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# TESE DE DOUTORAMENTO

## NOVEL PROCEDURES OF VIRAL QUANTIFICATION: DEVELOPMENT, OPTIMIZATION AND VALIDATION OF *EX VIVO* AND *IN VIVO* TECHNIQUES

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**NOVEL PROCEDURES OF VIRAL QUANTIFICATION:**
**DEVELOPMENT, OPTIMIZATION AND VALIDATION OF *EX VIVO***
**AND *IN VIVO* TECHNIQUES**

D. Diego Vázquez Rodríguez

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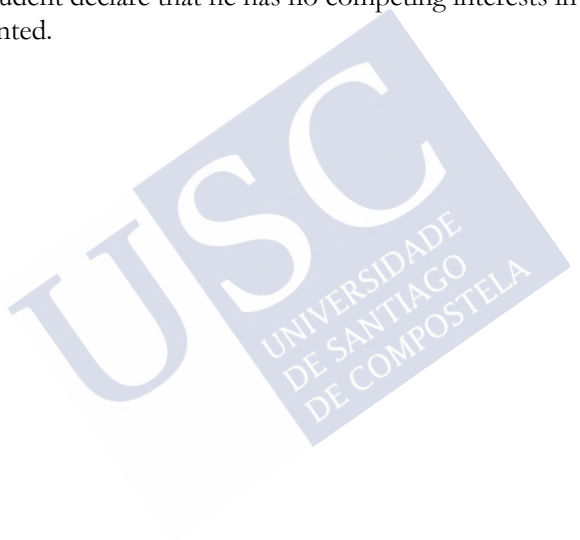

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A mis abuelos

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# Chapter 1

## Introduction and Objectives of this Thesis

# 1 INTRODUCTION

The worldwide development of aquaculture in the last decades is unquestionable. In 2016, the world aquaculture production reached 80 million tons, with an estimated first-sale value of US$232 billion, consisting of 54.1 million tons of finfish (US$138.5 billion), 17.1 million tons of molluscs (US$29.2 billion), 7.9 million tones of crustaceans (US$57.1 billion), and 938500 tones of other aquatic animals such as turtles, sea cucumbers, sea urchins, frogs and edible jellyfish (US$6.8 billion) (FAO, 2018). This situation has been promoted by the intensification of the culture processes.

However, the intensive fish and shellfish production is threatened by epidemic outbreaks affecting not only the "domestic" populations but also the wild stocks (Tompkins et al., 2015). Among the different pathologies affecting fish aquaculture, viral diseases have one of the highest economic impacts due to their virulence, rapid dispersion and absence of treatment and effective vaccines, what makes their complete eradication difficult. Therefore, research is focused not only to the development of prophylactic protocols and the design of new and more efficient vaccines, but also to the improvement of diagnostic procedures for the rapid control of outbreaks. Additionally, an increasing effort is being applied on health surveillances of cultured and wild fish populations, which has led to the discovery of several viruses which were not known to scientists; some of them are endemic among native populations and opportunistically spill-over to infect fish in aquaculture facilities (Walker and Winton, 2010). In addition, they have also provided important information on the distribution and virulence of other previously known viruses.

For the present study, focused on the development, optimization and validation of viral quantification procedures, any of those viruses could have been chosen to be used as a model. Why was the infectious pancreatic necrosis virus (IPNV) chosen among all? The same gap regarding the general aim of this study affects to all fish viruses in the literature, but for IPNV lower biosafety restrictions, together with the high availability and diversity of strains, make this pathogen a great candidate for these types of studies.

## 2 OBJECTIVES OF THE PRESENT STUDY

The purpose of the present study was the evaluation, design and validation of different techniques of virus quantification, using the infectious necrosis pancreatic virus (IPNV) as a general model. The final goal of the study was, in later stage, the transference of the technology validated to the standard operator procedures of the Aquaculture institute from the Universidad de Santiago de Compostela.

From this general objective the sub-goals were:

- The analysis of current methodologies available and revision of the literature to identify opportunities for improvement in the current processes of the laboratory.
- The design, optimization and validation of qPCR for detection and quantification of the Infectious Pancreatic Necrosis Virus
- To develop and validate a protocol for quantification of viral proteins expressed in salmon cell lines using fluorescence activated cell sorting.
- The design, optimization and validation of virus titration using micro flow cytometry.

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# Chapter 2

## Virus Quantification: A General Review

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# 1 THE IMPORTANCE OF VIRUS QUANTIFICATION

In most virology studies, one of the critical information is the concentration of total of infectious particles in a sample. Hence, it is crucial to know the replication cycle of a virus to study the level of virulence of new isolates, to assess the antiviral activity of chemotherapeutic agents, for monitoring the stage and efficiency of a virus purification in industrial processes or simply to evaluate virus pathogenicity (Hierholzer and Killington, 1996). In any experiment involving cells inoculation with virus, the multiplicity of infection (MOI; being the number of viral particles per cell) is crucial since irreproducible or unreliable MOIs, caused by inaccurate estimation of the viral titter of the inoculum, have a profound impact on the performance and batch consistency of the assay (Roldão et al., 2009). For this reason, accurate quantitative methods are mandatory.

The clinical utility of the viral quantification has been deeply reviewed in human medicine. However, in the veterinary field, and more precisely in fish virology, most techniques and methodologies have been adapted from previous advances in human virology. In spite of its relevance, just a couple of reviews have been published in recent years ( Heider and Metzner, 2014; Pankaj, 2013) . Following the classification made by Heider and Metzner (2014), virus quantification methods can be broadly classified in four different categories depending on what are they based on: i) in the levels of infectivity, ii) in detection of viral proteins, iii) in detection of viral nucleic acid, or iv) in counting of viral particles

# 2 METHODS FOR VIRUS QUANTIFICATION

## 2.1 Virus infectivity

The most basic and traditional procedures to know the viral concentration in a sample are based on the assessment of the viral infectivity. An infectivity assay measures the titer (the concentration) of an infective virus in a specimen or a preparation. These methods can be additionally classified in those based on the response of the inoculated subject to an infection –called quantal– and those relying on the detection and counting of foci of infection known as focal or quantitative assays.

a) Quantal assay

The end point dilution assays are definitely one of the most used methods for quantification of virus. Their objective is to find the viral dose yielding a response in 50 per cent of the inoculated subjects. Depending on the type of subject used for the inoculation, the final titer value could be named differently: tissue culture infectious dose 50 ($TCID_{50}$), if cell cultures are used; lethal dose 50

($LD_{50}$), using experimental animals, or egg infective dose 50 ($EID_{50}$), using embryonated chicken eggs. The general procedure always begins with serial dilution of the stock sample, and the subsequent infection or inoculation of the subjects.

The estimation of the titer by $TCID_{50}$ is, by far, one of the most used in the fish virology field. $TCID_{50}$ is generally defined as the lowest viral dilution where the 50% of a given batch of cells are demonstrated to be infected, demonstration being attained by the visualization of cytopathic effect (CPE) in the cell monolayer. Then the estimation of the titer can be performed using different nonparametric methods like Spearman and Karber (SK), Reed and Muench (RM), Dragstedt and Behrens (DA), Litchfield and Wilcoxon (LW), and moving average (MA) (Finney, 1952; Hamilton, 1991), or by parametric procedures as the maximum likelihood (ML) based on the single hit Poisson response (Myers et al., 1994). To answer the immediate question of which method should be chosen, Finney (1952); afterwards supported by Hamilton, 1991) argued that neither RM, DA or LW are as powerful as the SK procedure, and their continued use in some areas is "motivated only by tradition"; in fact, the Spearman-Karber method is recommended by FAO because " is statistically markedly superior and involves relatively simple calculation" (Litamoi et al., 1996) However, when the concentration is low but measurable (i.e titers below approximately 0.8 $\log_{10}$ of units per ml, using standard plates), SK method produces increasingly biased estimates of titers. Moreover, the methodology has an absolute requirement: that the serial dilutions provide both 100% and 0% infectivity, but this criteria is difficult to meet with low levels of virus in the sample (Darling et al., 1998). Therefore, either ML or SK methods have been recommended to be used at most virus concentrations; but, at low virus concentrations ML is preferred (Brownie et al., 2011). However, although spreadsheet algorithms are available to carry out the ML calculations one sample at a time, it is somehow harder to automate batch processing of multiple samples because the iterative solution of an equation is required. Therefore, the use of the SK method is more extended because calculations can be easily implemented in a spreadsheet format. This facilitates the standardization of computations across laboratories, when large numbers of assays are conducted (Brownie et al., 2011).

b) Quantitative assay

Quantitative assays are those in which foci of infection are counted somehow (Plaques, fluorescent foci, etc.). Plaques are localized as discrete foci of infection, denoted by zones of cell lysis or CPE within a monolayer of otherwise healthy tissue culture cells (Hierholzer and Killington, 1996). The plaque assay was firstly developed for animal viruses (the Western equine encephalomyelitis virus) by Renato Dulbecco in 1952 (Dulbecco, 1952), as an adaptation of the method already used for calculating bacteriophage stocks titers.

