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Second Cycle Degree (MSc)

In Biotechnologies for food sciences

"Does DNA extraction really matter? Validation and comparison of a standard and direct Real-Time PCR protocol for the rapid and economic diagnosis of Porcine circovirus type 2 (PCV-2) infection."

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Table of Contents

Abstract.

Porcine circovirus type *2* (PCV-2) is a virus of the *Circoviridae* family that since the first reports in the 90s has had an increasing impact on the swine industry in pig-producing countries all over the world causing multiple syndromes. Many diagnostic protocols have been developed and used for PCV-2 detection. Most of them are highly accurate but they can be costly and time-consuming, limiting their application, especially in developing countries.

To develop and validate a rapid and inexpensive protocol that can find application for research purposes and routine diagnosis, a direct Real-Time PCR (without DNA extraction) for the detection of PCV-2 was compared to a standard Real-Time PCR, requiring a preliminary DNA extraction step.

Optimization experiments for the direct Real-Time PCR were performed evaluating procedures on samples before loading and amplification like preheating, pipetting and centrifuging. Since no significant improvement in efficiency and sensitivity was detected after integrating these extra steps in the protocol, the final validation and comparison with the standard Real-Time PCR (with DNA extraction) was performed using the less time-consuming and easier direct protocol.

The parallel comparison was important for validating the accuracy of the protocol. Statistical analysis showed that the standard Real-Time PCR method provides higher PCR efficiency and repeatability and a lower variability in the viral titre estimation than the direct method. The overall higher efficiency of the standard Real-Time method could be explained by the process of extraction that helps to reduce potential inhibitors of amplification reaction.

The here validated direct Real-Time PCR method represents a potential alternative to the standard one for developing countries, guaranteeing a more affordable and rapid detection method to control PCV-2. On the other hand, the standard Real-Time PCR method still ensures higher efficiency and repeatability, thus more reliable results which are crucial when higher accuracy is needed.

The optimization of the direct method in order to enhance its performances is a prospect for future research.

Riassunto.

Il *Porcine circovirus 2* (PCV-2) è un virus appartenente alla famiglia *Circoviridae* che, sin dalle prime segnalazioni negli anni ’90, ha avuto un impatto crescente nella suinicoltura dei paesi produttori a livello globale essendo responsabile di molteplici sindromi. Diversi sono i protocolli diagnostici che son stati sviluppati ed utilizzati per l’identificazione di PCV-2. La maggior parte di essi forniscono risultati altamente accurati, ma il costo, il reagentario e la strumentazione necessari ne limitano l’applicazione, specialmente nei Paesi in via di sviluppo.

Con l’obiettivo di sviluppare e validare un protocollo rapido ed economicamente più accessibile che allo stesso tempo possa trovare applicazione nella ricerca e nella diagnosi di routine, è stata comparata una Real-Time PCR “diretta” (in cui i campioni non sono stati sottoposti ad estrazione del DNA) per l’identificazione di PCV-2 con il protocollo di Real-Time PCR standard, il quale richiede invece un processo preliminare di estrazione.

Durante la fase di ottimizzazione della Real-Time PCR diretta i campioni sono stati sottoposti a diversi trattamenti prima di essere testati, quali preriscaldamento, pipettaggio e centrifugazione. Poiché non è stato rilevato alcun miglioramento significativo dell'efficienza e della sensibilità dopo l'integrazione di queste fasi aggiuntive nel protocollo, la validazione finale e il confronto con la Real-Time PCR standard sono stati eseguiti utilizzando il protocollo diretto più pratico e meno dispendioso in termini di tempo.

Il confronto in parallelo è stato fondamentale per validare l’accuratezza del protocollo sviluppato. L’analisi statistica ha evidenziato come il metodo standard di Real-Time PCR garantisca una migliore efficienza e ripetibilità, così come una maggiore sensibilità e minore variabilità nella stima dei titoli virali rispetto al metodo diretto. Nel complesso, la maggiore efficienza registrata per il metodo standard di Real-Time potrebbe essere spiegata dal processo di estrazione che permette di ridurre potenziali inibitori della reazione di amplificazione.

Il metodo diretto di Real-Time PCR validato potrebbe rappresentare una potenziale alternativa a quello standard soprattutto per i Paesi in via di sviluppo, fornendo uno strumento rapido ed economico per il controllo del PCV-2. D'altra parte, il protocollo standard di Real-Time PCR assicura una maggiore efficienza e ripetibilità, garantendo quindi risultati più affidabili, fondamentali quando richiesta una maggiore accuratezza. L'ottimizzazione del metodo diretto per migliorarne le prestazioni rappresenta sicuramente una prospettiva di ricerca futura.

1. Introduction.

In the introduction chapter, a general overview of *Porcine circovirus* type 2(PCV-2) is provided, with a particular focus on its characteristics and epidemiology, current methods available for its identification, and the importance of developing and validating a new, more rapid, and affordable protocol for its detection.

1.1 Introducing *Porcine circovirus*es (PCVs): emergence, taxonomy and epidemiology.

Porcine circovirus 2 (PCV-2) is a virus affecting pig-producing countries worldwide. Being one of the main causes of pig losses, and thus economic losses, it is crucial nowadays to have a rapid, reliable, and affordable method for identifying the presence of this virus on a farm.

The first Porcine circovirus (later designated as PCV-1) was discovered in 1974 as a contaminant of porcine kidney-15 cell cultures (PK-15 cells) (Tischer et al., 1974). It belongs to the *Circoviridae* family and the genus *Circovirus*. Its presence was detected in several countries like Germany (Tischer et al., 1986), the USA (Hines & . Lukert, 1995), Canada (Dulac & Afshar, 1989), and England (Edwards & Sands, 1994).

In the late 1990s, a new PCV phylogenetically different from the PCV-1 was detected in pigs showing an emerging wasting disease, later termed as the post-weaning multisystemic wasting syndrome (PMWS). This new pathogen was named *Porcine circovirus type 2* (PCV-2) (Meehan et al., 1998).

Lately, in 2015, a new *Porcine circovirus* type 3, (PCV-3) was discovered thanks to recent developments in sequencing technologies, i.e. next-generation sequencing (NGS) (Palinski et al., 2017; Phan et al., 2016). However, similar to other circoviruses far more ancient origin and long-time circulation was thereafter demonstrated (Franzo et al., 2019; Opriessnig et al., 2020). It is still uncertain if PCV-3 directly induces any diseases or requires the co-infection with other pathogens or other co-factors as it has been detected in pigs with overt clinical disease (Kedkovid et al., 2018; Phan et al., 2016; Qi et al., 2019; Shen et al., 2018) but also in healthy animals (Klaumann et al., 2018, 2019).

Similarly, in 2019, a new *circovirus*, *Porcine circovirus* type 4 (PCV-4) was identified both in healthy pigs and in animals with severe disease including respiratory signs, enteric signs and porcine dermatitis and nephropathy syndrome (PDNS) in Hunan province, China (Zhang et al., 2019). PCV-4 was later detected also in other Chinese provinces and South-Korea (Chen et al., 2021; Ha et al., 2021; Hou et al., 2022; Nguyen et al., 2022; Sun et al., 2021) The knowledge and data pool around PCV-3 and, especially, PCV-4 continues to be limited and they can be considered as a prospective research field.

Porcine circoviruses have a simple architecture, featured by a single-stranded circular DNA (ssDNA) packed inside an icosahedral capsid measuring approximately 17 ± 1.3 nm in diameter (Grau-Roma et al., 2011) (see ***Figure 1***). The genome carries two major open reading frames (ORFs), ORF1 and ORF2, encoding the replicase (Rep) protein and the capsid (Cap) protein respectively (Hamel et al., 1998; Mankertz et al., 1998).

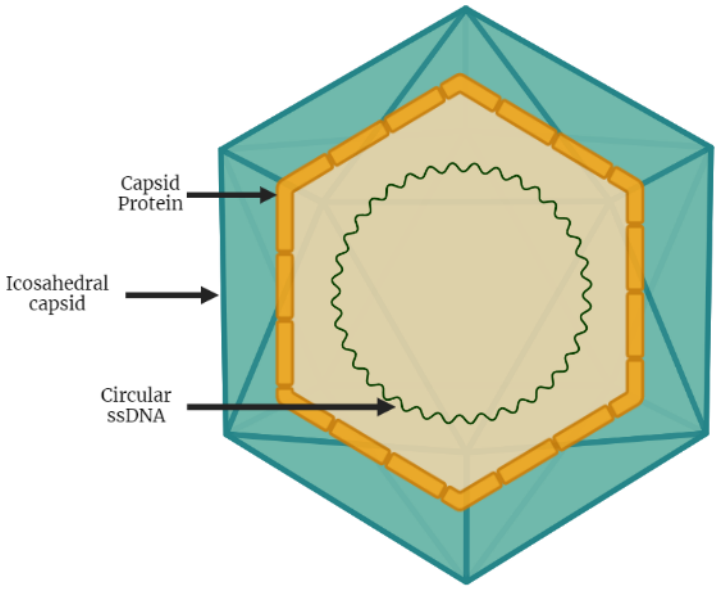

Figure 1. Circovirus structure (Created with BioRender.com).

The Rep is necessary for the replication of the circovirus genome, while the transcription of ORF2 gene produces a 233 amino acid capsid protein which is PCV-2 dominant immunogenic antigen (Cheung, 2003; Mankertz et al., 1998). In addition, several smaller ORFs have been identified, among which ORF3, ORF4 and ORF5 were characterized more in detail. The ORF3 gene encodes a 105-amino acid protein implicated in inducing apoptosis of infected cells and has been ascribed to have a role in PCV-2 pathogenesis (Chaiyakul et al., 2010; Juhan et al., 2010; Karuppannan et al., 2009; Liu et al., 2006), while the ORF4 seems to be involved in apoptosis inhibition. ORF5 may be involved in PCV-2 infection cycle (Lv et al., 2015).

