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PCR optimization and evaluation for molecular detection of *Renibacterium salmoninarum* in rainbow trout (*Oncorhynchus mykiss*)

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Abstract: The goal of this study was to optimize the molecular polymerase chain reaction technique (PCR) to detect *R. salmoninarum*, as well to evaluate and determine the sensitive limits, specificity, and predictive values. The methodology comprised two phases: first, was the implementation and optimization of nested PCR technique to detect *R. salmoninarum*, and second, was the technique evaluation through sensitive and specificity tests. In second phase, it was needed to make cycling and temperature modifications of primers alignment to optimize and suppressed to unspecific bands. Tested primers were P3, M21, P4, and M38 against two species phylogenetically associated: *A. salmonicida* and *M. luteus*. Decimals dilutions were applied to sensitive test. The calculated specificity was 100%, with positive predictive value of 100%, and negative value of 100%. With respect sensibility, based in 12.1 ng μL^{-1} concentration, was detected (0.121 ng μL^{-1}) in first step, and 1.21×10^{-4} ng μL^{-1} at second step. While at a 26.1 ng μL^{-1} concentration, was detected 2.61×10^{-3} ng μL^{-1} in first step, and 2.61×10^{-10} ng μL^{-1}. We can conclude that nested PCR technique was selective and effective for estimated detection of 6.5 cells of *R. salmoninarum.*

Keywords: Nested PCR, *Renibacterium salmoninarum*, evaluation

Introduction

One of the obstacles in aquaculture production is the appearance of diseases, which can be infectious type produced by viruses, bacteria, parasites, and fungi or those of non-infectious nature which may be environmental or nutritional origin. The environmental infectious type can produce high mortalities, and therefore economic losses that can affect the aquaculture sector (Sosa *et al*., 2000; Jiménez, 2006).

In this way, the bacterial kidney disease (BKD), is a systemic and chronic disease caused by positive Gram bacteria *Renibacterium salmoninarum,* which was widely studied with the purpose to prevent and control of BKD development. The BKD disease was recognized like highest prevalence disease which affect salmonids free-living and cultured populations, causing high mortalities in different life stages of salmon trout (Powell *et al*., 2005; Elliot *et al*., 2013). Without doubt, the successful of BKD control was supported in the efficient and opportune causal agent detection in infected fishes and biological material of importance.

That's why, that from first BKD report published in 1934, were developed different methods for their detection, which goes to specific bacterial culture substrate to immunological and molecular methods, which were faster and specific to detect antigens and unique bacterial sequences, like direct or indirect fluorescent antibody tests, FAT or ELISA, and last point and real time PCR (Pascho *et al*., 2002).

It's appropriate to mention that "gold star" method considered by World Organization for Animal Health (OIE), because of their sensitive and specificity to identified *R. salmoninarum*, was the nested polymerase chain reaction test (PCR) (OIE, 2017).

According to Pascho *et al*. (1998), it was observed discrepancies in the results when multiple diagnostic techniques were applied to same sample. This results to non-exist standardization method to detection tests regarding to specificity, sensibility, and repeatability, which make difficult to compare results. Although comparisons were made with respect to specificity and sensitivity between non-culture methods (immunologic and molecular), these were sufficient and rigorous to validate specific method (OIE, 2009; Pascho *et al*., 2002; Powell *et al*., 2005, Chase *et al*., 2006; Elliot *et al*., 2013). Because molecular techniques available today, must be continuously evaluated in the laboratories to be

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molecular techniques available today, must be continuously evaluated in the laboratories to be applied in detection to wide variety of pathogenic agents. Likewise, it's needing to standardize and implement improvements to other already stablished techniques (Abidi, 2015).

Therefore, the goal of this study was to evaluate and optimized the nested PCR test to *R. salmoninarum* detection from kidney tissue and to determinate sensitivity and specificity limits and the diagnosis of predictive limit values.