Since then, the method has been adapted for multiple animal virus including those affecting fishes, like the viral haemorrhagic septicaemia virus (VHSV), IPNV, the infectious haematopoietic necrosis virus (IHNV), the channel catfish virus (CCV), and the lymphocistis disease virus (LV) (Burke and Mulcahy, 1980; Espinoza and Kuznar, 2002; Wolf et al., 1973). Plaque assays are performed in two different ways: suspension assays (cells and virus are seeded at the same time after adsorption), or monolayer assay (where the virus suspension is added to a confluent tissue culture cell monolayer). Some viruses, such as herpes viruses and poxviruses, may be plaque-assayed under standard liquid culture medium, because direct cell-to-cell spread of these viruses ensures formation of localized plaques. However, many other viruses require a viscous overlay medium to prevent secondary infections. Whatever the method chosen, it should be adapted to the particular condition of the virus used, and some aspects in the optimization process should be taken into account since they could affect the final titer, as: (i) the ability of the virus to cause a detectable CPE in tissue culture, since some clinical isolates may fail to induce CPE or form plaques (Hammarlund et al., 2012); (ii) the time required for each specific virus to adsorb to the cells; (iii) the type of overlay used, because some can cause an inhibitory effect on the replication of the virus (Matrosovich et al., 2006); (iv) the incubation time needed for plaques formation; (v) the sensitivity to virus infection of the cell chosen (Lorenzen et al., 1999), and (vi) the cell concentration –or monolayer confluency– at the time of infection (Roldão et al., 2009). As a result of the infection of the cell by –theoretically, but not necessarily– a single virion, a plaque is finally formed, and the number of plaques formed in the cell monolayer with each dilution will be proportional to the concentration of infectious viral particles used for the infection. For statistical reasons, 20-100 plaques per monolayer are ideal for counting, although the actual number is often dependent on the size of the plaque and the size of the flask or plaque used for the assay (Hierholzer and Killington, 1996). The final titer is expressed as plaque forming units per milliliter (pfu/mL). Although this methodology, as the quantal assay described before, is relatively simple, due to the long incubations required to obtain results (normally between 3 and 10 days, depending of the virus and cells used) it is considered time consuming.

A direct equivalence between $TCID_{50}$ and pfu titers does not exist. In the publication headed by Bryan (1957), the author finds that the pfu/$TCID_{50}$ ratio must be around ln(2) ≈0.69, and using Monte Carlo simulation, it was demonstrated that the relation is about 0.56 when using the SK calculation method (Wulff et al., 2012). A comparative evaluation of both techniques for the titration of filovirus, suggested a relation value of around 0.1(PFU/$TCID_{50}$) (Smither et al., 2013). On the other hand, these authors also found a greater reproducibility with the plaque assay than with the end point dilution method, results which are in concordance with others in the literature reporting differences in terms of standard error between plaque assay (coefficient of

variation [CV] = 7%) and $TCID_{50}$ (CV=22-27%) (Roldão et al., 2009). However other authors reported higher variability values, between 5% and 44%, for the results obtained with plaque assay (Bae et al., 2003; Shurtleff et al., 2012).

Like $TCID_{50}$, the end-point dilution method can also be applied by inoculation of serial dilutions of the virus in test animals. Based on the symptoms used as end point, the results should be expressed accordingly: 50% paralytic dose ($PD_{50}$) per ml or 50% lethal dose ($LD_{50}$) per ml

An alternative approach of these methodologies, but also based on the lytic nature of the viruses, are those measuring the growth attenuation due to virus infection: microculture tetrazolium (MTT) and AlamarBlue™ assays (Janakiraman et al., 2006; Mena et al., 2003). Although both rely on the quantification of the absorbance variations due to the enzymatic transformation of the compound in the cells infected with serially diluted virus, AlamarBlue was reported to give better results in viability assays when compared to MTT (Hamid et al., 2004). The mean standard errors observed when both methods were applied to baculovirus quantification were 20% and 28%, respectively; despite the variability in the standard errors of the methods, viral titers were within the same order of magnitude, $10^7$ pfu/ml (Roldão et al., 2009). The incubation time, nonetheless, is much higher for the MTT assay (6 days of MTT against 24 hours of AlamarBlue).

## 2.2 **Viral proteins**

The classical procedures for virus quantification, based on the measurement of the infectivity mentioned before (quantal and plaque assay), require a susceptible cell monolayer developing a visible cytopathic effect (CPE). As a consequence, those procedures require between 3 and 12 days, depending on the virus being analyzed, and the required level of evaluation of the CPE; thus, both types of methods are considered time consuming. Moreover, some cells do not produce visible CPE, and some do not support plaque formation. For instance, some low passage clinical isolates of certain viruses of yellow fever may be more difficult to quantify because they do not elicit plaque formation or do not induce a measurable CPE. Similar challenges have been faced with other flaviviruses such as dengue virus, since clinical isolates often fail to induce CPE or to form plaques (Hammarlund et al., 2012; Li et al., 2011). To overcome these limitations, some alternatives have been developed. Some of the strategies detailed in the next lines are based on the assessment of viral production through the relative detection and quantification of specific viral antigens or proteins. This value could be correlated with the viral load, however it does not provide the actual concentration of infective viral particles, since the presence of some free antigens or dissociated proteins could affect the measurement.

a) Fluorescent Focus Assay

The fluorescent focus assay (FFA), an indirect immunofluorescence assay for quantitation of virus, is a variation of the plaque assay in which focal areas of infection are visualized by detection of viral antigen with polyclonal or monoclonal antibody (MAb). Since this method does not require the development of CPE in the infected cell monolayer, it can be used in a broader range of cell types (Payne et al., 2006). The procedure is quite similar to plaque assay but results are obtained faster (24 h to 72 h). In a study by Espinoza and Kuznar (2002), where the authors reported satisfactory results using FFA to quantify IPNV in CHSE cells after 16 h post infection (p.i.), a good correlation was observed between both methodologies. However, the same authors reported the possibility of secondary infections affecting the results; although they optimized the procedure reducing the time for the analysis to prevent this inaccuracy, the addition of a viscose overlay like the carboxy-methyl cellulose (CMC) have revealed to be a better choice for that purpose. The viscosity of the CMC prevents emerging viral particles from traveling beyond neighboring cells, thus avoiding secondary foci (Payne et al., 2006).

The success of the assay depends on the productive infection of cell monolayer and detection of the virus by an appropriate antibody. To this regard, one of the limitations of this technique is the high specificity of MAbs, which can actually represent a handicap because certain strains of a viral type might be miss-detected (Dopazo and Bandín, 2011). On the other hand, the lack of a suitable MAb could be solved by the use of polyclonal antisera. However, this option should be evaluated carefully due to possible unspecific interactions.

Although the use of this technique is quite common in human viruses like adenovirus, polyomavirus, rotavirus, Chrimea Congo hemorragic fever or dengue virus (Berber et al., 2013; Calgua et al., 2011; Iskarpatyoti et al., 2012; Schoepp and Beaty, 1984), and the results are highly correlated with those obtained with plaque assay, the quantification of fish viruses with this methodology has been more limited, probably linked to the limited development of suitable monoclonal antibodies (Chinchilla et al., 2013; Coll and Dominguez-Juncal, 1995; Espinoza and Kuznar, 2002; Lorenzo et al., 1996).

b) Enzyme-Linked Immunosorbent Assay

The basis of the enzyme linked immunosorbent assay (ELISA) relays upon the detection of antigen-antibody binding by an enzymatic reaction which develops a colorful signal. There are two basic ways of performing ELISA: "sandwich ELISA", where the bottom of multiwell plates are coated with antibodies against the specific viral antigen or protein, before the viral sample is added, and "indirect ELISA", in which the antigen is directly immobilized to the

bottom of the well. In both cases, after an incubation period to allow viral binding to occur, unspecific bounds are blocked using unspecific protein. A primary viral specific antiserum (Mab or PAb) and, afterwards, a secondary no virus-specific antibody, conjugated to an enzyme (horseradish peroxidase [HRP] or alkaline phosphatase [AP]) are used to detect the presence of the target. The quantity of bounded target is then quantified by colorimetric changes using an adequate substrate for the specific linked enzyme. This method has been widely used for the detection and quantification of human viruses, and is also well described for diagnosis -not so well for quantitation- of viruses in aquaculture (Chen et al., 2002; Dixon and Hill, 1983; Fenner et al., 2006; OIE, 2015; Way and Dixon, 1988; Whittington and Steiner, 1993). A variation of the previous methods is the competitive ELISA, in which after the immobilization of the antibodies in the plate, a "competition" reaction is performed between known quantities of enzyme-linked antigen and native antigen present in biological samples for binding to them. The concentration of antigen in the biological sample is inversely proportional to the colorimetric signal detected. The main advantage of this approach, is that it does not require the use of species-specific enzyme-conjugated antibodies for the quantification (Song et al., 2009).