As mentioned before, the ORF2 encoded capsid protein is the main target of the immune system, the selective pressure acting on this protein likely explains the higher variability of this gene (Segalés et al., 2008; Xiao et al., 2015a).

Studies and protocols implemented on ORF2 amplification allowed to investigate PCV-2 epidemiology and diversity, which is crucial considering its important economical role. Over time, several PCV-2 variants emerged, different both from an epidemiological and biological perspective. A classification of these in genotypes was proposed in 2018 (Franzo & Segalés, 2018), which has been allowing to define nine genotypes: from PCV-2a to PCV-2i (Wang et al., 2020).

Since its detection, major changes in the prevalence of circulating genotypes of PCV-2 have been observed. From 1996 to the early 2000s the most prevalent genotype was PCV-2a; a "genotype shift" to PCV-2b occurred in the mid-2000s, accompanied by increased virulence (Beach & Meng, 2012; Carman et al., 2006, 2008; Constans et al., 2015; Cortey et al., 2011; Franzo et al., 2016; Timmusk et al., 2008; Wiederkehr et al., 2009).

A second "genotype shift" is occurring globally, from PCV-2b to PCV-2d, thought to be a result of the worldwide use of PCV-2 vaccines (Franzo et al., 2016; Kwon et al., 2017; Xiao et al., 2015).

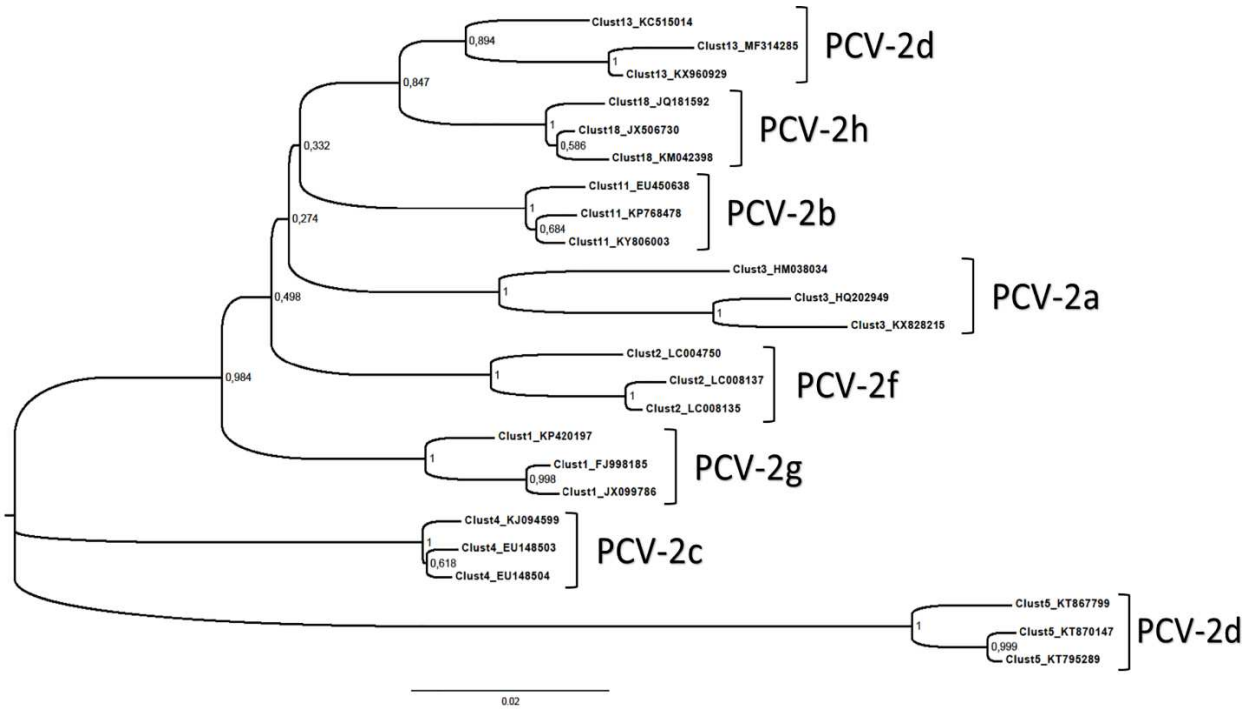

Figure 2. PCV-2 Neighbor-Joining phylogenetic tree. Neighbor-Joining phylogenetic tree reconstructed based on row genetic distances (i.e. pairwise p-distance) calculated on a collection of strains representative of the proposed PCV-2 genotypes. Both the cluster and genotype nomenclature are reported. Bootstrap support is displayed near the corresponding node (Franzo & Segalés, 2018).

Unlike PCV-1 which is apathogenic, PCV-2 is known to be the causing agent of some porcine diseases directly or in synergy with other pathogens or environmental factors. PCV-2 associated syndromes are collectively named Porcine circovirus diseases (PCVD) (see ***Figure 3***).

Figure 3: PCV-2 can cause uneven growth in pigs, creating economic loss for producers (Hancox, 2021).

The most common clinical conditions are:

- postweaning multisystemic wasting syndrome (PMWS), now named PCV-2 systemic disease (PCV-2-SD),
- porcine respiratory diseases complex (PRDC),
- porcine dermatitis and nephropathy syndrome (PDNS),
- enteritis,
- reproductive disorders (PCV-2-RD) (Ladekjaer-Mikkelsen et al., 2001).

We will use PCVD in this dissertation to refer to all clinical manifestations related to PCV-2 infection.

Interesting and still unsolved is the PCV-2 emergence, since PCVD symptoms emerged very unexpectedly and almost simultaneously in swine populations all over the world and increased in frequency over the years. However, retrospective studies have revealed the PCV-2 circulation for decades before its first detection. Some theories advocate that the emergence of other cofactors might have influenced the evolution of PCV-2 epidemiology (e.g. management practices, facilities, nutrition, etc.) (Grau-Roma et al., 2011). In addition, the development of global markets has enhanced the movement of animals or animal products between countries, thus favoring the spreading of infectious agents (Drew et al., 2011) (see ***Figure 4***).

As evidence of its wide distribution, this virus has reached also underdeveloped/developing countries or even small farmers around the world that have very limited access to the new technologies for the detection of these viruses. Similar consideration can be drawn for other pathogens that can affect also other plants, animals, and, even humans (Barman et al., 2018; Deka et al., 2021; Franzo et al., 2022; Park & Chae, 2021; Xu et al., 2021). To provide adequate surveillance also in developing countries, we must continuously work on optimizing our protocols to make them easy to use and more affordable to those with fewer economic resources. An "easy-to-use protocol" would facilitate the identification of PCV-2 in pig-producing farms leading to better control and prevention of disease development.

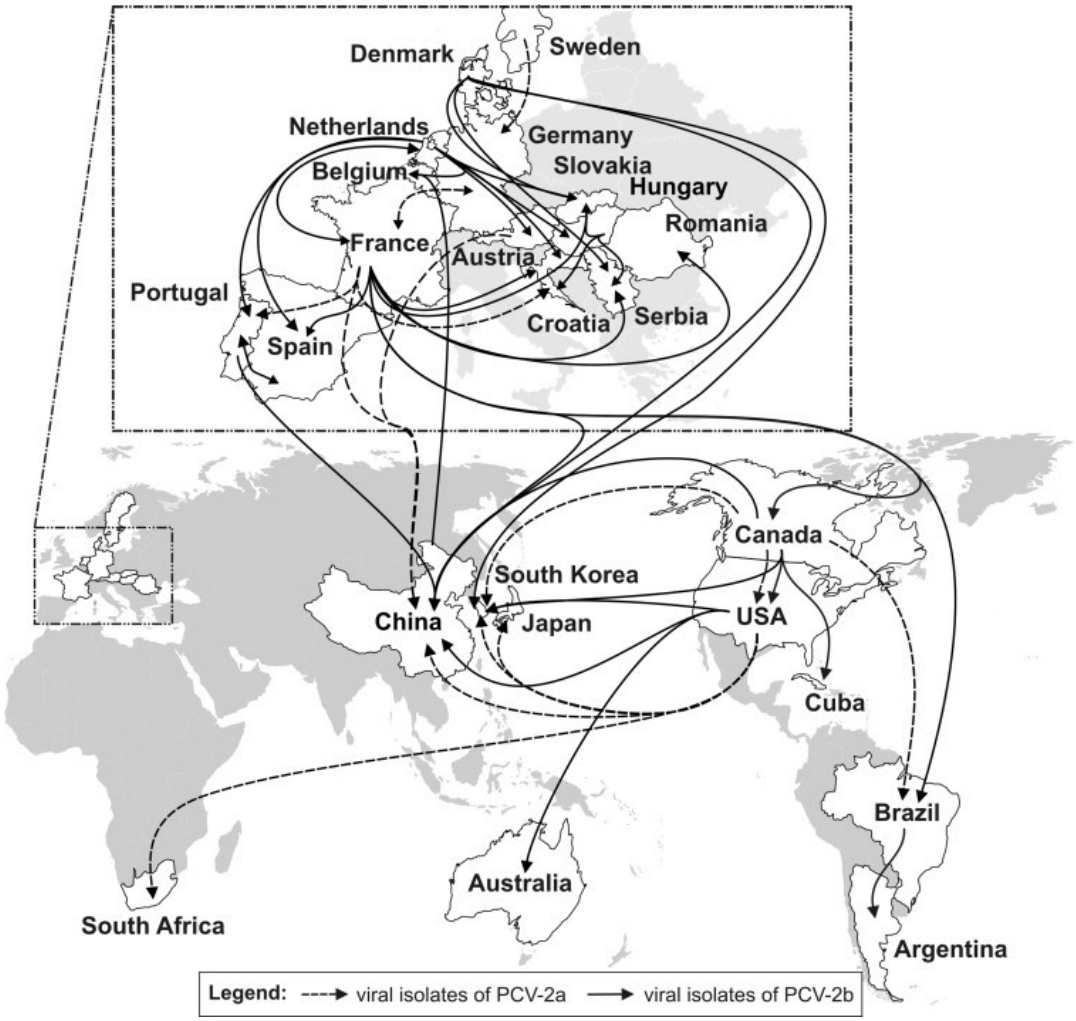

Figure 4. Prediction of the main routes of dispersal of PCV-2 in the swine industry. These routes were predicted from the haplotype network and considered the groups of viral isolates that were identified in more than one country and the statistics on the international trading of live pigs (Vidigal et al., 2012).