Materials and Methods

The methodology comprises two phases. First one, was the implementation and optimization of nested PCR technique to detection *R. salmoninarum,* and second, technique evaluation trough sensitivity and specificity tests.

Biological material for PCR implementation and optimization

The positive (Rs+) kidney tissue was donated from Western Fisheries Research Center (USGS) Laboratory from Seattle, Washington, USA. Bacterial cultures were used of *Aeromonas salmonicida* and *Micrococus luteus*; donated by Live Food Chemical Analysis Laboratory of Universidad Autónoma Metropolitana-Xochimilco.

DNA extraction

It was used Blood and Tissue Kit from QUIAGEN, Inc., following the manufacturer specifications for positive Gram bacteria, as is the case of *R. salmoninarum* and *M. luteus,* and for negative Gram bacteria, as is the case of *A. salmonicida.*

Quality, concentration and purity of DNA

DNA quality was evaluated using electrophoresis in agarose gel (2%), dyed with ethidium bromide, also was quantified in a nanodrop equipment (Thermo scientific 1000), and determined the purity from each sample calculating lecture proportions to 260 nm and 280 nm. The samples were adequate to process, obtaining a proportion of A260/A280 between 1.8-2.0 range.

Nested PCR standardized

Implementation of nested PCR technique was made following the protocol of National Wild Fish Survey described by Barbash (2004), according with methodologies of Ronald J. Pascho and Chase from USGS in Seattle, Washington, Reference Laboratory of OIE, 2016. The standardization of PCR technique was made starting from DNA obtained from the samples to 26.1 ng μL^{-1}concentration, using P3, M21, P4, M38 primers, referred in OIE (2008), protocol. These primers were designed to amplifying the gen fragment that codified p57 (Jandaand Abbott, 2007; Chase and Pascho, 1998; Barbash, 2004).

The primers used in first step were: forward (P3), 75-93 (5'-AGC-TTC-GCAAGG-TGA-AGG-G-3'; and reverse (M21), 438-458 (5'-GCA-ACA-GGT-TTA-TTT-GCCGGG-3; designed to obtain a 384 bp product. The primers used in second step were: forward (P4), 95-119 (5'ATT-CTTCCA-CTT-CAA-CAG-TAC-AAG-G-3',) and reverse (M38), 394-415 (5'-GAT-TAT-CGTTAC-ACC-CGA-AAC-C-3') to obtain 320 bp product.

First step of DNA amplification of *R. salmoninarum*

The first step of PCR was made with a total volume of 25 µL. Taq PCR Core de QUIAGEN. Inc. Kit was used and 24 µL of reaction mixture, which their concentrations and conditions were: 20 pMole from each primer, 1.5 mM $MgCl_2$, Buffer PCR 1X, 0.2 mM dNTPs and 2 U Taq polymerase, plus 1 µL of DNA sample. The thermocycler (Biometra 9700) was programed with 30 cycles, with pre-heating sample to 94 °C for two minutes, followed by denaturalization to 94 °C for 30 seconds and alignment to 60 °C for 30 seconds extension at 72 °C for 1 minute.

Nested PCR. Second DNA amplification of *R. salmoninarum*

At second step, volume was maintained to reaction mixture of 25 µL: it was included 24 µL of reaction mixture: 20 pM from each primer, 1.5 mM $MgCl_2$, 1X PCR Buffer, 0.2 mM dNTPs and 2 U Taq polymerase plus 1 µL of DNA obtained from first amplification step. The conditions were: 35 cycles in thermocycler (Biometra 9700), with sample pre-heating to 94 °C, denaturalized to 94 °C for 30 seconds; alignment at 60 °C for 30 seconds; and the extension to 72 °C for 1 minute (OIE, 2008; Barbash, 2004).

How they were not reached the expected results under laboratory conditions, we proceeded to criteria application to optimized nested PCR, according to Dieffenbach and Dveksler (2003), and Eguiarte (2007), considering the parameters and conditions showed in (Tab.1).