c) Single Radial Immunodiffusion assay

Probably due to its simplicity, the single radial immunodiffusion assay (SRID), originally reported by Fahey and McKelvey (1965) and Mancini and col. (1965), is one of the most popular and traditional methods for quantification of influenza virus. Moreover, it is the unique method of quantification of influenza vaccine release (Transfiguracion et al., 2015). The basis of this method is the formation of disks of antigen-antibody precipitation in agarose gel. Although it is the traditional assay used to measure the effectiveness of inactivated influenza vaccines (Schmeisser et al., 2010), one of its drawbacks is the length of the incubation time, 4.5 higher than ELISA (Lee et al., 2008) for achieving results. Same authors reported less sensitivity and a higher cost compared with the ELISA, and the assay format requires large amounts of strain-specific reagents (Schmeisser et al., 2014).

d) High Performance Liquid Chromatography

High performance liquid chromatography (HPLC) has been developed to separate, identify and quantify components in a liquid mixture. The technology has been adapted for the quantification of reovirus, baculovirus, adenovirus and orthomyxovirus. Reverse phase HPLC (Rp-HPLC) has been developed for the isolation and quantification of the subunits of hemagglutinin (HA), cleaved into HA1 and HA2 (Kapteyn et al., 2006). The method has yielded a good correlation with the SRID and showed a limit of quantification of 0.25 μg HA/mL (Lorbetskie et al., 2011). To the author knowledge the use of this technology for quantification of fish viruses has not been reported yet. In spite of the fact that

this technology can be largely automated, HPLC does require highly trained personnel and necessitates considerable expenditure for reference material to be used as controls.

e) Hemagglutination assay

The ability of some viruses to aggregate red blood cells has been used since the decade of 1940, to assess the concentration of some viral particles in a given sample. The hemagglutination assay (HA) is a tool used to screen cell culture or amnio-allantoic fluid harvested from embryonated chicken eggs for hemagglutinating agents, such as type A influenza. The HA assay is not an identification assay, as other agents also have hemagglutinating properties (Killian, 2008), neither a measure of infectivity due to the inactivated virus could retain their agglutination capacity. The titer is calculated by end point dilution titration, and interpreted as the last dilution showing complete agglutination or by the dilution which shows 50% of agglutination (Hierholzer and Killington, 1996) and the titer expressed as one HA unit per volume used. However, despite its common use, in most reports the precision of the assay showed to be low, providing measurement errors of about 0.15 log units (+41%/-29% on a linear scale) (Kalbfuss et al., 2008). Moreover, its sensitivity has been proved to be lower than other methods as ELISA, IFA of quantitative PCR. Fish orthomyxovirus, like ISAV or paramyxovirus affecting salmon gills, have been quantified by their agglutination properties. However in the case of ISAV this activity has been only observed in fish erythrocytes (Falk et al., 1997), while paramyxovirus agglutinates erythrocytes from mammals, birds or fish (Kvellestad, 2003). Nonetheless, the use in fish virology is not as frequent as for other viruses from mammals or birds.

f) Flow Cytometry

Flow cytometry (FCM) is based on the measurement of the physical and/or chemical characteristics of cells while they pass through a measuring apparatus in a single row in a fluid stream (Shapiro, 2003). The uses of flow cytometry in virology have been deeply reviewed by Mcsharry in 1994 and 2000. Quantification of virus using flow cytometry has evolved exponentially due to the improvements in the technology. The procedures published in the literature can be broadly classified in two groups: on one hand, the direct quantification of viruses in solution using fluorescent dyes and, on the other, those strategies based on the detection and quantification of viral antigens or proteins in cells or tissues, using immunofluorescence techniques.

Although in the early years of this technology its sensitivity was quite low, in the last decades it has been proved to be reliable for viral detection and quantitation of different viruses using SYBR Green I (Brussaard et al., 2000; Chen et al., 2001). FCM has been used for the quantification of algal viruses.

Although the FCM counts for *Heterocapsa circularisquama* virus (HcV) were significantly higher than that by the epifluorescence microscope (EFM) (ANOVA, $p < 0.05$), they were consistent at 1.5-3.7 × $10^8$ particles/mL, and the authors concluded that the number of small viruses in marine environment could be underestimated by FCM analysis (Tomaru and Nagasaki, 2007).

Another approach used for quantification of the viral load in a given sample is the immunofluorescence staining of viral antigens or proteins expressed in the infected cells. To this regard, a high correlation between the percentage of infected cells and the viral load of a sample has been proved (Drayman et al., 2010; Grigorov et al., 2011; Lonsdale et al., 2003). Normally, the titrations are performed over culture cells infected with a serially diluted viral sample. Then, the infected cells are incubated with polyclonal or monoclonal antibodies against some viral antigen, expressed extra or intracellularly. These antibodies can be labelled with some fluorescent dye or can be indirectly detected by a fluorescent labelled secondary antibody (McSharry, 2000). Finally, the obtained titers are generally expressed as fluorescent infectious units/mL, infectious units/mL or infected cells/mL. The detailed mathematical approaches of the different assays published to the date are beyond the scope of this review, but in general: the titer is calculated by the formula T = [% positive cells]x[number of infected cells]/[volume of infecting virus sample]. The reproducibility of the method, determined from the CV (as percentage) has been shown between 7% to 31% (Hammarlund et al., 2012; Roldão et al., 2009). The FCM assay can only be used with samples with a titer higher than $1x10^4$ infectious units/mL (Grigorov et al., 2011; Lambeth et al., 2005) or $5x10^3$ pfu/mL (Gates et al., 2009). This is a limitation of the FCM assay compared to the plaque assay, which is more sensitive (Lambeth et al., 2005).

The last strategy is known as quantitative flow cytometry (QFCM). It has been recognized that the intensity of the fluorescent signal is proportional to the amount of antibody bound per cell and therefore related to the number of antigen sites expressed. This relationship makes flow cytometry, at least theoretically, capable of quantifying antigen expression in terms of molecules per cell (Maher and Fletcher, 2005). Liu et al. (1997) demonstrated the utility of QFCM analysis of CD38 as a prognostic marker in HIV infection. Although many methods have been developed for standardizing QFCM measurements, the easiest ones are those using bead-based standards (Maher and Fletcher, 2005). The use of fluorescence standard provide the key for converting arbitrary relative intensity units to standard quantitative fluorescence units, reporting quantitative molecules of equivalent soluble fluorochrome (MESF) intensities and even numbers of antibodies bound to the cell (antibody binding capacity; ABC) (Schwartz et al., 1998). The quantitative detection of equine influenza virus infection in MDCK cells calibrated particles using the transformation of arbitrary fluorescence intensities into the standardized units MESF, allows comparisons

of signal intensities not only within samples stained in a group but also within different staining groups, e.g. measurements of different bioprocess batches (Schulze-Horsel et al., 2008). Nevertheless, there are several issues to be solved in flow cytometric quantitation of antigen expression; these extend to the level of instrumentation, reagents and the cells to be examined (Gratama et al., 1998). The utility and interest in the use of QFCM in the clinical setting are well documented in the literature and underscore the necessity for continued development of control procedures and materials for this purpose (Maher and Fletcher, 2005). Owing to its fast analysis and reliability, flow cytometry immunotitration has revealed as a valid and efficient alternative to infectivity assays like plaque assay or $TCID_{50}$. However, the cost of the equipment and reagents, added to the need of highly trained personnel difficult the general use of these procedures.

## 2.3 **Virus genome**

### a) Real time Polymerase Chain Reaction

It is unquestionable that one of the greatest revolutions of molecular biology in the past century was the polymerase chain reaction (PCR), and thereafter it was powered with the possibility to follow the evolution of the amplification on real time (Real Time PCR) and its capacity to produce true quantitative data qPCR (Higuchi et al., 1992, 1993; Wittwer et al., 1997). Although most of the PCR diagnostic protocols reported so far are qualitative, nowadays the real-time qPCR technology, based on the detection and quantitation of a fluorescent reporter (Bustin, 2000), is progressively replacing the traditional one. The literature about this topic is legion and have been deeply reviewed (Bustin et al., 2009; Hoffmann et al., 2009; Kubista et al., 2006; Mackay et al., 2002).