1.2 PCV-2 Vaccinations.

PCV-2 is now known as an endemic and highly prevalent virus, increasing the need to control its spread and clinical manifestations. Both in developed and in developing countries, management plays a pivotal role in preventing the devastating consequences of the PCVDs, and it consists in:

- disinfection;
- limiting animal contact,
- mixing of batches and cross-fostering;
- isolating or euthanasing of diseased pigs;
- temperature, airflow and pens space management;
- anti-parasitic treatments and vaccination. (Madec et al., 2000)

Conventional vaccines were developed and became available in 2004 in Europe and in 2006 in North America, and later they were introduced by pig farmers worldwide. As predicted, the usage of these vaccines was accompanied by a decrease in morbidity and improved production efficiency (Ellis et al, 2014).

Up to recent days, scientists have gathered a lot of knowledge about various aspects of PCV-2 such as its evolution, phylogeny, immune response, interaction with host cellular proteins and efficacy of the vaccines. The most used commercial vaccines that are now found on the market derive from the PCV-2a genotype and its capsid protein (Opriessnig et al., 2007). They can induce cell-mediated and humoral immunity against PCV-2 and have proven to be successful in decreasing the disease burden (Fort et al., 2009; Fort et al., 2012; Kekarainen et al., 2010).

Unfortunately, despite the health benefits and improved production parameters, PCV-2 infection is still widespread even among the vaccinated population. The PCV-2 genotypes continue to evolve and this is reflected by the recent change in the prevalence of PCV-2d (Xiao et al., 2016).

Circovac, Merial was the first commercial PCV-2 vaccine and was based on the classical technique of inactivated oil-adjuvanted vaccine.

Circovac was followed by four other commercial PCV-2 vaccines in the international market that were licensed only for use in piglets. Three of them (Circoflex, Boehringer Ingelheim; Circumvent, Intervet/Merck; Porcillis PCV, Schering-Plough/Merck) are based on ORF2 capsid protein because of its potential to induce a protective immune response (Blanchard et al., 2003; Nawagitgul et al., 2002).

The fourth vaccine (Suvaxyn PCV2 One Dose, Pfizer Animal Health/Fort Dodge Animal Health) is based on a chimaeric PCV-1/ 2 virus containing the genome of PCV-1, with the ORF2 capsid gene replaced by that of PCV-2 (Fenaux et al., 2004).

Vaccine	Antigen	Adjuvant	Animals	Dosage
Circovac	Inactivated PCV-2	Light paraffin oil	Sow	2mL
			Piglet	0.5mL
Circoflex	Capsid	Aqueous polymer	Piglet	1mL
Circumvent	Capsid	D1-a-tocopherol + liquid paraffin	Piglet	2mL
Porcillis PCV	Capsid	D1-a-tocopherol + liquid paraffin	Piglet	2mL
Fostera PCV	Inactivated chimaeric PCV1/2	Sulpholipo-cyclodextrin in squalane	Piglet	2mL

Table 1: Commercial porcine circovirus type 2 available in the international market (Chae, 2012).

1.3 Diagnostic methodologies.

Until recently many different assays were used for the detection of PCV-2 targeting the viral antigens or genome. For example, during the early studies performed in Canada to identify the causative agent of PMWS, direct detection of the viral DNA or antigen detection with *in situ* hybridization (ISH) and immunohistochemistry (IHC) respectively was carried out in formaline fixed tissues (McNeilly et al., 1999).

ISH is a method that uses sequence specific probes (labelled with either radio- or fluorescent-, labeled bases) to detect a specific sequence or region of the DNA/RNA. One of the most beneficial aspects of using ISH is the matrix optimization, since it is possible to perform different hybridizations on the same tissue. On the other hand, one of its drawbacks is the low sensitivity, making difficult the identification of low levels of DNA and RNA copies (Jensen et al, 2014).

IHC uses the antigen-antibody reaction to localize specific antigens in cells or tissues and is a widely used method. It can provide good results but it requires multiple steps and the development of a "gold-standard" assay for calibrating test results (Goldstein & Watkins, 2008; Hofman et al., 2002; Hofman & Taylor, 2013).

Another diagnostic methodology that is really important also from a surveillance and control perspective is the *enzyme-linked immunosorbent assay* (ELISA), an immunological assay commonly used to measure antibodies, antigens, proteins and glycoproteins in biological samples. For the detection of PCV-2, ELISA targets the well-characterized recombinant PCV-2 capsid protein (Ge et al., 2012; Lian et al., 2021). Nevertheless, ELISA sensitivity is lower than that of nucleic acid amplification tests because it does not amplify the target in the samples tested, potentially leading to higher false negative results (Martín et al., 2021).

In summary, these techniques have a limited sensitivity, low throughput and require expensive laboratory instruments and specialized personnel for sample processing and results interpretation, compared to other diagnostic methodologies.

Among the methods targeting the viral genome, polymerase chain reaction (PCR) is undoubtedly the most rapid and sensitive method for its identification. One of the earliest applied approaches involved a combination of methods used of nested polymerase chain reaction (nested PCR) with restriction fragment length polymorphism (RFLP) (Ciacci-Zanella & Morés, 2003).

It was only later in the early 2000s that Real-Time PCR (Chung et al., 2005) and loop-mediated isothermal amplification (LAMP) PCR (Zhou et al., 2011) started to be regularly used for the identification of PCV-2. More recently, DNA microarrays and multiplex ligase detection–PCR were used for the simultaneous detection of several swine viruses (Jiang et al., 2011), as well as isothermal recombinase polymerase amplification (RPA) assays (Yang et al., 2017).

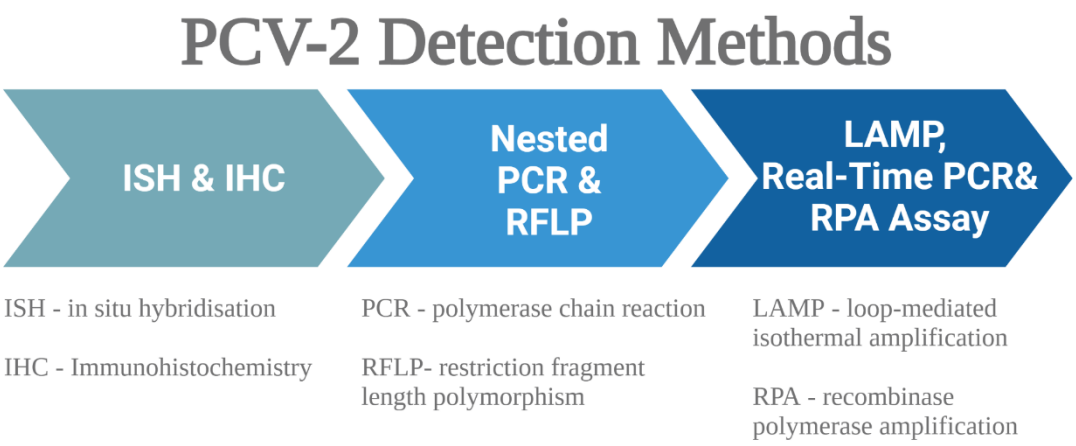

Figure 5. Schematic representation of methodology development from the first detection of porcine circovirus 2 (PCV-2) until today (Created with BioRender.com).

1.3.1 Introducing Real-Time PCR.

Real-Time PCR is currently the most used method for the identification of PCV-2 because it allows the sensitive, specific, and reproducible detection and quantification of nucleic acids. The assays are fast and easy to perform; the risk of carry-over contamination is minimal because of the closed-tube formats of the analyses; post-PCR processing is not required, and the results obtained have high precision, provided that the evaluation is done correctly (Wilhelm & Pingoud, 2003).

Real-Time PCR is a technology that has various applications such as:

- Gene expression analysis
- Detection of genetically modified organisms (GMOs)
- Quantification of viral load
- Genotyping of Single Nucleotide Polymorphisms (SNPs)
- Allelic discrimination.

This method has played a key role in the detection, quantification, and typing of viral pathogens due to its higher sensitivity and specificity (Hoffmann et al., 2009) for diagnostic purposes, compared to previous techniques (e.g. culture methods) which are more time-consuming and hard to perform for some viruses. Moreover, in combination with ELISA tests used to assess animal antibody coverage, it allows for effective monitoring measures which are crucial for surveillance and control plans.

The Real-Time PCR functioning is based on the monitoring of the generated fluorescence by using two main strategies:

1. Non-specific fluorescent DNA dyes (Higuchi et al., 1992) and
2. Oligonucleotide probes labelled with a fluorescent dye (Holland et al., 1991).

***Figure 6**. LightCycler® 96 Instrument used in our study* (Roche Molecular Systems).

In a probe based Real-Time PCR the knowledge of the target sequence that we want to amplify is pivotal because the primers and the probe must effectively bind that sequence. In our experiment ORF1 gene, a highly conserved region of the viral genome, was the target sequence (Opriessnig et al., 2003). ***Figure 6*** illustrates the Real-Time machine used in this study.

