Electronic Journal of Biotechnology 28 (2017) 20–26

Contents lists available at ScienceDirect

Electronic Journal of Biotechnology

Research article

Inter-laboratory ring trial to evaluate real-time reverse transcription polymerase chain reaction methods used for detection of infectious pancreatic necrosis virus in Chile

David Tapia [a,*,1], Yoanna Eissler [b,*,1], Juan Carlos Espinoza [b], Juan Kuznar [b]

[a] Doctorado en Acuicultura, Programa Cooperativo Universidad de Chile, Universidad Católica del Norte, Pontificia Universidad Católica de Valparaíso, Chile
[b] Centro de Investigación y Gestión de Recursos Naturales, Instituto de Química y Bioquímica, Facultad de Ciencias, Universidad de Valparaíso, Gran Bretaña 1111, Valparaíso, Chile

ARTICLE INFO

Article history:
Received 27 December 2016
Accepted 24 May 2017
Available online 30 May 2017

Keywords:
Aquaculture
Diagnosis
Diagnostic techniques
Fish diseases
IPNV
Pathogens
qRT-PCR
Ring test
RNA virus
Salmonids
Validation

ABSTRACT

Background: Infectious Pancreatic Necrosis Virus (IPNV) is the etiological agent of a highly contagious disease that affects salmonids. In Chile, the second worldwide salmon producer, IPNV causes great economic loss and is one of the most frequently detected pathogens. Due to its high level of persistence and the lack of information about the efficiency of its diagnostic techniques, the National Reference Laboratory (NRL) for IPNV in Chile performed the first inter-laboratory ring trial, to evaluate the sensitivity, specificity and repeatability of the qRT-PCR detection methods used in the country.
Results: Results showed 100% in sensitivity and specificity in most of the laboratories. Only three of the twelve participant laboratories presented problems in sensitivity and one in specificity. Problems in specificity (false positives) were most likely caused by cross contamination of the samples, while errors in sensitivity (false negatives) were due to detection problems of the least concentrated viral sample. Regarding repeatability, many of the laboratories presented great dispersion of the results (Ct values) for replicate samples over the three days of the trial. Moreover, large differences in the Ct values for each sample were detected among all the laboratories.
Conclusions: Overall, the ring trial showed high values of sensitivity and specificity, with some problems of repeatability and inter-laboratory variability. This last issue needs to be addressed in order to allow harmonized diagnostic of IPNV within the country. We recommend the use of the NRL methods as validated and reliable qRT-PCR protocols for the detection of IPNV.

1. Introduction

Infectious pancreatic necrosis (IPN) is a highly contagious disease caused by a non-enveloped, bi-segmented, double-stranded RNA virus (infectious pancreatic necrosis virus; IPNV) that affects salmonids reared in intensive culture systems [1,2]. IPN is considered as one of the most important diseases in salmon aquaculture worldwide because it causes high mortality rates in first-feeding fry and in post-smolts shortly after transfer to seawater [3,4]. Additionally, survivor fish from an outbreak can become lifelong asymptomatic carriers of the virus, transmitting it horizontally to other susceptible fish or vertically to their progeny; perpetuating the disease in the population [5]. In Chile, the second major producer and exporter of salmon worldwide [6], IPN is considered an endemic and prevalent disease, that affects mainly Atlantic salmon (*Salmon salar*) fry, producing great economic loss to the salmon farming industry [7,8]. To date, the isolates reported in the country have been classified within genogroups 1 and 5, that correspond to strains from Europe and North America, respectively [9,10,11]. Despite the high prevalence of the disease and the magnitude of its impact in salmon farming, there is not a specific health surveillance and control program for IPN in the country. It is only included in the general health program for fish, which establishes the screening of breeders to identify possible IPNV carriers, with the consequent elimination of their ova, in an effort to prevent vertical transmission [12]. Consequently, rapid diagnostic methods for the control of IPN are needed that ought to be specific and accurate, in order to detect any possible variant of the virus from different sources (e.g. fish in different stages of growth and from both freshwater and marine farms), as well as highly sensitive, to detect the low levels of virus present in asymptomatic carriers when screening valuable broodstock.

Traditionally, the diagnostic method recommended for IPN by the World Organization for Animal Health (OIE) in its Manual of Diagnostic Tests for Aquatic Animals was the isolation of the virus in

* Corresponding authors.
E-mail addresses: david.tapia.e@mail.pucv.cl (D. Tapia), yoanna.eissler@uv.cl (Y. Eissler).
[1] Both are first authors and contributed equally to this work.
Peer review under responsibility of Pontificia Universidad Católica de Valparaíso.

http://dx.doi.org/10.1016/j.ejbt.2017.05.008

cell culture, followed by antibody-based identification of the agent [13]. Nevertheless, the chapter on IPN was last updated in 2003 without including any molecular methodology. Moreover, because the disease is considered enzootic in most of the regions where salmonid fish are cultivated it is no longer considered in the OIE list, so it has been removed from the last editions of the manual (2009 and forward). Currently, several reverse transcriptase polymerase chain reaction (RT-PCR) based techniques have been described for the detection of IPNV [14,15,16,17], most of which use primers against the capsid protein of the virus (VP2). In Chile, it is well known that diagnostic laboratories use mostly real time RT-PCR (qRT-PCR) for routine diagnosis of IPN, because its application is simple, accurate and offers fast results. However, since there is no standardized methodology, laboratories use different in-house qRT-PCR procedures, with sets of primers and probes targeting different regions of the IPNV genome.