The development of real-time PCR has brought true quantitation of target nucleic acids out from the research to the diagnostic laboratories (Mackay et al., 2002). Quantitation of the amount or concentration of a template (RNA or DNA) can be performed following two strategies: absolute or relative qPCR. Absolute quantification relates the PCR signal to input copy numbers using a calibration curve, whereas relative quantitation is based on the expression levels of a target gene versus a reference or control gene (Pfaffl, 2004). Generally, relative quantitation provides sufficient information and is simpler to develop. However, when monitoring the progress of an infection, absolute quantification is useful in order to express the results in units, which are common to both scientists and clinicians and across different platforms. Absolute quantitation may also be necessary when there is a lack of specimens with a known stage of the infection to demonstrate changes in virus levels, when no suitably standardized reference reagents are available, or when the viral load is used to

differentiate active versus persistent infection (Mackay et al., 2002). Many real time-qPCR protocols for the detection of microbial targets have been published, and commercial assays are available for a number of clinically important human viruses (Ratcliff et al., 2007). In veterinary diagnostics, and more specifically in fish virology, this technique has been successfully implemented for detecting and quantifying many viral pathogens, including VHSV (Garver et al., 2011; Jonstrup et al., 2013), IHNV (Overturf et al., 2001; Purcell et al., 2013), ISAV (Snow et al., 2006; Workenhe et al., 2008), or IPNV (Bowers et al., 2008; Calleja et al., 2012). However, there is still a lack of properly validated standards to be implemented in fish virus quantification. Commercial kits are usually validated using a large cohort of clinical samples. Development of in-house assays may be achieved if an appropriate structured validation procedure is implemented (Ratcliff et al., 2007).

b) Digital PCR

The first reference of digital PCR (dPCR)was in the 1990s (Sidransky et al., 1992; Vogelstein and Kinzler, 1999). The struggle with the design, validation and preservation of the standards needed for the previous technique has been overcome with the advent of the dPCR. One of the greatest benefits of this technology is the direct measurement of viral nucleic acid concentration, providing the absolute number of copies/ml without the need for a standard curve. The chemistry used in digital PCR is the same as qPCR, but the amplification is detected by a different method. The fluorescent quantification is made by diluting the sample and partitioning it into individual reactions (droplets), in a manner that each reaction ideally contains one (never more than 2) or no copies of the DNA of interest (Sedlak and Jerome, 2014). The absolute number of target nucleic acid molecules in the sample is calculated directly from the ratio of positive to total partitions using binomial Poisson statistics (Pinheiro et al., 2012). In a recent study, the comparative evaluation between RT-dPCR and RT-qPCR detecting the main viruses responsible for foodborne outbreaks human Noroviruses (NoV) and Hepatitis A virus (HAV), revealed that the RT-dPCR is 1 $\log_{10}$ more sensitive. Moreover, RT-dPCR may provide more accurate measurements than RT-qPCR as it is not dependent on amplification efficiency (Coudray-Meunier et al., 2015). In other study, the authors quantified GB Virus Type-C (GBV-C), an occult RNA virus associated with HIV-1 infection. They observed that, even after removing the potential error of a mass-based standard curve using a digital PCR calibrated standard curve, RT-qPCR had, on average, a lower repeatability; more precisely, dPCR had an average coefficient of variation of 11.7±2.2% for the viral load tested, while standard qPCR had an average CV of 25.8±4.9% (White et al., 2012). Another key advantage of this technology is that the efficiency of the reverse transcription and the kinetics of

the amplification, relevant principles in qPCR, have no impact on the final digital PCR quantification. In a work published by Veach et al. (2015), the authors observed that the extracted RNA does not need to be purified from the reagents used to lyse the virus; they found a high correlation of the dPCR method with other quantification procedures of influenza virus like ELISA, HA and Virus Counter. RT-dPCR demonstrates higher precision and repeatability for quantifying waterborne viruses, at their characteristic low concentrations: 54.4±2.6 and 6±1 rotavirus copies/10 µL reaction (Rački et al., 2014). Digital PCR is unlikely to supplant qPCR in the short term, but instead will be a complementary approach in certain applications (Rutsaert et al., 2018; Sedlak and Jerome, 2014). Recently, its reliability in quantification of important pathogens for the aquaculture industry like VHSV and IHNV (Jia et al., 2017; Pavšič et al., 2016) has been proven.

## 2.4 **Virus particles**

Although the technical advances in Virology has provided a diverse variety of methodologies for viral quantification, the truth is that counting of viral particles was traditionally performed by the use of the transmission electron microscope (TEM). In a recent review by Heider and Metzner (2014), the authors include description of the atomic force microscopy (AFM), laser light scattering applications such as multiple-angle laser light scattering (MALLS) (or nanoparticle tracking analysis; NTA), tunable resistive pulse sensing (TRPS)– a method based on the Coulter principle–, and flow cytometry (FC) variants like the Virus Counter (VC). In addition, liquid chromatography has been evaluated for the quantification of virus particles (Transfiguracion et al., 2015).

a) Electron microscopy

Although it has been gradually replaced by more sensitive methods such as PCR and immunofluorescent assays (IFA), transmission electron microscopy (TEM) has certainly represented a major contribution to virology, because it facilitates the diagnosis of viral infections, supports the investigation on virus–host cell interactions, and has led to the discovery of new viruses (Roingeard, 2008). The basic viral quantification procedure using TEM is built upon negative staining (mainly with uranyl acetate, phosphotungstic acid or ammonium molybdate) of the viral particles and its counting along with standard latex particles of known concentrations (Reid et al., 2003; Watson et al., 1963; Zheng et al., 1996). TEM quantitation is far quicker than infectious titration and it also allows a qualitative observation of the viral morphology. However, the detection limit do not allows it to be used with samples with a concentration lower than $10^5$ particles/mL (Malenovska, 2013; Reid et al., 2003).

b) Virus Counter

Based on a prototype reported some years ago by Stoffel et al.,(2005), ViroCyt (2013; http://www.virocyt.com) has developed a specialized "flow virometer" suitable for rapid virus particle quantification in liquid samples. The method of detection is based on a dual labelling system, one label specific for proteins and another for the viral genome. Intact virus particles are quantified by detecting fluorescence from particles containing colocalized proteins and nucleic acids (Stoffel and Rowlen, 2005). Both, a certain size of virus (>25 nm) and a certain length of viral genome (49000 nt/bp) are necessary to guarantee a sufficient level of staining to be detected (Heider and Metzner, 2014). The substitution of a multistep antibody staining procedure by a non-specific staining increases the simplicity of the procedure for the operator, and reduces the assay costs (Arakelyan et al., 2013; Ferris et al., 2011). The reliable range of measurement reported by Heider and Metzner,( 2014) was between $5 \times 10^5$ to $1 \times 10^9$ virus particles/ml. In a recent evaluation of the method for filovirus quantitation, the authors observed that the CV ranged from 9.4% to 31.5% for those samples that fell within the linear range of the instrument ($2.8 \times 10^6$ to $1.0 \times 10^9$ VP/mL)(Rossi et al., 2015). These limits are similar to those obtained for H1N1 influenza virus ($9.8 \times 10^4$ VP/mL) (Stepp et al., 2011). High correlation of the method with TEM, qPCR or $TCID_{50}$ was observed, but a big gap between infectious and total virus counts was detected (Heider and Metzner, 2014). In quantification of particles directly from samples with the high levels of contaminant proteins typical of clinical samples (whole blood, serum, plasma) or stock preparations with fetal bovine serum concentrations higher than 10%, the level of background negatively affects the counting; therefore, previous dilution or purification of the sample is mandatory (Rossi et al., 2015).

Other technologies like AFM, MALLS or NTA have been recently reviewed in detail by Heider and Metzner (2014). Although promising in their results, only a few research groups have reported their evaluation. The viruses tested are reduced to influenza virus, adenovirus and lentivirus. All of them have their pros and cons, but it is too early to determine if their use will be generalized for research with other viruses including those affecting aquaculture.

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# Chapter 3

# Design and Validation of a RT-qPCR Procedure for Diagnosis and Quantification of Most Types of Infectious Pancreatic Necrosis Virus Using a Single Pair of Degenerated Primers



The data reported in this chapter was published in: *Design and validation of a RT-qPCR procedure for diagnosis and quantification of most types of infectious pancreatic necrosis virus using a single pair of degenerated primers. Journal of Fish disease, 2017 Volume 40, Issue 9 Pages: 1129-1252 doi:10.1111/jfd.12590.*

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# Chapter 4

# Quantitative Flow Cytometry to Measure Viral Production Using Infectious Pancreatic Necrosis Virus as a Model: A Preliminary Study



The data reported in this chapter was published in: *Quantitative Flow Cytometry to Measure Viral Production Using Infectious Pancreatic Necrosis Virus as a Model: A Preliminary Study Applied Sciences. 2018, 8, 1734; September 2018 doi:10.3390/app8101734*

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# Chapter 5

# A novel procedure of quantitation of virus based on microflow cytometry analysis



The results of this study were published in: *A novel procedure of quantitation of virus based on microflow cytometry analysis*. *Applied Microbiology and Biotechnology, Volume 100, Issue 5, pp 2347–2354. March 2016, doi: 10.1007/s00253-015-7228-3*

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# Chapter 6

## Final Discussion and Conclusions

# 1 FINAL DISCUSSION

Whether it is for routine evaluation of a biopharmaceutical product, for vaccine production, or for validation of antiviral substances, virus quantification is a critical step in any of these processes. Therefore, the main objective of the whole study constituting the present doctoral thesis was to provide the Fish Virus Unit at the Institute of Aquaculture, USC, with a set of standardized and validated techniques for reliable quantification of fish viruses. In addition, because in the last years EU researchers have aimed their efforts to the development of diagnostic and quantification procedures for the viral haemorrhagic septicaemia virus (VHSV) and the viral nervous necrosis virus (VNNV), leaving apart another important virus: the infectious pancreatic necrosis virus (IPNV); it was the goal of the present study to provide additional tools to fight this disease.