Real- Time PCR is a 3 steps reaction, whose temperature and duration depend on the kit used and primers and probe designed:

1. Activation
2. Denaturation
3. Primers Annealing and DNA polymerase Extension.

The *Activation* step, which can be also called "the initial denaturation step", activates the hot start DNA polymerase, whose enzymatic activity is suppressed at room temperature to avoid nonspecific amplifications prior to thermal protocol. Hence, temperature and timing depend on the polymerase enzyme used. The activation step is followed by denaturation, annealing and extention that are repeated in cycle (e.g. 45 cycles for our protocol).

During *Denaturation* temperature is again increased to roughly 95 °C to separate the double stranded DNA helix into two single-stranded DNA templates, preparing the nucleic acid for the annealing of primers and probe. Denaturation approximately lasts just 10 seconds.

During *Annealing and extension* temperature lowers roughly to 60 °C for 15-30 seconds (according to primers and probe designing, amplicon size and polymerase) to induce the binding of primers and probes to their target sequence in the template. DNA polymerase binds to the primers (forward and reverse) and adds the complementary nucleotides (dNTPs) , in the 5'-3' direction, one by one following the 3'-5' strands as a template. When the DNA polymerase reaches the probe location in the strand, it cleaves the probe, allowing fluorescence emission and detection, and displaces it with new dNTPs continuing the strand extension.

A probe is an oligonucleotide double-labelled with a reporter fluorophore at the 5' end and with a quencher at the 3' end. The quencher dye absorbs the fluorescence of the reporter dye due to its proximity, which prevents fluorescence emission. The two primers (reverse and forward) allow amplification of the product, and both the primers and the probe hybridize to the target sequence. During the target sequence amplification, the polymerase digests the probe, and the quencher is therefore separated from the fluorophore, which now emits fluorescence after excitation (see ***Figure 7***). The polymerase can cleave the probe only while it remains hybridized to its complementary strand, that is why the temperature conditions of the polymerization phase should be adjusted to ensure probe binding (Navarro et al., 2015).

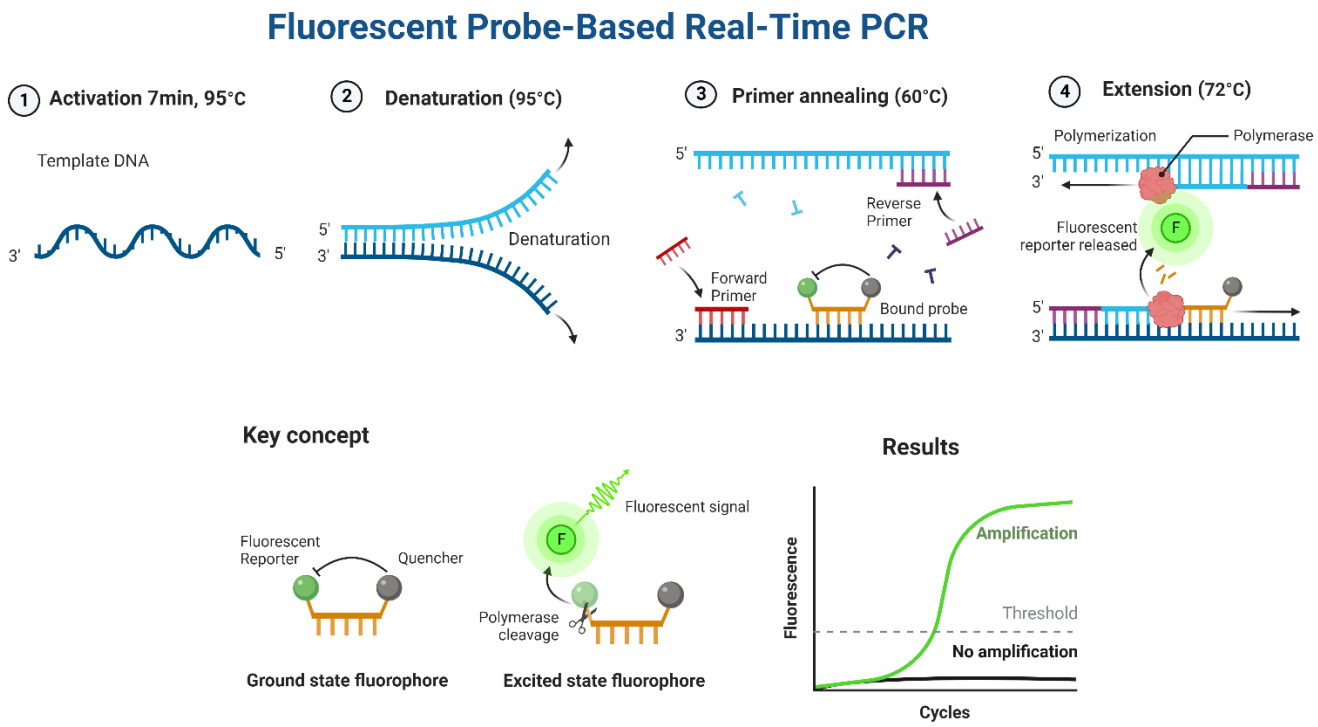

Figure 7. Concept illustration of Real-Time PCR steps with temperatures and timing used in our experiment and key concept illustration on how fluorescent probes work (Created with BioRender.com).

What makes Real-Time PCR unique and different from classic PCR is that the measurement of fluorescence after each cycle provides information about the amount of DNA amplicons in the sample in Real-Time. With each PCR cycle the amount of PCR product doubles which can be detected with an increase in fluorescence. This generates a classic Real-Time PCR curve, a plot of the amplification process in which the PCR product accumulation follows a sigmoidal pattern. During the first few cycles we have what we call *"initiation phase"* which means that the fluorescence cannot yet be distinguished from the baseline; then the PCR product increases exponentially and the intensity of fluorescence overcomes the background.

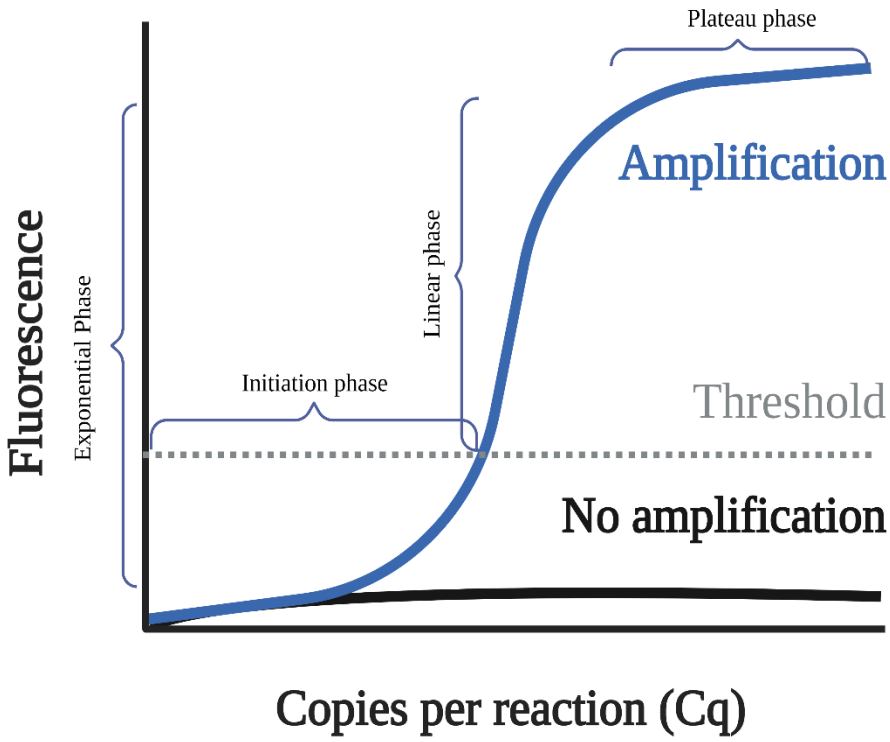

Figure 8. Classic Real-time PCR amplification curve. (Created with BioRender.com)

The fractional cycle when the fluorescence emerges above the background is called quantification cycle or "Cq value" (can be found in other literature also as Ct, meaning threshold cycle) and it is inversely proportional to the amount of the target. The greater the amount of target nucleic acid in the sample, the lower the Cq level will be as it will reach the detection level more quickly.

When the fluorescence reaches the threshold level the cycle enters the *"linear phase"*, in which PCR product, thus the fluorescence, increases linearly, roughly 1000 times every 10 cycles. When reagents start to run out, the curve reaches a "*plateau phase*" after which no increase in fluorescence can be observed.

It is during the linear phase that the information necessary to evaluate the efficiency of the reaction is obtained.

The efficiency value (E-value) of a PCR amplification is normally expressed as a percentage and gives the rate at which our PCR product is generated. In an ideal experiment, the product will duplicate after each round, and in this case the efficiency value is 100%. The E-value is calculated from the slope of a standard curve, generated through a ten-fold dilution, by the following equation:

$E = -1+10^{(-1/slope)}$.

The exponential amplification of the PCR reaction is obtained with the following equation: Exponential amplification= $10^{(-1/slope)}$.

The optimal efficiency value and exponential amplification value would be between 90 and 100% and between 1.9 – 2 respectively, which correspond to a slope between -3.6 and -3.3, that represents a difference of 3.6 - 3.3 cycles between a sample and its 10-folds dilution. A lower efficiency can be caused by:

Low Real-Time PCR Efficiency causes	
Causes	Effect
Bad primer/probe design	Unspecific alignment of primers/probe to their target Formation of secondary structures such as hairpins or primer dimers. Inadequate melting temperature of primers and probe
Reagent concentration	Not accurate reagent concentrations in the master mix.
Reaction conditions	Non-optimal reaction conditions.

Table 2: Cause-effect table of factors that can lead to a low efficiency of PCR (Čepin, 2017).

1.3.2 Standard versus Direct Real-Time PCR.

To perform a standard Real-Time PCR the nucleic acid needs to be extracted and isolated before the amplification.