Inter-laboratory comparison trials, or ring trials, are studies in which the performance (sensitivity, specificity, repeatability, reproducibility) of a diagnostic method is evaluated using identical samples in several laboratories under control of a supervising laboratory. Ring trials are useful for validation of PCR based diagnostic methods, providing a way for the standardization and harmonization of assay protocols between laboratories [18]. Several ring tests have been carried out to evaluate the performance of different animal pathogen detection methods, as well as the technical competence of the participant laboratories [19,20,21,22,23,24,25,26]; however, at the moment of this study, there are no published reports of ring test for diagnostic assays of salmon viruses.

The Laboratory of Virology from the University of Valparaíso, as the National Reference Laboratory (NRL) for IPN in Chile [27], is responsible for the technical evaluation and standardization of diagnostic methods for the virus in the country. In this context, the NRL carried out the first inter-laboratory comparison trial to evaluate the performance of the in-house qRT-PCR assays used by diagnostic laboratories in Chile to detect IPNV. This paper describes the development of the ring test carried out by the NRL, from the production of the samples, to the evaluation of the laboratories that participated in the trial, assessing the sensitivity, specificity and repeatability of their methods. The aim of this study is to contribute in the standardization and validation of a reliable qRT-PCR protocol for the detection of IPNV that can be recommended for general use in Chile.

2. Material and methods

2.1. Participants

Twelve diagnostic laboratories participated in the trial; their participation was a requirement to continue performing the diagnostic of the IPN disease under the Chilean National Fisheries and Aquaculture Service (SERNAPESCA) authorization. All of the laboratories remained anonymous to avoid any conflict among the participants. The laboratories used their own in-house methods for the detection of IPNV, a summary of the information provided by each one and their identification number is shown in Table 1. The NRL is listed with the number 13 and used two qRT-PCR assays during the trial, 13SG and 13T, which correspond to methods with SYBR-Green and Taqman chemistries, respectively.

2.2. Sample panel composition and distribution

The sample panel was prepared entirely in the NRL and distributed to each participant laboratories. Sets of 8 samples in culture media were prepared. Each set consisted of 5 positive samples and 3 negative samples. The positive samples consisted in viral suspensions at different concentrations obtained from cell culture infected with two strains of IPNV. These strains belonged to genogroup 1, VR299 type strain (UV84 GenBank accession number HQ738519) and genogroup 5, Sp. type strain (ALKA3 GenBank accession number KF954912), and were isolated and sequenced in the NRL. The negative controls were culture medium, cells in culture medium and a suspension of infectious salmon anemia (ISA) virus (Table 2).

Table 1
Information of the qRT-PCR methods used by the laboratories.

Laboratory	Detection chemistry	Real time RT-PCR platform	Genomic target
1	Taqman	MX3000P, Stratagene	VP2 protein
2	Taqman	MX3000P, Stratagene	VP2 protein
3	Taqman	MX3000P, Stratagene	VP2 protein
4	Taqman	StepOne Plus, Applied Biosystems	VP1 protein
5	Taqman	StepOne Plus, Applied Biosystems	VP2 protein
6	Taqman	StepOne Plus, Applied Biosystems	VP2 protein
7	Taqman	LightCycler 480 II, Roche	VP2 protein
8	Taqman	LightCycler 480 II, Roche	VP2 protein
9	Taqman	StepOne Plus, Applied Biosystems	Not informed
10	Taqman	LightCycler 480 II, Roche	Not informed
11	Taqman	MX3000P, Stratagene	Not informed
12	Taqman	MX3000P, Stratagene	VP1 protein
13	Taqman & SYBR	StepOne Plus, Applied Biosystems	VP1 & VP2 proteins

The positive samples were obtained by amplification of the IPNV strains in Chinook salmon embryo cells (CHSE-214) derived from *Oncorhynchus tshawtyscha* embryonic tissue. Once a massive cytopathic effect (CPE) was observed, the cells were subjected to two cycles of freezing and thawing, and centrifuged at 3000 × g for 15 min at 4°C. Supernatants were collected and serially diluted with L-15 (Leibovitz) culture medium, supplemented with 10% fetal bovine serum (FBS, HyClone) and 50 $\mu g \cdot mL^{-1}$ gentamicin, to obtain the different concentrations of each viral strain.

The negative controls were prepared as follows: Atlantic salmon kidney (ASK) cells were infected with ISA virus, and once a CPE was observed, the same procedure described above was followed to harvest the viruses. In addition, CHSE-214 cells free of infection, were subjected to the same procedure. The third control was L-15 (Leibovitz) culture medium supplemented with FBS and gentamicin.

Aliquots of 0.5 mL of all negative and positive samples were distributed in centrifuge tubes and immediately stored at -20°C. Negative controls and positive samples, were produced and aliquoted in different days to avoid crossed contamination. Finally, all samples were encoded, to blind the trial, and sent refrigerated to each laboratory.

2.3. Results report

The information about the ring trial and specific instructions about reporting the results, were sent via email to all the laboratories two weeks in advance to provide enough time to answer any doubts about the trial. The sample panels were sent to each laboratory via currier and consisted in 3 sets of 8 samples (24 samples in total), to analyze one set per day and thus to measure intra-laboratory repeatability over the three days of the trial.

Laboratories were informed to report the results for each sample set every day via email in a standard form given by the NRL.

Table 2
Samples included in the ring test.

Sample ID	Type of sample	Expected result
M01	IPNV genogroup 5	Positive
M02	IPNV genogroup 5 dilution 10^{-2}	Positive
M03	L-15 Cell culture medium	Negative
M04	CHSE-214 cells in culture medium	Negative
M05	ISAV	Negative
M06	IPNV genoproup 1	Positive
M07	IPNV genoproup 1 dilution 10^{-2}	Positive
M08	IPNV genoproup 1 dilution 10^{-4}	Positive