After the extensive review of the current methods used for quantification of viruses performed in Chapter 1, it was concluded that, for most of them the gold standard is, still the cell based methods like plaque assay or 50% tissue culture infectious dose estimation by TCID50. In the particular case of viruses affecting the fish aquaculture industry, cell culture is also the gold standard method chosen by the World Organization for Animal Health (OIE). However, that technique is time consuming and exhibits low sensitivity which, in some cases, makes virus quantification difficult (Dopazo and Bandín, 2011). Therefore, although this one was used as reference, other techniques were selected to be evaluated for viral quantification after previous optimization: the quantification of *i)* genomic units, by means of quantitative Polymerase Chain Reaction (qPCR) (Chapter 2), *ii)* and viral proteins (Chapters 3 and 4).

Based on the large experience of the Institute team with the PCR based technologies, both for diagnosis and quantification of viruses (Cutrín et al., 2009; Dopazo and Bandín, 2011; Lopez-Vazquez et al., 2015; Olveira 2006), qPCR was the first technique selected to be optimized and validated for diagnosis and quantification of IPNV. The qPCR is a well-recognized technology for nucleic acid quantification and has been –and nowadays is– widely employed for viral quantification, as well as to quantify gene expression. Unfortunately, in some cases those studies reporting its use did not include the validation of the procedure, which compromise, to our understanding, the reliability of the results.

The *in vitro* validation of the qPCR procedure developed was performed using, as reference standards, crude virus (titrated by the plaque assay and the end-point dilution $TCID_{50}$ methods) and several *in vitro* transcribed RNA standards obtained from representative reference strains. The standards tested were found in all cases highly reliable based on the correlation values obtained ($R^2$ always $\geq 0.95$) (Broeders et al., 2014). In addition, its high repeatability and reproducibility confirms the reliability of the procedure for viral quantification.

terms of quantification of IPNV protein synthesis. Although qFC is not a new technique (Schwartz et al., 2002; Serke et al., 1998; Wang et al., 2002) its application to quantitatively study virus replication has been limited (Gates et al., 2009; Schulze-Horsel et al., 2008) . To this regard, in the present study we have demonstrated that, thanks to the MARIS method (Hrvatin et al., 2014), the developed procedure can also be applied to study the viral replication, since it can assess the capsid VP2 protein production significantly related to the RNA synthesis.

As already reported by other authors (Mizrahi et al., 2018), qFC requires careful standardization in order to get meaningful and reproducible results. Such standardization must include every item in the whole process: Sample handling – even type of sample: virus and cells – calibration beads, the cytometer itself, or even data analysis. This was the complete process followed in chapter 3 to set up our procedure. However, it has been evaluated for IPNV on BF-2 cells with anti-VP2 protein antibody. Therefore, the procedure should be validated for each particular virus to stablish individual protocols suitable for each different case.

On the other hand, in spite of the fact that flow cytometry has been revealed as a valuable tool for virus quantification, it requires highly experience operators, and very expensive equipment and reagents to obtain reliable data. Therefore, to evaluate other alternatives, an easy to use approach based on the "Lab on a Chip" technology – (microfluidic glass chips) was designed and evaluated for viral quantification. The optimization of the procedure described in chapter 4 has demonstrated microflow cytometry (μFCM) to be an affordable and reliable alternative to traditional qFC when simple analysis of virus infected cells is required. The designed procedure provided a repeatable and reproducible viral titration in a minimum incubation time of 16h, with a dynamic range of 5 logs, and a minimum detectable titer of $10^4$ $TCID_{50}$/ml. It reached an improved limit of $10^2$ TCID50/ml when the incubation time was increased to 24h. In addition, with this method, 6 samples can be analyzed within the same glass chip, which reduces time and costs in reagents and materials.

In the study performed for chapter 4, we have observed that, as for qFC, μFCM is highly influenced by several parameters, which must be optimized to standardize the procedure for each virus-cell-sera set. In our case, the first parameter which we realized that influenced the efficiency of the procedure was the virus-cell incubation time, as already indicated above. Moreover, the high specificity of the method, which relies on the affinity of the monoclonal antibodies for an specific epitope of the viral protein, makes the initial optimization of the test conditions critical. It is important to highlight that despite the high specificity of the mAb (against VP2 epitopes) employed for both FACS and μFCM, it was possible to detect different types of IPNV.

**Table 1.-** Summary of parameters and characteristics of the 3 techniques developed

| Technique | LOD (Inf/ml) | DR | Reliability of titration | Time | Operator Experience |
|---|---|---|---|---|---|
| qPCR | 31 $TCID_{50}$<br>50 pfu<br>60 RNA copies | 2-9 lg $TCID_{50}$ **q**<br>1.5-6.5 lg $TCID_{50}$ **I**<br>$10^{2.5}$-$10^{10}$ copies | Underestimation | ½ day | Medium |
| qFC | 10 $TCID_{50}$<br>26 pfu | 1-6 lg $TCID_{50}$<br>1.4-6.4 pfu | 2nd-degree polynomial relationship | 24h | High |
| µFCM | 200 $TCID_{50}$<br>501 pfu | 2-7 lg $TCID_{50}$<br>2.7-7.7 lg pfu | Overestimation under $10^5$ $TCID_{50}$ | 16h for $>10^4$ $TCID_{50}$<br>24h for $>10^2$ $TCID_{50}$ | Medium |

*LOD, limit of detection; DR, dynamic range; R&R, repeatability and reproducibility; Replic, the virus is detected associated to replication in cell culture; Stand., the technique has been standardized;* ***q****, 8 logs if a second-degree polynomial regression is applied;* ***I****, 5 logs if a lineal regression is applied*

Which of the procedures developed would be advisable for diagnosis and quantification? Following, we will compare the validated parameters and the characteristics of the three procedures and technologies, their advantages and disadvantages (Table 1).

Regarding the detection capacity in terms of limit of detection (LOD), the best method was the qFC, which was able to detect as little as 10 $TCID_{50}$/ml (or 26 pfu/ml) of virus. This value is 1 log lower than the data reported for other viruses like measles (Grigorov et al., 2011) or varicella (Gates et al., 2009). Among the three technologies, the worst sensitivity was exhibited by µFCM, with a LOD=$2\times10^2$ $TCID_{50}$/ml (50 pfu/ml). The LOD obtained with qPCR (around 30 $TCID_{50}$/ml or 50pfu/ml) revealed a sensitivity close to the qFC; however, we must say that this value was obtained with just one replica, and the LOD data with 3 replicas (480 pfu/ml) was quite similar to that provided by µFCM.

The dynamic range (DR) for quantification in terms of $TCID_{50}$ or pfu was the same for the 3 techniques: 6 log, an optimum data, as reported in previous guidelines (Bustin et al., 2009). However, in the case of qPCR, the use of *in vitro* transcribed RNA (*iv*RNA) provided a 9 logs DR; also with this technology, the DR reached 9 logs of $TCID_{50}$ when a second-degree regression was applied.

A perfect equivalence between traditional titration and quantification by any of the three procedures was not observed. For instance, with µFCM an overestimation (from 1.5 to 3 logs) was observed at the lowest titers (from $10^2$ to $10^5$), and almost no differences at the highest ones. To this regard, It has been

reported that the concentration values obtained by plaque assay or any other infectivity method are lower than methods based in nucleic acid or protein (Heider and Metzner, 2014). However, in the case of qPCR, the use of *iv*RNA as standard implied an underestimation of the real titer in the whole DR, from 1.5 log of difference at the lowest titers to 0.5 at the highest, what, as discussed in Chapter 2, could be due to the degeneration of several bases in the sequence of the primers. This effect has also been observed in IPNV by Jorquera et al., (2016), although they also stated that degenerated primers is the best solution to compensate polymorphic sites and overcome the high mutation rate of these viruses. Only with qFC a clear relationship between titration and protein quantification was observed, but just using a second-degree polynomial regression.

Using these parameters as criteria and considering that the 3 methods showed high repeatability and reproducibility, the extremely high sensitivity of the qFC, and the high sensitivity and wide dynamic range of the qPCR, makes them the best candidates to be used for viral quantification. However, other criteria must be considered, for instance, the requirements of level operator's experience is an important aspect to ensure the reliability of the results. To this regard, the qFC technology requires a highly qualified technician to operate the equipment and provide raw data for analysis; even, the analysis of the raw data must also be performed with a dedicated software, which requires previous training and experience. In terms of simplicity, the μFCM has been found to be suitable for operators with low experience (Chapter 4) because the equipment and method designed are, once standardized, of low complexity for preparation and performance. In addition, in spite of the fact that highly trained operators obtained better results, the μFCM have shown to be robust when performed by unexperienced technicians, as observed from the assessment of the reproducibility of the method, with coefficients of variation (CV) lower than 10%. Regarding the qPCR, the standardization of the procedure itself is complex, as it is for qFC and μFCM but, once standardized, it is expected to be reproducible between different laboratories and technicians of different levels of experience (Dopazo and Bandín, 2011).

There are two additional parameters that must also be considered: *time* and *viral activity*. Taking the second one under consideration, qPCR should not be taken into account because only qFC and μFCM rely on the infection of cell monolayers by viruses. However, considering the first criteria –*time*–, qPCR exceeds the other two techniques in speed to perform detection, identification and quantification.