A well-performed extraction is believed to provide more reliable results in Real-Time PCR because it prevents or lowers amplification inhibition or false-negative results from undesired contaminants.

On the other hand, DNA/RNA extraction is a challenging and time-consuming task, not to mention that the required reagents are usually quite expensive.

Moreover, the extraction phase introduces other variables that we need to account for to obtain a good and reliable results:

- Operator skills (laboratory technician, researcher, student, etc.),
- cross-contamination,
- extraction kit quality and accuracy
- cost of reagents.

Stating so, the standard Real-Time PCR method is not ideal for usage when needed in developing countries where local diagnostic laboratories often do not have the necessary facilities and staff to perform complicated and time-consuming steps like those involved in DNA extraction. A direct Real-Time PCR could allow saving on costs of reagents and time that are needed for nucleic acid extraction.

To overcome the disadvantages associated with extraction, without losing the advantages of Real-Time PCR, several direct methods were developed, above all for those pathogens whose rapid detection became suddenly urgent. To put the benefits of the direct Real-Time PCR method under this perspective, the outbreak of SARS-CoV-2 and, thus, the Covid-19 pandemic is a perfect example. Real-Time PCR proved to be one of the gold standard methods for the SARS-CoV-2 detection but the urgent need for rapid identification and the shortage of reagents and extraction kits due to the sudden spread of the disease, led to the need for a protocol that does not include the RNA extraction. Plenty of research was made to develop and optimize such protocols which could guarantee to the world a more rapid, cheaper and not less efficient detection method for the virus (Buchan et al., 2021; Lübke et al., 2020; Rajh et al., 2021; Smyrlaki et al., 2020).

Although the PCV-2 epidemiology is not as alarming as that of SARS-CoV-2, it still affects on daily bases the pig industry causing big economical damage both in developed and developing countries, thus justifying the need of developing rapid and easy to use protocols. With this purpose, my thesis aimed to develop, optimize, and validate a direct protocol allowing to skip the single-stranded DNA (ssDNA) extraction step.

1.4 Overview of thesis dissertation.

In this dissertation, we will refer to the Real-Time PCR where we initially performed nucleic acid extraction as “standard Real-Time PCR/ standard method” while to the Real-Time PCR performed directly without the extraction step as “direct Real-Time PCR/direct method”.

The goal of the experimental work is to develop and optimize a rapid protocol for the detection of PCV-2 allowing direct sample analysis with Real-Time PCR without performing DNA extraction.

To achieve this, we initially evaluated a series of different protocols in order to optimize results (i.e. maximize Ct values, number of dilutions, efficiency, handling, number of required steps). Different dilutions of the same sample were tested under different heating temperatures, extra pipetting and centrifugation before loading to the Real-Time PCR machine.

After assay optimization and results evaluation, we chose the protocol with which we obtained the best results with the lowest number of steps required.

Moreover, the data gathered were statistically analyzed and then interpreted.

Advantages and disadvantages of the direct method were briefly discussed, as well as problems faced along the assay optimization and why they might have happened.

Lastly we discussed the importance of developing a direct method emphasizing its potential impact in the pig producing industry.

2. Materials and Methods.

The standard and most used protocols for performing a Real-Time PCR involve the nucleic acid extraction from the tissue/cells we want to test. A variety of standard kits are already available for performing an efficient extraction protocol depending on the type of matrix and the type of nucleic acid (DNA/RNA). In our experiments, the "Viral DNA/RNA kit (A&A Biotechnology)" was used according to the manufacturer's instructions.

2.1. Reference sample processing

To evaluate and compare the analytic sensitivity of the considered diagnostic protocols, a serial dilution of a positive sample in a negative one was performed.

Samples consisted of lungs and lymph nodes from pigs regularly slaughtered for food consumption. After being screened for PCV-2 with an in-house Real-Time PCR (see section "Real-Time PCR protocol"), one positive and one negative sample were selected for this study.

To create the reference matrix to test, negative and positive tissues were separately homogenized using respectively 20 and 4 ml of PBS, in a ratio of 1g/10ml. The supernatants were then aspirated and collected in two distinct Falcon tubes.

Nucleic acids were extracted from 100 µL of both positive and negative reference samples using the Viral DNA/RNA kit (A&A Biotechnology), according to the manufacturer's instructions. Extracted reference samples were tested using Real-Time PCR to confirm their negativity and positivity status and quantify the viral titer. The analyte concentration obtained in the positive sample was used to set the number of dilutions to test with the standard method (with DNA extraction) and with the direct method (without DNA extraction).

Finally, the ten-fold dilution of the positive sample homogenate in the negative matrix was performed and several aliquots of each dilution were prepared and stored at -80°C until further processing.

2.2. Real-Time PCR protocol

The DyNAmo Flash Probe qPCR Kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to perform all the Real-Time PCR.

Each reaction was performed on a total volume of 10 µL of a standard mix containing:

- 5 µL of 2X DyNAmo Flash Probe qPCR master mix,
- 0.4 µM of each PCV-2-specific primer (P1570F 5'-TGGCCCGCAGTATTCTGATT-3' and P1642R 5'-CAGCTGGGACAGCAGTTGAG-3') (Opriessnig et al., 2003)
- 0.2 µM of probe (P1591 5'-6FAM-CCAGCAATCAGACCCCGTTGGAATG-IBFQ-3') (Opriessnig et al., 2003)
- 2 µL of extracted DNA or non-extracted sample.
- Sterile water for molecular biology added up to 10 µL of the final volume.

Reactions were performed on a LightCycler® 96 Instrument thermal cycler (Roche) (***Figure 6)***. The cycling conditions were 7 min at 95°C for initial activation, followed by 45 cycles of denaturation at 95°C for 10 s, and annealing and extension at 60° C for 30 s. The fluorescence signal was acquired at the end of each cycle extension phase.

2.3. Assay optimization and validation

Seven reference samples to be tested for the optimization of the assay were obtained by 10-fold dilution of the original positive supernatant using the negative one as the solvent, as previously mentioned. The panel for both the standard method and direct method included the original positive supernatant and its dilutions, and the negative supernatant. Positive and negative controls, previously validated, were also added to the Real-Time PCR.

To avoid tissue frustules potentially inhibiting the extraction process (standard method) or the amplification reaction (direct method), each sample was centrifuged at 2000 rpm for 2 minutes before extraction or before sample loading in the Real-Time PCR plate.

The following sample processing conditions were attempted and compared with the reference method, to evaluate their potential benefit in maximizing reaction performances on non-extracted samples. In summary, samples extracted with minicolumns were compared to:

- unprocessed samples;
- unprocessed samples heated over a range of temperature from 55 to 100 °C;
- unprocessed samples heated and centrifuged or pipetted.

The heating process was performed using the laboratory thermo-mixer T-shaker EuroClone ®.

To assess the repeatability within and between runs, five logarithmic dilutions were obtained and tested in triplicate for each method.

2.4. Statistical analysis

During the assay optimization phase, estimated viral titers, the limit of detection (LOD), number of detected dilutions, and reaction efficiency were selected as parameters of interest and used to evaluate the assay performances and eventually adjust the conditions of the tested method.

Real-Time PCR results were collected in a database classifying single results according to the method, dilution, and run. Separate subsets of only positive results according to dilution and sample processing method were also created.

The repeatability of the Real-Time PCR assays was assessed by analyzing the mean value, standard deviation, and coefficient of variation of estimated logarithmic concentration values of the analyte. Reaction efficiencies and relative variation coefficients, LOD, and several detected dilutions were also considered to evaluate repeatability from a descriptive point of view.

An exploratory data analysis was performed graphically:

- Normality of data classified by method and dilution was assessed using a Q-Q plot.
- Multiple boxplots were graphed to compare different dilutions in terms of estimated logarithmic concentration values both within sample processing methods and overall without stratifying per method.
- Effect of the interaction between method and run using estimated logarithmic concentration values for each dilution subset was assessed by interaction plots.

Thereafter, the presence of statistically significant difference among estimated logarithmic concentration values (dependent variable) was formally evaluated through ANOVA for each dilution, or the corresponding non-parametric test Kruskal-Wallis, taking into consideration the sample processing method, the run, and interaction between them as independent variables playing as potential sources of variability. For this purpose, dedicated data subsets according to the dilution level were generated.

ANOVA assumptions of normal data distribution and homogeneity of variance were preliminary and formally tested by fitting a linear model and performing Levene's test. Fligner-Killeen's test was performed as a non-parametric equivalent of the latter.

Tukey's honest significant test or Dunn's test with Bonferroni correction (for non-normally distributed data) were performed as post-hoc analysis to account for multiple comparisons.

To provide a global picture and to simultaneously account for the effect of the different explanatory variables, a generalized linear model (GLM) was fitted, selecting as explanatory variables the dilution level, the sample processing method, the run, and the interaction between the method and run.

Datasets were created using Microsoft Excel©. Subset management and statistical analysis were performed using R. The level of statistical significance for all considered tests was set at $p<0.05$.

3. Results and Discussions

3.1 Optimization Experiments.

During laboratory activity seven dilutions of a positive sample in a negative one were prepared and used for both the direct and standard method. We performed ssDNA extraction from 100 µL of each point of the standard curve, whereas theirs DNA extraction was not required for the direct Real-Time PCR and the samples were used directly as templates.

The first part of our work consisted of exploring different preprocessing approaches allowing to optimize efficiency (i.e. E-value) and sensitivity (i.e. Cq values and number of detected dilutions) of the direct Real-Time PCR.

We initially performed a Real-Time PCR on the unprocessed samples and the extracted samples (see ***Table 3***). This is the easiest protocol because it requires fewer steps to be performed.