DNA electrophoresis and display

DNA display was made from 5 µL aliquots, using

electrophoresis of agarose at 1.6% (Promega #v3121), Buffer TAE 1 × (40 mM Tris acetate, 1 mM EDTA), with programming 90 V per 1:20 hours in power source (Labnet, Inc. 300). DNA was dyed with an ethidium bromide solution (J.T. Baker) (0.5 mg mL^{-1}). PCR products size were estimated for comparison with molecular weigth markers of 100 - 3000 pb (Axygen Biosciences, 10 mM EDTA, 10% glycerol, 0.015% blue bromophenol and 2.8 M urea); the display and capture were made with a AlphaImager™ Gel Imaging System (Alpha Innotech DE 400). First PCR products size 384 bp and 320 bp in second step.

Tab. 1: Modification to nested PCR protocol, amplification second step.

Parameters	Conditions
Cycles	20, 30, 35
$MgCl_2$	0.0, 0.2, 0.3, 0.5, 1.0, 1.5, 2.1
Primer	0.5
Alignment temperature (°C)	59, 59, 59.4, 60.5, 62.4, 64.6, 67.2, 69, 69.9, 72.6, 75
Sol. Q (QUIAGEN. Inc.)	With/without

Evaluation

Evaluation was made considering two parameters: analytic specificity and sensitivity.

Specificity

In this test, primers designed by Pascho *et al*. (1998) for *R. salmoninarum* were tested against DNA from two bacteria strains phylogenetically associated: *A. salmonicida* (Elliot *et al*., 2013) and *M. luteus* (Mc Intosh *et al*., 1996), following the protocol resulting from the standardization test with five replays.

Sensitivity

Sensitivity tests were made from positive DNA known concentration of *R. salmoninarum*: 12.1 ng $\mu L^{-1}$and 26.1 ng μL^{-1}. For each concentration, were made a ten decimal dilutions serial, followed by PCR and products visualization at two steps of amplification.

Analysis

Regarding to standardization, it was used a matrix to identify the adequate conditions to optimized PCR products. For specificity and sensitivity results analysis were made 2 × 2 analysis table for qualitative type values, which response was sustained in the presence or absence of analyte, directly or indirectly detected at sample. This analysis calculated the probability of tests results with a confidence range of 95% (Trullols *et al*., 2004; Capielli *et al*., 2006; Kenneth *et al*., 2009).

Sensitivity = True positive/ all organisms with diseases S= A/(A+C)

Negative predictive value = True negative/all organisms with negative test VPN= D/(C+D),

Positive predictive value = True negative/all organisms with positive test VPP= A/(A+B), (Tab. 2).

Tab. 2: Gold test 2 x 2 table for diagnosis test.

diagnosis test results	With disease	Without disease	Total
Positive test	A (True +)	B (False +)	(A+B)
Negative test	C (False -)	D (True -)	(C+D)
Totals	(A+C)	(B+D)	(A+B+C+D)

Results

PCR standardization

Cycle concentrations and conditions from the first step were adequate, because it was achieved the 384 bp products like described by OIE (2008) and Barbash (2004) (Fig. 1).

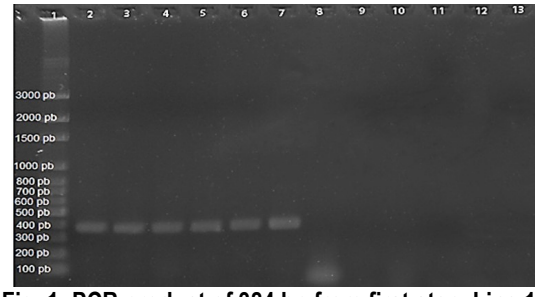

Fig. 1: PCR product of 384 bp from first step. Line 1: Molecular weight marker (MWM); Line 2-7: Positive samples of *R. salmoniarum* (*Rs*+); Line 8 and 9: kidney samples, negative control (*Rs*-); Line 10 and 11: (MTN-) of rainbow trout kidney negatives; Line 12: Negative extraction control (NEC) Line 13: Negative amplified control (NAC).