Which method should be advised?

This discussion seems to introduce us more in a sea of confusion, instead of clarifying which technique should be used in each situation. Therefore, it is important to recapitulate, and to apply a summary of the summary, to advise on which technology must be used in each case –and why:

1/ μFCM should be used for viral titration when quantification of active virus and how quick the results are provided is important. In this case, it must be considered that, as demonstrated in chapter 4, for medium titers ($\geq 10^4$ $TCID_{50}$/ml) the method is reliable after a minimum incubation time ≥16h; but, for lower viral titers ($\geq 10^2$ $TCID_{50}$/ml) ≥24h are needed. The considerable reduction in time makes this method a great alternative to infectivity method like Plaque assay, because the reading of results is reduced from days to hours. Vaccines production and quantification of virus stock or inoculums could benefit by this approach, because, although qPCR is one of the most frequent methods used for that purpose, the addition of infectivity information is crucial in processes of production of biopharmaceutical products.

2/ qPCR should be applied for viral quantification when total virus is needed but active virus is not crucial. It is also important when high sensitivity (because low viral loads are expected) and speed are needed.

3/ qFC and qPCR, combined, are advised on viral replication studies, since both provides complementary data: protein and RNA synthesis, what provides important information to understand the whole viral cycle.

What else should be done?

As already stated in the present study, the procedures reported here have been evaluated for a specific virus-cell set. Therefore, the first issue we should assume as crucial is that, if this methodology is to be applied, research should be extended to other viruses and/or cell lines to cover most needs in research and diagnosis of virus. Robustness of the protocols in collaborative trials with other laboratories is encouraged because that will provide valuable inputs on the performance of the methods concerning the detection and quantification of other viruses.

## 2 FINAL CONCLUSIONS

1- The importance and singularity of the RT-qPCR procedure proposed in this study for IPNV lies in that, for the first time, a method based in a single pair of primers has been demonstrated to be reliable not only for the diagnosis of most IPNV types, but also for simultaneous quantification of the viral load, both *ex vivo* and *in vivo*.

   It will dramatically simplify and reduce the cost –maintaining the reliability– of surveillance and monitoring programs, and of any study requiring diagnosis and quantification of IPNV-type viruses without the need of a previous knowledge of the expected type of IPNV present in the population.

2- The results from the present study support the use of the Flow cytometry in combination with qPCR technology, not only for the quantification of the optimal virus infectivity rate, but also to study viral replication from a quantitative approach

3- Our results strongly support the proposal of the micro flow cytometry (μFC) method, as a reliable alternative to the traditional methods of viral titration because, even when using incubation times of 24h, the reduction of the time needed to quantify the viral concentration of a sample is unquestionable.

4- Regarding the applicability of the methodologies, *i)* μFCM should be used for viral titration when quick quantification of *active* virus is an unavoidable requirement; *ii)* qPCR should be applied for viral quantification when knowledge of total virus is needed but active virus is not crucial; it is also important when high sensitivity (because low viral loads are expected) and speed are needed; *iii)* qFC and qPCR, combined, are advised on viral replication studies, since both provide complementary data (protein and RNA synthesis) to understand the whole viral cycle.

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# RESUMEN

## CAPÍTULO 1: Introducción

El desarrollo mundial de la acuicultura en los últimos años es incuestionable. En 2016, la producción mundial de acuicultura alcanzó 80 millones de toneladas, con un valor de primera venta estimado de US $ 232 mil millones, que consiste en 54,1 millones de toneladas de peces (US $ 138,5 mil millones), 16.1 millones de toneladas de moluscos (US $ 29,2 mil millones) , 7,9 millones de toneladas de crustáceos (US $ 57,1 mil millones) y 9385000 toneladas de otros animales acuáticos (US $ 6,8 mil millones) (FAO, 2018). Esta situación ha sido promovida en gran parte, por la intensificación de los procesos de cultivo.

Sin embargo, la producción intensiva de peces y mariscos está amenazada por brotes epidémicos que afectan no solo a las poblaciones "domésticas" sino también a las poblaciones silvestres (Tompkins et al., 2015). Entre las diferentes patologías que afectan a la acuicultura de peces, las enfermedades virales tienen uno de los mayores impactos económicos debido a su virulencia, rápida dispersión y ausencia de tratamiento y vacunas efectivas, lo que dificulta su erradicación completa. Por lo tanto, la investigación se centra no sólo en el desarrollo de protocolos profilácticos y el diseño de nuevas y más eficientes vacunas, sino también en la mejora de los procedimientos de diagnóstico para facilitar el control de los brotes. Además, se está aplicando un esfuerzo cada vez mayor en las inspecciones del estado de salud de poblaciones de peces cultivados y silvestres, lo que ha llevado al descubrimiento de virus de peces que la ciencia desconocía hasta el momento; siendo alguno de ellos endémicos entre las poblaciones nativas y propagándose de manera oportunista para infectar peces en instalaciones de acuicultura (Walker y Winton, 2010). Además, esa vigilancia, también ha proporcionado información importante sobre la distribución y la virulencia de otros virus conocidos previamente.

## CAPÍTULO 2: Cuantificación viral: Una revisión general

En la mayoría de los estudios de virología, uno de los principales datos a conocer es la concentración de partículas totales o infecciosas en una muestra. Por lo tanto, la cuantificación del virus es crucial para conocer entre otras cosas, el ciclo de replicación de un virus, para estudiar el nivel de virulencia de nuevos aislados, para evaluar la actividad antiviral de los agentes quimioterapéuticos, para monitorizar la etapa y la eficiencia de una purificación de virus en procesos industriales o simplemente para evaluar la virulencia de un determinado agente viral (Hierholzer y Killington, 1996). En cualquier experimento que involucre la infección de células con virus, la multiplicidad de la infección (MOI por sus siglas en Inglés, que es el número de partículas virales por célula) es el factor más crítico, ya que las MOI irreproducibles o imprecisas, causadas por una estimación

inexacta del título viral del inóculo, tienen un profundo impacto en el rendimiento y la consistencia del lote o del ensayo (Roldão et al., 2009). Por esta razón, es muy importante contar con métodos cuantitativos precisos.

La utilidad clínica de la cuantificación viral ha sido profundamente revisada en medicina humana. Sin embargo, en el campo veterinario, y más concretamente en la virología de peces, la mayoría de las técnicas y metodologías se han adaptado de anteriores avances en virología humana. A pesar de su relevancia, solo un par de revisiones se han publicado en los últimos años (Heider y Metzner, 2014; Pankaj, 2013).

En esta línea, y siguiendo la clasificación realizada por Heider y Metzner, los métodos de cuantificación de virus pueden clasificarse en cuatro categorías diferentes dependiendo de en qué se basen: i) en los niveles de infectividad, ii) en la detección de proteínas virales, iii) en la detección de ácido nucleico viral, y iv) en la enumeración de partículas virales.

En este capítulo se han revisado en detalle las siguientes técnicas de cuantificación basándonos en la clasificación descrita anteriormente:

- Infectividad: Ensayos quantales y cuantitativos.
- Detección de Proteínas Virales: Ensayo de Foci Fluorescentes, ELISA, Ensayo de inmunodifusion Radial Simple (SRID), Cromatografía liquida de alta precisión (HPLC), Ensayos de Hemaglutinación, Citometría de Flujo.
- Detección de ácido nucleico viral: PCR en tiempo real PCR digital
- Enumeración de partículas virales: Microscopia electrónica, Contador de Virus

Para el presente estudio, centrado en el desarrollo, la optimización y la validación de los procedimientos de cuantificación viral, cualquiera de los virus que afectan actualmente a la industria de la acuicultura podría haberse elegido como modelo. ¿Por qué se eligió entre todos el virus de la necrosis pancreática infecciosa (IPNV, por sus siglas en Inglés)? La misma brecha con respecto al objetivo general de este estudio afecta a todos los virus de peces en la literatura, pero las restricciones de bioseguridad del IPNV, junto con la alta disponibilidad y diversidad de cepas, hacen de este patógeno un gran candidato para este tipo de estudios. Por lo tanto, el propósito del presente estudio fue la evaluación, diseño y validación de diferentes técnicas de cuantificación de virus, utilizando el virus de necrosis infecciosa pancreática (IPNV) como modelo general. El objetivo final del estudio fue, en una etapa posterior, la transferencia de las tecnologías desarrolladas en esta tesis a los protocolos estándar del Instituto de Acuicultura de la Universidad de Santiago de Compostela.