	Extracted Samples	Non-extracted samples
Dilutions	**Cq values**	
PS	24.05	30.93
PS-1	28.46	31.74
PS-2	31.01	35.99
PS-3	37.54	37.54

Table 3 Cq values obtained from the Real-Time PCR performed on extracted and non-extracted samples. (PS=abbreviation for "Positive Sample" followed by a number representing its factor of dilution. Here only the dilutions in which we detected viral presence are shown).

We were able to identify viral presence in the first 4 dilutions for both the extracted and non-extracted samples. The difference in Cq values between the two methods decreases in more diluted samples (see ***Table 3***).

Thereafter other optimization runs were compared to this one and to the standard method results to evaluate if preprocessing steps were worthy in terms of cost-benefit.

In a second run we decided to pretreat the non-extracted samples heating them at two different temperatures (Alcoba-Florez et al., 2020; Hamatani et al., 2006): 100ºC and 55ºC (see ***Table 4***). We noticed that the samples that were heated at 100ºC had a blurrier view than usual and also some precipitate under a gelled supernatant which made the sample difficult to handle (see ***Figure 8***). We suspect that this can be due to the denaturation and jellification of proteins that might have been present in greater quantity in the unprocessed/non-extracted samples. It has been confirmed from previous studies that bovine and porcine plasma proteins when heated can lead to heat induced denaturation and aggregation of the proteins leading to the jellification. This can be described as an intermediate state between a solution and a precipitate. Solutions of porcine and bovine blood plasma have a viscosity that exhibits Newtonian behavior in the range 20°C to 73°C and then the viscosity exponentially increases forming a gel structure that is reversible when the solution is further heated up to 76°C and irreversible at 79°C (Hermansson et al., 1982; Howell & Lawrie, 1987; Howell & Lawrie, 1983).

	Non-extracted samples preheated at 55ºC	Non-extracted samples preheated at 100ºC
Dilutions	**Cq values**	
PS	29.05	30.1
PS-1	34.13	38.07
PS-2	37.09	

Table 4: Cq values obtained from the Real-Time PCR of our non-extracted samples preheated at 55ºC and 100ºC.

After the Real-Time PCR run we observed that the number of positive samples detected decreased for both preheating protocols compared to those obtained with the direct method without preheating. Only 2 and 3 dilutions were detected for the samples pretreated at 100ºC and 55ºC respectively. Since only the original positive sample and the first dilution pretreated at 100ºC were detected, it was not possible to calculate the efficiency of this reaction and thus make a comparison on this parameter.

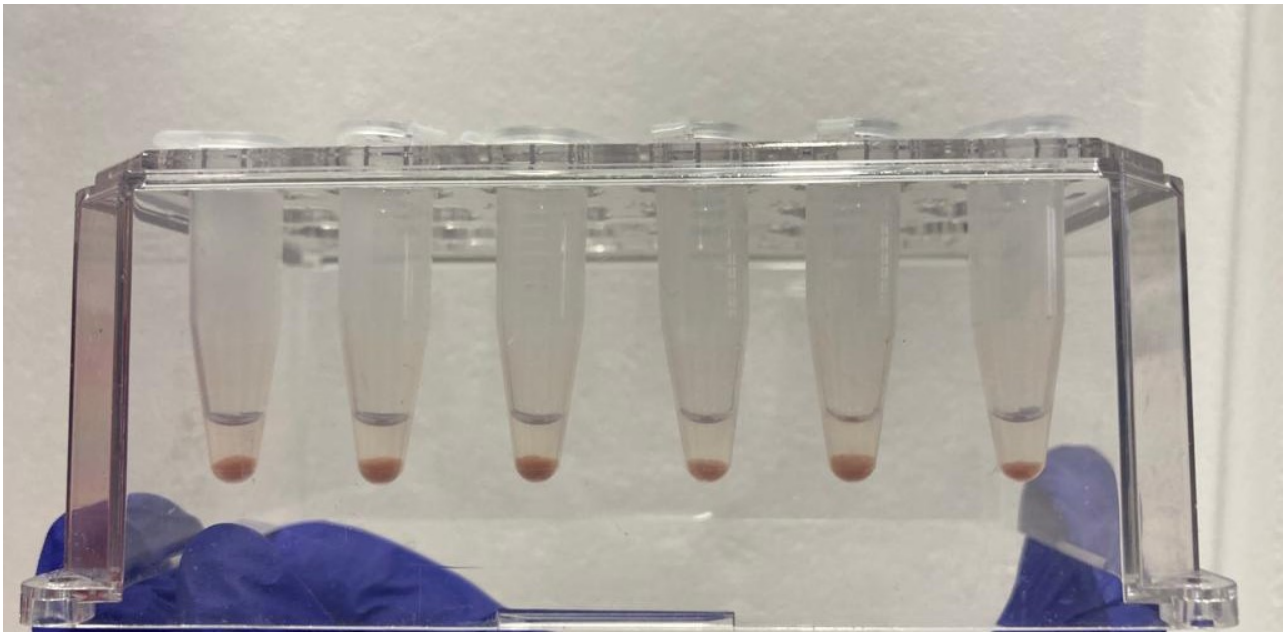

Figure 9: Picture taken during our laboratory work of the samples pretreated at 100ºC where blurriness and jellification of the supernatant and the precipitate can be easily observed.

To evaluate if intermediate temperatures could provide better results, the first dilution of the positive sample was pretreated at four other temperatures within the range of 55ºC and 100ºC (i.e. 60ºC, 70ºC, 80ºC and 90ºC).

Interestingly we noticed that also the sample that was heated at 90ºC showed the same gelled supernatant and manipulation difficulty as the one we treated on the previous experiment with 100ºC, confirming what was described by Howell and Lawrie, and Hermansson (Hermansson et al, 1982; Howell & Lawrie, 1987; Howell & Lawrie, 1983). After running the Real-Time PCR, viral detection was observed only for the samples heated at the first three temperatures while the sample treated at 90ºC tested negative (see ***Table 5***).

Temperature test on the non-extracted samples	
Temperatures	**Cq values**
60ºC	33.69
70ºC	35.59
80ºC	34.03
90ºC	NEG

Table 5*:* Cq values obtained from the Real-Time PCR of non-extracted sample under different temperatures to evaluate the role of heating in the reaction efficiency

For the samples treated with 60ºC, 70ºC and 80ºC there was an increased Cq values compared to untreated samples. Therefore, we decided to continue the assay optimization further implementing steps on the 55°C preheating protocol.

In attempt to maximize results of 55°C preheating protocol, the effects of centrifugation and pipetting were evaluated testing in the same run:

A. PS-1 preheated at 55ºC;
B. PS-1 preheated at 55ºC and pipetted before loading;
C. PS-1 preheated at 55ºC and centrifuged before loading.

Preheated, pipetted and Centrifuged samples.	
Sample	**Cq value**
A	33.3
B	34.65
C	33.55

Table 6. Cq values obtained from the Real-Time PCR of our non-extracted positive samples.

As we can observe from the ***Table 6***, no significant differences in Cq values were observed. Therefore, for a final evaluation, we decided to compare the positive sample and its dilutions using the standard Real-Time PCR, the direct Real-Time PCR with and without 55°C preheating step.

	Extracted Samples	Non-extracted Samples	Non-extracted Samples preheated
Dilutions		**Cq values**	
PS	24.81	33.1	31.52
PS-1	28.32	32.22	33.32
PS-2	31.26	35.19	34.46
PS-3	34.42	41.6	
PS-4	35.77		

Table 7: Cq values obtained from Real-Time PCR performed on extracted samples, non-extracted samples and non-extracted preheated samples.

At the end of the Real-Time PCR run we detected viral presence in:

- 5 dilutions for the extracted samples
- 4 dilutions for the non-extracted samples, and,
- 3 dilutions for the non-extracted and preheated (55ºC) samples.

Not only fewer dilutions were detected with preheating, but also there was no significant difference between the Cq values of the heated and the non-heated samples.

After experimenting different additional processing steps (preheating, pipetting or centrifuging) we came to the conclusion that using all these extra steps does not considerably affect the efficiency and sensitivity of the reaction. Therefore, we reasonably decided to perform the repeatability evaluation by comparing the less time-consuming and easy protocol for the direct Real-Time PCR to the standard method.

3.2 Repeatability descriptive assessment

To fully validate the developed protocol, it is important to assess its repeatability and if it does provide standardized and reliable results.

As mentioned in the previous paragraph, in order to build a protocol as easier and efficient as possible, we decided to test the repeatability of the direct protocol without preheating since there were no significant differences worth the effort of any additional step.

After testing our samples in triplicates, in parallel both for the standard and the direct method in three different runs (Run A, B and C), the following results were obtained (see ***Table 8***):

Extracted Samples				
	Run A	Run B	Run C	CV
Efficiency	2.33	2.53	2.43	4%
Slope	-2.7185	-2.4808	-2.5906	-5%
Detected dilutions	5	5	5	
Non-extracted Samples				
	Run A	Run B	Run C	CV
Efficiency	2.53	1.81	1.77	21%
Slope	-2.4836	-3.8667	-4.0131	-24%
Detected dilutions	4	4	4	

Table 8: Table representing the results obtained from the Real-Time PCR of the samples from the "Standard Method" (Extracted samples) and the samples from the "Direct Method" (Non-extracted samples). For each run the table shows the values of the efficiency, the slope, relative coefficient of variation (CV) expressed in percentage and number of detected dilutions.

Overall, the standard method provides greater repeatability of the reaction efficiency than the direct method, which shows more than 5-fold variability. Ideally, the number of cycles that should occur between a sample and the following 10-fold dilution is 3.32. It is interesting to observe that in each run, the standard method deviates more from 3.32 than the direct method. Except from Run A, a higher efficiency has been observed for the standard method (see ***Table 8***). This could

be explained by the process of extraction that helps to reduce potential inhibitors of amplification reaction.

In general, therefore, the direct method manifests lower efficiency, with greater variability, which from the perspective of a protocol to be used in routine diagnosis is not ideal.

