that VC5 and TX7 fully meet the requirements for further research and development of vaccines against porcine parvovirus infection.

CHAPTER IV
CONCLUSION AND RECOMMENDATIONS

4.1. CONCLUSIONS

1. The study successfully isolated 32 PPV strains from clinical samples collected in six provinces: Thai Binh, Thanh Hoa, Binh Dinh, Dak Lak, Dong Nai, and Binh Duong.

2. All 32 isolates were classified as type PPV1. NS1 gene sequences showed no variation among the isolates, and these isolates were genetically closely related to PPV1 strains previously reported in China. The VP2 gene sequences of the 32 isolates were 100% identical, exhibiting no sequence differences. Phylogenetic analysis indicated that the isolates in this study are most closely related to the Kresse strain (Canada, 1996) and the PPV1-0225-L-SD strain (China, 2022).

3. The optimal culture conditions for the parvovirus isolates in this study were determined as E-MEM supplemented with 5% FBS and 1% antibiotics, incubated at 37°C with 5% CO_2. Under these conditions the virus replicated rapidly from day 1 to day 4, reached peak replication on day 5, and remained stable from day 5 to day 7. Viral titers of the isolates ranged from 4.5 to 6.3 $\log_{10}$ $TCID_{50}$/1 ml. Among them, strains VC5 and TX7 exhibited the highest titers and were selected for further evaluation of stability, virulence, and immunogenicity.

4. Strains VC5 and TX7 demonstrated both biological and genetic stability over 15 serial passages. Both isolates exhibited high virulence, causing fetal death in 100% of experimentally infected sows, with 71% (TX7) to 80% (VC5) of fetuses showing characteristic lesions (mummification). The two strains induced robust humoral responses in pigs and in guinea pigs, with HI titers rising after vaccination and peaking at day 21 following the second dose. Importantly, VC5 and TX7 conferred substantial protection in pigs, with protection rates of 81.82% and 82.35% (proportion of live fetuses) following virulent challenge

4.2. RECOMMENDATIONS

The VC5 and TX7 strains exhibited high TCID50 titers and demonstrated stable biological as well as genetic characteristics over 15 serial passages, along with high virulence and strong immunogenicity. It is recommended that further studies be conducted in the following directions:

1. Conduct in-depth studies to complete the viral characterization database: This includes detailed morphological investigation using transmission electron

microscopy (TEM), full-genome sequencing, and advanced analyses of the genetic and molecular structural features of the VP2 gene (based on 3D capsid modeling or antibody docking approaches).

2. Carry out subsequent studies to improve the evaluation protocol for candidate vaccine strains towards the pilot production of an inactivated PPV vaccine for swine: This involves determining appropriate storage conditions and assessing viral stability over time; selecting suitable adjuvants to enhance immunogenic responses; and comparing the protective efficacy and immune responses of experimental vaccines derived from VC5 and TX7 strains with those of currently available commercial vaccines.

Implementation of these recommendations will contribute to the completion of the viral characterization database and provide a comprehensive evaluation of vaccine candidate strains, thereby supporting pilot production and facilitating future application in the development and commercialization of effective PPV vaccines for the swine industry in Vietnam.

SUMMARY OF NEW CONTRIBUTIONS OF THE DISSERTATION

The dissertation provides several novel findings that contribute to the field of parvovirus isolation and the selection of potential strains for vaccine development, as follows:

1. This study represents the first successful isolation of porcine parvovirus in Vietnam using the continuous PK-15 cell line.

2. The research systematically identified both the biological and genetic characteristics of PPV strains isolated in Vietnam, thereby providing a comprehensive understanding of the currently circulating strains in the country.

3. Two PPV strains with stable biological and genetic characteristics over multiple passages, high virulence, and strong immunogenicity were successfully selected. These strains constitute promising candidates for the development of vaccines against porcine mummification. Importantly, no vaccine against porcine mummification is produced or commercialized domestically in Vietnam— all available porcine parvovirus vaccines are imported—the outcomes of this study establish a scientific foundation for the development of an indigenous PPV vaccine, addressing a critical gap in veterinary disease prevention and control in Vietnam.

LIST OF PUBLISHED WORKS

1) TT Hang Trinh, V. Tan Do, V. Khien Do, and Hung Vu-Khac, (2024), “Isolation and characterization of porcine parvovirus in Vietnam”, Veterinary World, 17(7), pp. 1530–1537.

2) Trinh Thi Thu Hang, Vu Khac Minh Duong, Do Van Tan, Pham Trung Hieu and Vu Khac Hung, "Determination of some biological characteristics and immunogenicity evaluation of two parvovirus isolates in Vietnam", Vietnam Journal of Science and Technology, version B (Acceptance letter)