## CAPÍTULO 3: Diseño y validación de un procedimiento RT-qPCR para el diagnóstico y cuantificación de la mayoría de los tipos de virus pancreáticos infecciosos utilizando un solo par de cebadores degenerados

Es incuestionable que una de las mayores revoluciones de la biología molecular en el siglo pasado fue la reacción en cadena de la polimerasa (PCR), y posteriormente se impulsó con la posibilidad de seguir la evolución de la amplificación en tiempo real, PCR en tiempo real o qPCR (por sus siglas en Inglés); y su capacidad para producir verdaderos datos cuantitativos (Higuchi et al., 1992, 1993; Wittwer et al., 1997). Aunque la mayoría de los protocolos de diagnóstico de PCR publicados inicialmente son cualitativos, en la actualidad, la tecnología de la PCR en tiempo real, basada en la detección y cuantificación de un amplicón o sonda fluorescente (Bustin, 2000), está reemplazando progresivamente al uso de la PCR tradicional. La literatura sobre este tema es muy abundante y se ha revisado en profundidad en anteriores publicaciones (Bustin et al., 2009; Hoffmann et al., 2009; Kubista et al., 2006; Mackay et al., 2002).

El desarrollo de la PCR en tiempo real ha llevado la verdadera cuantificación de los ácidos nucleicos virales a los laboratorios de diagnóstico (Mackay et al., 2002). La cuantificación de la concentración de una muestra (ARN o ADN) se puede realizar siguiendo dos estrategias: qPCR absoluta o relativa. La cuantificación absoluta relaciona la señal de PCR con los números de copia de entrada utilizando una curva de calibración, mientras que la cuantificación relativa se basa en los niveles de expresión de un gen objetivo frente a un gen de referencia o control (Pfaffl, 2004). En general, la cuantificación relativa proporciona información suficiente y es más fácil de desarrollar. Sin embargo, cuando se realiza el seguimiento del progreso de una infección, la cuantificación absoluta es útil para expresar los resultados en unidades, que son comunes tanto para los científicos como para los clínicos en diferentes plataformas. La cuantificación absoluta también puede ser necesaria cuando faltan muestras con una etapa conocida de la infección para demostrar cambios en los niveles de virus, cuando no hay disponibles reactivos de referencia estandarizados adecuadamente, o cuando se usa la carga viral para diferenciar la infección activa frente a la persistente ( Mackay et al., 2002). Se han publicado muchos protocolos de qPCR en tiempo real para la detección de enfermedades microbianas, y se dispone de kits comerciales para varios virus humanos clínicamente importantes (Ratcliff et al., 2007). En diagnósticos veterinarios, y más específicamente en virología de peces, esta técnica se ha implementado con éxito para detectar y cuantificar varios patógenos virales, incluyendo (en sus siglas en Inglés): VHSV (Garver et al., 2011; Jonstrup et al., 2013), IHNV (Overturf et al., 2001; Purcell et al., 2013), ISAV (Snow et al., 2006; Workenhe et al., 2008), o IPNV (Bowers et al., 2008; Calleja et al., 2012). Sin embargo, todavía faltan estándares validados

adecuadamente para ser implementados en la cuantificación de virus de peces. Los kits comerciales generalmente se validan utilizando una gran cohorte de muestras clínicas. El desarrollo de ensayos internos se puede lograr si se implementa un procedimiento de validación estructurado apropiadamente (Ratcliff et al., 2007).

Hoy en día, la PCR cuantitativa está reemplazando a otros métodos tradicionales porque proporciona información adicional sobre la carga viral, lo cual es importante para tener una mejor comprensión del nivel de replicación del virus y de la etapa de la infección y su nivel de riesgo. El principal problema radica en la gran diversidad del virus de necrosis pancreática infecciosa, que puede comprometer la fiabilidad del diagnóstico. Como parte de esta tesis doctoral, hemos diseñado un procedimiento RT-qPCR para el diagnóstico y la cuantificación de IPNV basado en un único par de cebadores dirigidos al segmento B del genoma del virus. El procedimiento ha sido validado, *in vitro* e *in vivo*, probando dos tipos diferentes de estándares frente a siete genotipos de referencia y 23 aislados de campo de diferentes genotipos.

El protocolo presentado en el Capítulo 3 es apropiado para la detección de cualquier genotipo del virus IPNV, con un límite de detección de 31 TCID50/mL, 50 pfu/mL o 66 copias de RNA/mL, según el estándar con que se realice la cuantificación. Todas las curvas estándar mostraron una alta fiabilidad ($R^2$> 0,95). Los resultados presentados en el capítulo 3 respaldan la alta fiabilidad de este nuevo protocolo para el diagnóstico y cuantificación de IPNV.

**CAPÍTULO 4: Citometría de flujo cuantitativa para medir la producción viral utilizando el virus de la necrosis pancreática infecciosa como modelo: un estudio preliminar.**

Los procedimientos clásicos para la cuantificación de virus, basados en la medición de la infectividad descritos en detalle en el Capítulo 1 de esta tesis (ensayo infectividad y cuantificación de placas), requieren que en el cultivo de células susceptibles se desarrolle un efecto citopático visible (CPE , en sus síglas en Inglés). Como consecuencia, esos procedimientos requieren entre 3 y 12 días, dependiendo del virus que se esté analizando y del nivel requerido de evaluación del CPE; Por lo tanto, se considera que, hasta obtener un resultado final mediante ambos tipos de cuantificación, es necesario invertir mucho tiempo. Además, algunas células no producen CPE visible, y algunas no admiten la formación de placa. Por ejemplo, algunos aislamientos clínicos de ciertos virus como la fiebre amarilla pueden ser más difíciles de cuantificar porque no provocan la formación de placa o no inducen un CPE medible. Se han observado problemas similares con otros flavivirus como el virus del dengue, ya que los aislamientos clínicos a menudo no inducen efecto citopático, ni forman placas (Hammarlund et al., 2012, Li et al., 2011). Para superar estas limitaciones, se han desarrollado algunas alternativas a los métodos tradicionales.

Algunas de las estrategias se basan en la evaluación de la producción viral mediante la detección relativa y la cuantificación de antígenos o proteínas virales específicas. Este valor podría correlacionarse con la carga viral, sin embargo, no proporciona la concentración actual de partículas virales infecciosas, ya que la presencia de algunos antígenos libres o proteínas disociadas podría afectar la medición.

La citometría de flujo (FCM) se basa en la medición de las características físicas y / o químicas de las células mientras pasan a través de un aparato de medición en una sola fila en una corriente de fluido (Shapiro, 2003). Mcsharry revisó a fondo los usos de la citometría de flujo en virología en 1994 y 2000. La cuantificación del virus mediante citometría de flujo ha evolucionado enormemente debido a las mejoras en la tecnología. Los protocolos de cuantificación viral usando citometría de flujo publicados en los últimos años, pueden clasificarse en dos grupos principalmente: por un lado, la cuantificación directa de virus en solución usando tinciones fluorescentes y, por otro, las estrategias basadas en la detección y cuantificación de antígenos o proteínas virales en células o tejidos, utilizando técnicas de inmunofluorescencia.

La estrategia descrita en el Capítulo 4 se conoce como citometría de flujo cuantitativo (qFCM en sus siglas en inglés). Se ha reconocido que la intensidad de la señal fluorescente es proporcional a la cantidad de anticuerpo unido por célula y, por lo tanto, está relacionada con la cantidad de antígeno expresados. Esta relación hace que la citometría de flujo, al menos teóricamente, sea capaz de cuantificar la expresión del antígeno en términos de moléculas por célula (Maher y Fletcher, 2005). Liu y colaboradores en 1997 demostraron la utilidad del análisis mediante citometría de flujo cuantitativa de CD38 como marcador pronóstico en la infección por VIH. Aunque se han desarrollado muchos métodos para estandarizar las mediciones de QFCM, los más fáciles son aquellos que utilizan estándares basados en microesferas fluorescentes (Maher y Fletcher, 2005). El uso del estándar de fluorescencia proporciona la clave para convertir unidades de intensidad relativa arbitrarias en unidades de fluorescencia cuantitativas estándar, informando moléculas cuantitativas de equivalencias de fluorocromo soluble (MESF, por sus siglas en Inglés) e incluso números de anticuerpos unidos a la célula (capacidad de unión del anticuerpo; ABC) (Schwartz et al. al., 1998). Como ejemplo, la detección cuantitativa de la infección por el virus de la influenza equina en las partículas calibradas de células MDCK mediante la transformación de intensidades de fluorescencia arbitrarias en las unidades estandarizadas MESF, permite comparaciones de las intensidades de señal no solo dentro de las muestras teñidas en un grupo sino también dentro de diferentes grupos de tinción.

Hemos diseñado un estudio preliminar para evaluar la fiabilidad de qFCM para estudiar la replicación viral, utilizando IPNV como modelo. Además, hemos adaptado la metodología FACS descrita por Hrvatin y colaboradores en

2014 para la cuantificación de la replicación viral en las células clasificadas positivas.

En el capítulo 4 antes de evaluar el uso de qFCM para la cuantificación de componentes virales, primero realizamos unas pruebas preliminares del protocolo para garantizar su fiabilidad y rendimiento para discriminar entre las concentraciones virales, y hemos confirmado la correlación entre las dosis virales (MOI) y fluorescencia (MFI), y entre estos y el número de copias de ARN en las células positivas.

Para ello hemos usado microesferas con una emisión de fluorescencia conocida (que se usan como referencia para transformar las unidades de fluorescencia arbitrarias en MESF), y se demostró que son fiables basándonos en los valores de CV por debajo del 10%, en todos los casos, y en los valores de correlación significativos ($R^2 > 0,99$).