Logarithmic Concentrations								
	Extracted Samples				**Non-extracted Samples**			
Dilutions	Run A	Run B	Run C	**Overall**	Run A	Run B	Run C	**Overall**
PS	2.47%	4.62%	4.45%	3.67%	5.40%	6.82%	4.23%	8.81%
PS-1	7.62%	11.13%	6.74%	8.93%	41.25%	3.41%	6.10%	23.04%
PS-2	5.66%	3.47%	13.23%	10.52%	42.76%	10.75%	4.33%	42.25%
PS-3	44.27%	37.45%	19.39%	32.86%	498.76%	-500.48%	-326.24%	-655.47%
PS-4	11.95%	9.35%		9.74%	5.40%	6.82%	4.23%	8.81%

Table 9: Table representing the coefficient of variation (CV) of the logarithmic concentrations calculated for each run of the standard and direct method.

Considering the logarithmic concentration values obtained for each dilution in each run (see ***Table 9***), in the overall, all PS (positive sample) dilutions show higher repeatability (lower variability) with extraction than with the direct method. In both methods, more diluted samples show lower within-method repeatability: this difference between dilutions is even more evident in the case of the direct method, further confirming the standardization that extraction confers. The fact that we loaded the non-extracted samples from the same eppendorf in each run further accentuates the importance of extraction in ensuring greater repeatability of the standard method and thus less susceptibility to the variability that possibly greater manipulation might generate. Moreover, using 100 microliters in the standard method against the 5 microliters of sample used in the direct method could have "spread" any difference within sample variability over a larger volume, further explaining the greater repeatability of the standard method. The use of a smaller amount of sample in the direct method might make it more susceptible to manipulation.

It seems there is no significant difference between runs within the same dilution and method. Looking at the difference between dilution PS-3 and the previous dilutions, the increase in variability is evident. Except for run B, in both methods, the concentration value registered for the original positive sample is always less variable than its dilutions. Overall, the closer the dilution is to the LOD, the more the repeatability decreases. Although dilution -4 shows less variability than some previous dilutions, since it did not result as a dilution in run C in sufficient numbers to calculate the standard deviation, an evaluation against the other dilutions would not be reliable.

3.3 Repeatability statistical assessment.

Only data for the original positive sample and of its first three dilutions were considered for statistical analysis since they were constantly detected by both of the two tested methods.

Boxplots in ***Figure 10*** show the results of the exploratory data analysis aimed to compare different dilutions in terms of logarithmic concentration values within the standard method (***10***A) and the direct method (***10***B) and the overall without stratifying per method (***10***C). In the standard method (***10***A), higher viral titers have been estimated compared to the direct method (***10***B).

The application of the direct method (***Figure 10***B) seems to be associated with a wider distribution (i.e. higher variability) of the estimated logarithmic concentration values. For both methods (***Figure 10***A and ***10***B) a greater variability can be observed for samples at the highest dilution (1/1000, named as -3).

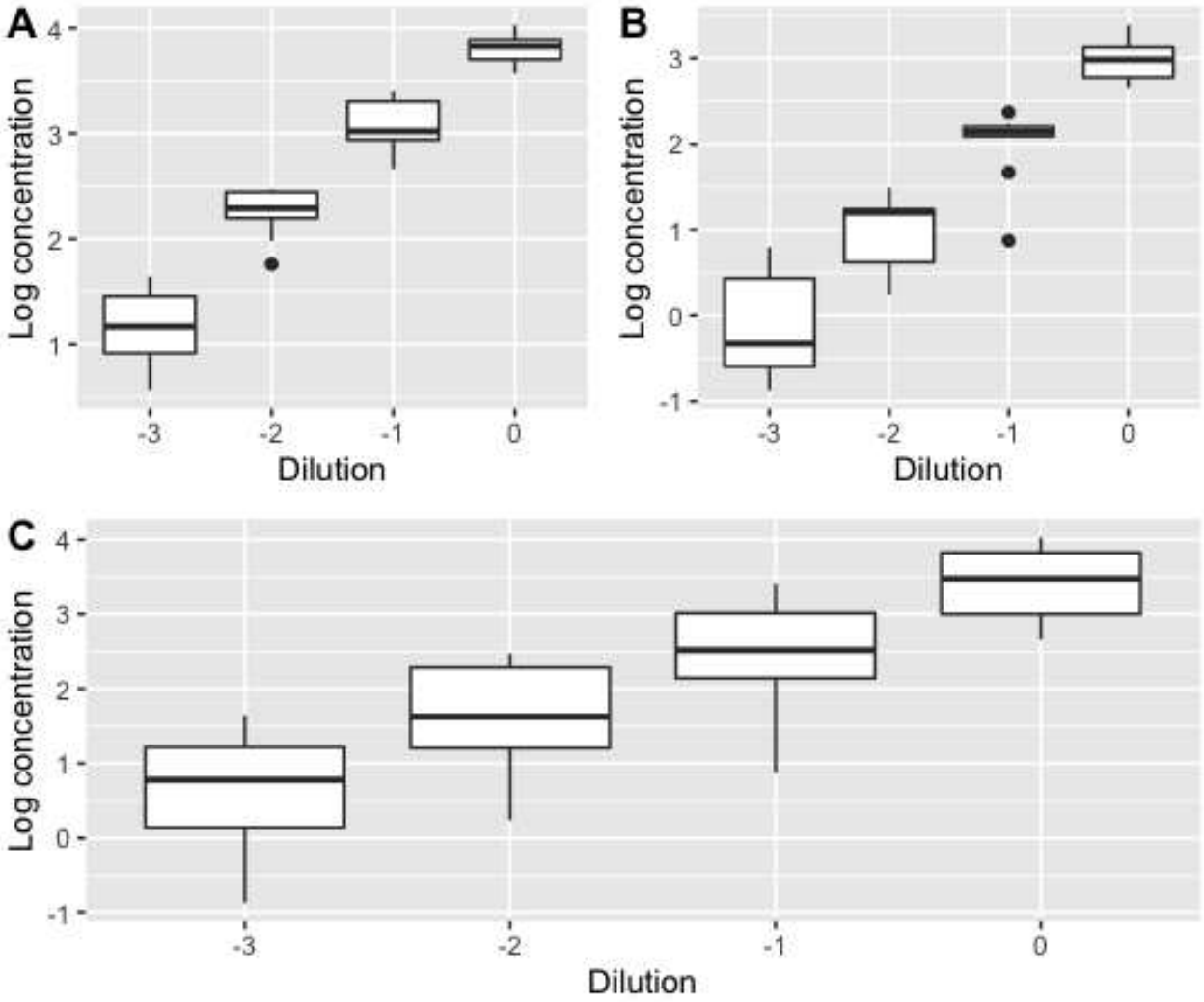

Figure 10: *Comparison of the logarithmic concentration value distributions between dilutions A) with DNA extraction, B) without DNA extraction, and C) overall. Dilution labels refer to the original positive sample (0), diluted 10 (-1), 100 (-2) and 1000 (-3) times.*

The greater variability and the lower estimated logarithmical concentration values observed in the direct method boxplots (***10***B) suggest an association between the method and the estimated concentration.

For both methods (***10***A and ***10***B) and also in the overall (***10***C), boxplots showed a decrease of 1 logarithm in the concentration values over the serial dilution levels, which is indicative of a proper dilution process in samples preparation.

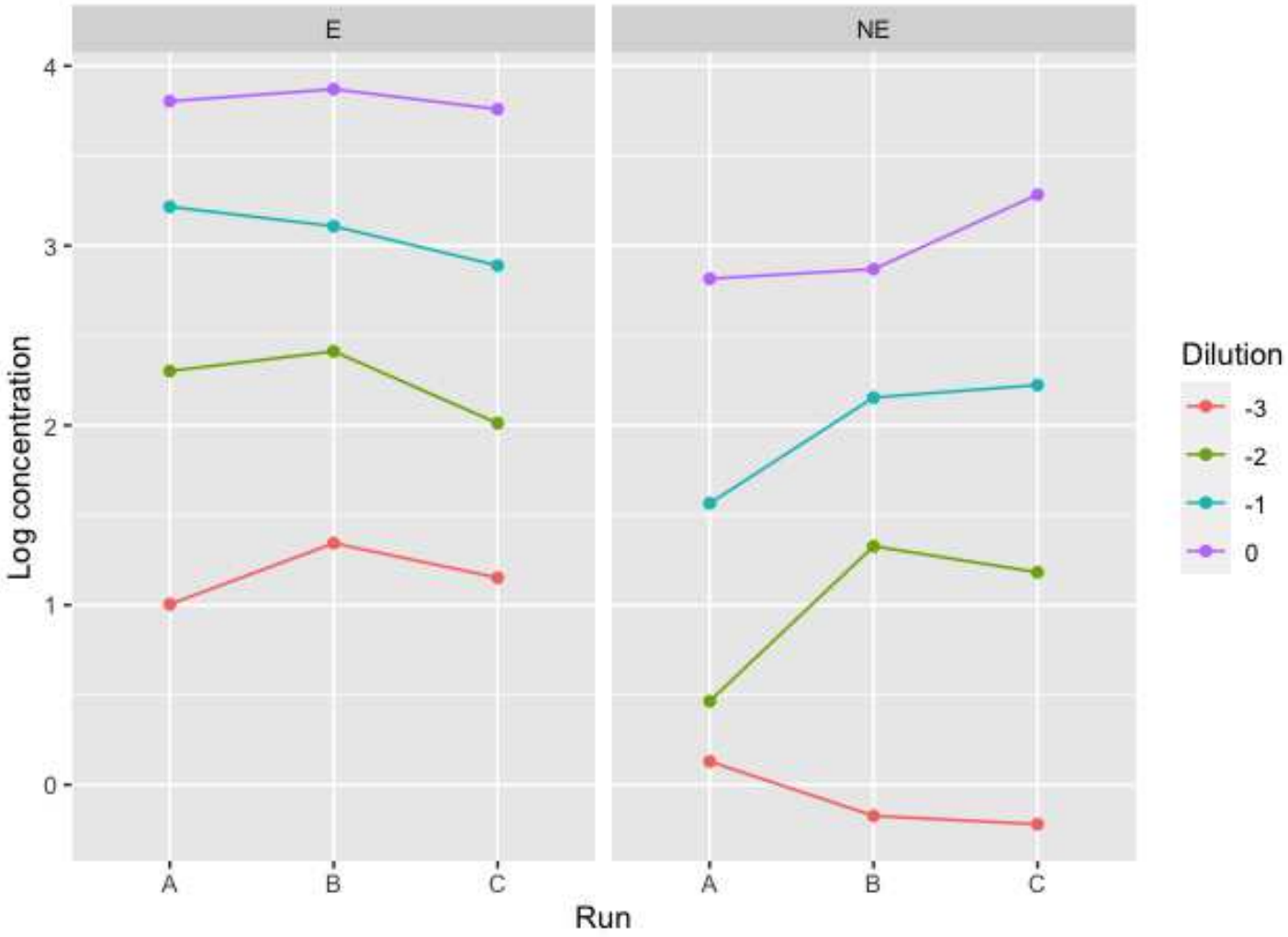

__Figure 11__: Interaction plots considering the run as trace factor of the logarithmic concentration values according to the method (E: with DNA extraction; NE: without DNA extraction) applied. Results for each dilution data subset are presented with different colors for each method.

Figure 11 illustrates the variation of the estimated concentration values according to the run for each method, thus providing a graphical representation of both the repeatability within the method for each dilution and a comparison between the two applied methods.

For both methods, an increase in titer heterogenicity was observed at higher dilutions (***Figure 11***), thus indicating lower repeatability between runs at lower viral titers.

Comparing the two methods, within dilution level, lower estimated concentration values of about 1 logarithmic unit were detected for the direct method, similarly to what is illustrated in ***Figure 10***A compared to ***10***B.

Moreover, dot-connecting lines are less parallel in the direct method, suggesting a greater variability between runs without sample extraction.

Accordingly, the distance between dilution lines is less constant within the direct method, thus signaling a greater variability of the reaction efficiency when samples are not extracted. Therefore, it is plausible that the extraction process guarantees a lower variability both between different runs and in the efficiency of amplification reactions, as well as a lower inhibition in the Real-Time PCR reaction.

Statistical analysis performed confirmed what was shown by the graphs.

For the undiluted sample (0), estimated logarithmic concentration values were normally distributed for both method data subsets, and all the method-group and run-group variances were equal. ANOVA test identified a significant difference in the logarithmic concentration values between the two methods, between methods according to the run but not between runs. Post-hoc analysis for multiple comparisons revealed that the interaction between method and run identified by ANOVA was due to a significant difference between methods in any combination of runs, and between some runs within the direct method.

Logarithmic concentration values of samples diluted 1/10 (-1) were normally distributed only when extraction was performed, whereas homogeneity of variances was respected. Kruskal-Wallis test identified a significant difference in the estimated logarithmic concentration values between the two methods, between methods according to the run and not between runs. As observed with the undiluted samples (0), post-hoc analysis for multiple comparisons showed that the identified interaction between method and run was due to a significant difference between methods in any combination of runs, and between some runs within the direct method.

Normal distribution and homogeneity of variances were observed for logarithmic concentration values of samples diluted 1/100 (-2) for both method data subsets. ANOVA test identified a significant difference between the two methods, between methods according to the run and, unlike what was observed for previous dilutions, also between runs. An explanation of the spotted difference between runs was provided performing the post-hoc analysis: in the pairwise comparison between specific combinations of method and run, a significant difference between the two methods in any run comparison and between some runs within the direct method was identified, similarly to what described for the logarithmic concentration values of the 0 and -1 dilution data subsets. Thus, the difference within the direct method for 0 and -1 diluted samples was narrower than that observed for -2 diluted samples, which is why no statistically significant between-run difference was identified for previous dilutions.

Having identified a significant difference between runs within the direct method in the mean logarithmic concentration values of 0, -1, and -2 diluted samples suggests that the direct method doesn't guarantee repeatability as strong as the standard method does (***Figure 11***).

Considering logarithmic concentration values of samples diluted 1/1000 (-3), a significant difference between methods was estimated when the results of all the runs were considered; however, no difference was detected comparing the methods within each run. The greater variability registered for more diluted samples could explain this phenomenon: the concentration estimation approximating the LOD occurred with a greater variability that led to a higher "noise", thus decreasing the statistical power that was instead sufficient for other dilutions in which the

variability was lower. A larger sample number for higher dilution levels could have counterbalanced the lower statistical power, thus increasing the chances of confirming the difference between methods within each run that was instead observed in less diluted samples (***Figure 10***).

The fitted GLM has further confirmed what ***Figure 10*** and ***Figure 11*** graphically show and the statistical associations described by ANOVA and Kruskal-Wallis test.

GLM dilution, method, and interaction between method and run as potential meaningful predictors of the estimated mean logarithmic concentration value of the analyte. According to this model, for each 10 folds' dilution, the mean logarithmic concentration value decreases by almost 1 unit, confirming the proper execution of dilution at the time of sample preparation (***Figures 10*** and ***11***).

The application of the direct method against the standard method on the same samples led to an underestimation in average concentration values of 1.37 logarithmic units (***Figure 10***A compared to ***10***B). The significant role of the interaction between method and run as a predictor of the mean logarithmic concentration value was demonstrated while the effect of run alone was not significant.

Therefore, GLM results confirm what is described by ANOVA and Kruskal-Wallis test and post-hoc analysis per each dilution data subset, consistently suggesting lower repeatability of the direct method compared to the standard one, thus providing a global and summed explanation of the difference observed in the estimated logarithmic concentration values.

Overall, the standard method provides greater repeatability of the reaction efficiency than the direct method, which shows more than 5-fold variability. Ideally, the number of cycles that should occur between a sample and the same sample diluted 10 times is 3.32. It is interesting to observe that in each run, the standard method deviates more from 3.32 than the direct method, which on average deviates less. The overall lower efficiency of the standard method could be also explained by the process of extraction that could lower itself the Real-Time PCR efficiency: in the standard method efficiency it's included also the efficiency of the extraction process.

In general, therefore, the direct method manifests higher efficiency, albeit with greater variability, which from the perspective of a protocol to be used in routine diagnosis is not ideal.

4. Conclusions.

PCV-2, which is currently known to have 9 genotypes, is a widespread virus that has a big impact on the pig industry. Besides all the benefits that vaccination has on controlling disease development and spreading of PCV-2, they can still disrupt the equilibrium among the pathogen and its host and alter the competitive hierarchy between viral genotypes (Williams et al, 2010) leading to the continuous emergence of new variants (Constans et al., 2015; Opriessnig et al., 2020; Segalés, 2015; Timmusk et al., 2008; Wiederkehr et al., 2009; Xiao et al., 2015a). This contributes to concerns regarding the effectiveness of these vaccines, considering that some failures have been reported recently. (Opriessnig et al., 2013; Seo et al., 2014; Xiao et al., 2012). Thus, the rapid identification of these pathogens can still help maintain the disease under control and prevent as more economical damages as possible.

Additionally, the effort of developing rapid and cheaper protocols is necessary especially for those countries whose economical resources are limited and where access to vaccination, advanced technologies or extraction kits is not as easy and affordable as it is for other countries (Ariyama et al., 2021; Barman et al., 2018; Chae, 2012; Deka et al., 2021; Franzo et al., 2022; Xiao et al., 2015a).

Moreover, even though performing a standard Real-Time PCR can provide a higher diagnostic sensitivity, for farmers, above all in developing countries, what is important is to have an effective method accurately identifying the presence or absence of a pathogen that at the same time guarantees reduced costs and simplified procedures. Performing a direct Real-Time PCR reduces both costs and time needed for experiments, requiring less reagents and no extraction kits (which have a considerable price). A summary of the main differences in advantages and disadvantages between direct and standard Real-Time PCR is presented in ***Table 10***.

Method Comparison	
Direct Real-Time PCR	**Standard Real-Time PCR**
Rapid.	Time-consuming.
No extra reagents needed except the Real-Time PCR reaction kit.	Expensive extraction kits needed, leading to supply chain gaps.
Real-Time PCR sample preparation only. Less manual handling.	Extra manual handling potentially enhancing experimental errors.
Potential inhibitors interfering with target amplification.	Lessening of potential inhibitors interfering with target amplification.
Higher variability and lower efficiency.	Lower variability and higher efficiency.

Table 10: Comparison table of direct Real-Time PCR and standard Real-Time PCR.

Considering results obtained by the experimental work here presented, better results were observed in terms of sensitivity and repeatability for the standard method.

Nevertheless, the direct Real-Time PCR methods for the detection of PCV-2, and not only, can still be considered a good tool for rapid detection when resources are limited although there is still need for further research to be done in order to optimize this method and increase its accuracy. When it comes to diagnostic procedures the direct method can have both some advantages and drawbacks depending on the reason Real-Time PCR is performed. In case of need of accurate results and high risk or serious consequences involved, then the standard method will be more appropriate because it guarantees higher efficiency and a lower result variability.

On the other hand, the direct method can still provide results, that in certain scenarios can be a good compromise between its lower accuracy and the need of a detection method when resources are limited and urgency emerges. Moreover, in presence of clinical signs, PCV-2 viral load is supposed to be higher, thus guarantying positive results allowing to ignore sensitivity and repeatability deficiencies observed mainly at lower concentrated samples.

An optimized direct method overcoming sensitivity and repeatability limitations could have even a wider usage in the future for the rapid detection of already known and emerging pathogens.

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