El método fue probado en el capítulo 4 para estudiar la replicación viral. Para este propósito, se estudió la cinética de expresión de la proteína VP2 durante las primeras 24 h de la infección por IPNV analizando MFI a partir de eventos detectados FITC +. Para estudiar la producción de ARN viral, se aplicó el método MARIS descrito por Hrvatin et al. (2014)

Los resultados revelaron evidencia de la internalización de las partículas de IPNV, ya que las células analizadas justo después de la adsorción mostraron un aumento de fluorescencia con respecto a las células infectadas simuladas. Luego, se observó un pico importante de producción de VP2 a las 12 h post infección (p.i). Curiosamente, este pico en la producción de VP2 coincide claramente con el de la producción de ARN, y debemos destacar que los cebadores de PCR fueron diseñados para la secuencia de VP2.

Espinoza y colaboradores publicaron previamente resultados similares usando microscopía de fluorescencia y anticuerpos monoclonales contra VP2 en el año 2000, quienes observaron una alta acumulación de grandes grupos esféricos de proteínas VP2 en una ubicación perinuclear, aparecieron señales fuertes a las 8 h p.i. Sin embargo, el resultado más importante de este ensayo fue el hecho de que hemos demostrado que los datos de qPCR se correlacionaron significativamente ($R^2 = 0.9514$) con la producción de ARN (secuencia VP2).

El protocolo descrito en el capítulo 4 ha demostrado ser repetible y reproducible a un nivel aceptable, aunque para garantizar la reproducibilidad, el uso de un estándar en cada análisis es inevitable. Respecto a su uso para la cuantificación viral, se observó una relación directa (por una regresión polinómica de segundo grado) entre los títulos víricos y las Moléculas de Fluorocromo Soluble Equivalente (MESF). Los resultados apoyan el uso de esta tecnología, no solo para cuantificación de virus, sino también para estudiar la replicación viral desde un enfoque cuantitativo.

Sin embargo, como ya se indicó previamente en este trabajo, los protocolos descritos en esta tesis se han evaluado para un conjunto específico de células y virus. Por lo tanto, el primer punto que deberíamos asumir como crucial es que, si esta metodología desea aplicase en otro ámbito, la recualificación debería extenderse a otros virus y / o líneas celulares para cubrir la mayoría de las necesidades en investigación y diagnóstico de virus.

**CAPÍTULO 5: Un nuevo procedimiento de cuantificación de virus basado en análisis de citometría de microflujo**

Otro enfoque utilizado para la cuantificación de la carga viral en una muestra dada es, mediante la tinción de inmunofluorescencia de antígenos virales o proteínas expresadas en las células infectadas, establecer una correlación entre la concentración de antígeno o proteína viral medida, y la concentración real de virus. A este respecto, se ha demostrado una alta correlación entre el porcentaje de células infectadas y la carga viral de una muestra (Drayman et al., 2010; Grigorov et al., 2011; Lonsdale et al., 2003). Normalmente, las titulaciones se realizan sobre células de cultivo infectadas con una muestra viral diluida en serie. Luego, las células infectadas se incuban con anticuerpos policlonales o monoclonales contra algún antígeno viral, expresado extra o intracelularmente. Estos anticuerpos pueden marcarse con algún colorante fluorescente o pueden detectarse indirectamente por un anticuerpo secundario marcado fluorescente (McSharry, 2000). Finalmente, los títulos obtenidos se expresan generalmente como unidades infecciosas fluorescentes por mL de solución, unidades infecciosas / mL o células infectadas / mL. Los detalles matemáticos de los diferentes ensayos publicados hasta la fecha están fuera del alcance de esta revisión, pero en general: el título se calcula mediante la fórmula T = [% de células positivas] x [número de células infectadas] / [volumen de infección] muestra de virus]. La reproducibilidad del método, determinada a partir del CV (como porcentaje), se ha demostrado entre el 7% y el 31% (Hammarlund et al., 2012; Roldão et al., 2009). El ensayo FCM solo se puede utilizar con muestras con un título superior a $1x10^4$ unidades infecciosas / ml (Grigorov et al., 2011; Lambeth et al., 2005) o $5x10^3$ pfu / mL (Gates et al., 2009). Esta es una limitación del ensayo FCM en comparación con el ensayo de placa, que es más sensible (Lambeth et al., 2005).

Aunque debido a la rapidez de análisis y fiabilidad, la titulación por citometría de flujo se puede convertir en una alternativa rápida y eficiente a los ensayos de infectividad como el análisis de placa o $TCID_{50}$, el coste del equipo y los reactivos, sumado a la necesidad de personal altamente capacitado, dificultan la generalización de esta tecnología. Recientemente, los dispositivos integrados "*Lab-on-a-chip"* han llevado a cabo la miniaturización de este equipo, convirtiendo la citometría de flujo en una alternativa asequible y fácil de usar, en comparación

a los citómetros tradicionales. En el presente estudio, hemos diseñado un procedimiento de citometría de microflujo (μFC) para la cuantificación de virus, utilizando el virus de la necrosis pancreática infecciosa (IPNV) como modelo. La optimización de las condiciones y la validación del método se informan en detalle en el Capítulo 5 de esta tesis.

La fiabilidad del protocolo descrito en el capítulo 5 ha quedado demostrada por la alta repetibilidad y reproducibilidad, ya que los valores de coeficiente de variación observados siempre fueron inferiores al 10%. De hecho, aunque los resultados obtenidos por el operador más experimentado parecen proporcionar la mayor repetibilidad y reproducibilidad, los obtenidos por técnicos con poca experiencia fueron lo suficiente precisos como para ser considerados fiables. Además, la frecuencia de los falsos positivos (es decir, la sobreestimación del título viral debido a la detección de virus no infeccioso) se ha descartado claramente porque la muestra viral inactivada se cuantificó con títulos y porcentajes extremadamente bajos de células infectadas, como se esperaba. Finalmente, el procedimiento se evaluó con varias muestras de diferentes tipos y títulos de IPNV, y los resultados demostraron que los valores proporcionados por el procedimiento μFC están altamente correlacionados con los de los ensayos tradicionales de dilución y placa.

**CAPÍTULO 6: Discusión Final y Conclusiones**

Debido a la aplicabilidad y la fiabilidad de los métodos presentados en líneas anteriores, estudios de validación adicionales podrían derivarse de esta tesis. Se recomienda evaluar la robustez de los protocolos en un ensayo de colaboración con otros laboratorios y proporcionará información valiosa sobre el rendimiento de los métodos relacionados con la detección y cuantificación de otros virus. Además, sería valioso incluir un análisis y evaluaciones comparativas con el número de partículas obtenidas para un título definido.
Como se indicó anteriormente (Heider y Metzner, 2014; Roldão et al., 2009), no es posible definir una técnica única para la cuantificación de la carga de virus que se adapte a todos los virus y que se espere obtener la misma eficiencia y precisión en todos de ellos. Por lo tanto, un enfoque general para la cuantificación de la carga de virus no es factible y se recomienda seguir un solo método, completar una validación cuidadosa de las condiciones para cada caso particular y evaluar los resultados frente a un panel de estándares calificado.

Las conclusiones de esta tesis son las siguientes:
1- La importancia y singularidad del protocolo de RT-qPCR propuesto en este estudio para IPNV radica en que, por primera vez, se ha demostrado que un método basado en un solo par de cebadores es fiable no solo para el diagnóstico

de la mayoría de los genotipos de IPNV, sino también para la cuantificación simultánea de la carga viral, tanto *ex vivo* como *in vivo*.
Este protocolo puede ayudar a simplificar y reducir drásticamente el costo, manteniendo la fiabilidad, de los programas de vigilancia y monitorización, y de cualquier estudio que requiera diagnóstico y cuantificación de virus de tipo IPNV sin la necesidad de un conocimiento previo del tipo esperado de IPNV presente en la población.
2- Los resultados del presente estudio respaldan el uso de la citometría de flujo en combinación con la tecnología qPCR, no solo para la cuantificación de la tasa óptima de infectividad del virus, sino también para estudiar la replicación viral desde un enfoque cuantitativo
3- Nuestros resultados respaldan firmemente la propuesta del método de micro-citometría de flujo (μFC), como una alternativa fiable a los métodos tradicionales de titulación viral porque, incluso cuando se usan tiempos de incubación de 24 h, la reducción del tiempo necesario para cuantificar la concentración viral de una muestra es incuestionable.
4- Con respecto a la aplicabilidad de las metodologías, i) μFCM debería usarse para la titulación viral cuando la cuantificación rápida del virus activo es un requisito inevitable; ii) qPCR debería aplicarse para la cuantificación viral cuando se necesita el conocimiento del virus total pero el virus activo no es crucial; también es importante cuando se necesita alta sensibilidad (porque se esperan bajas cargas virales) y velocidad; iii) qFC y qPCR, combinados, se aconsejan en estudios de replicación viral, ya que ambos proporcionan datos complementarios (síntesis de proteínas y ARN) para comprender todo el ciclo viral.