With respect to second amplified step with the conditions cited by OIE (2016), were obtained unspecific bands in addition to expected products bands of 320 bp, (Fig. 2).

The fragments sequences obtained from first and second steps (Macrogen-Korea), showed identity of 98% to *R. salmoninarum* (ATCC 33209) (ID: NC_010168.1) at first step and 99% at second step (ID: NC_010168.1) in (BLASTN 2.6.1).

From evaluation from each reagent that compose the reaction, and the cycle number and temperature of second step, was determined the use of 20 pMole from each primer, 1.5 mM $MgCl_2$, 1× PCR Buffer, 0.2 mM dNTPs, 2U Taq polymerase and primer alignment temperature (P4, M38) of 69.9°C (Fig.3) with an

increase at 35 cycles of amplification to obtain final product of 320 bp at the second step of PCR. In case of initial DNA concentrations up to 20 ng μL^{-1}, was obtained better amplification product when in second step make an increase only of one tenth of the first reaction product with the same concentrations from reaction mixture (Fig.4).

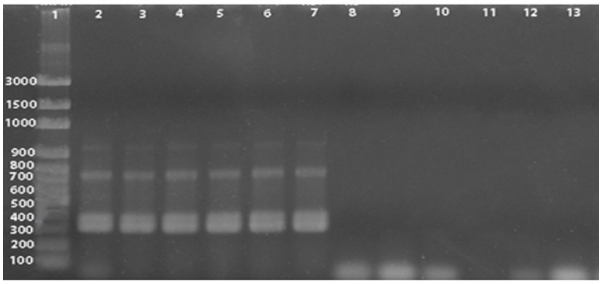

Fig. 2: PCR product of 320 bp from second step. Line 1: MWM, Line 2-7 positive samples of *R. salmoniarum* (*Rs*+); Line 8 and 9: Negative control of kidney (*Rs*-); Line 10 and 11: (MTN-) rainbow trout kidney negative samples; Line 12: Negative extraction control (NEC) Line 13: Negative amplified control (NAC).

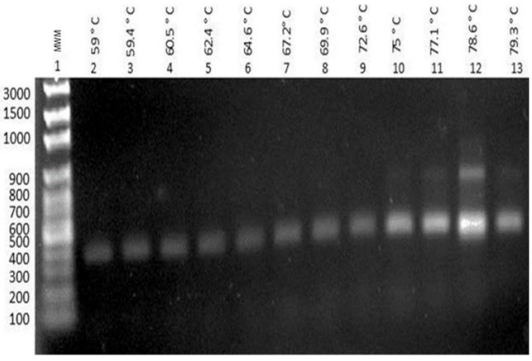

Fig. 3: Temperature gradient to amplification of second step (320 bp). Line 1: MWM, Line 2-13: without modifications at reaction mixture.

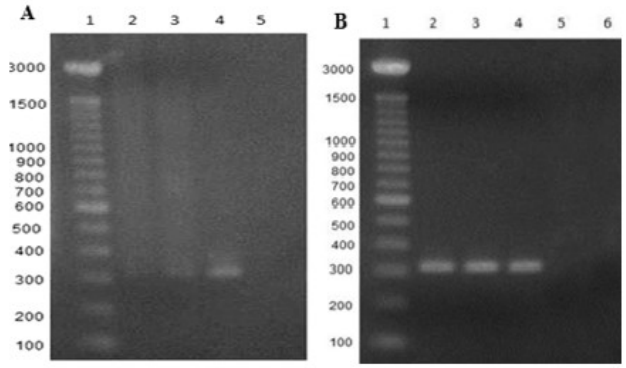

Fig. 4: Electrophoresis in agarose gel at 1.6%. A) Different volumes of first step products atsecond amplification. Line 1: Molecular weight marker (MWM); Line 2: 1 µL; Line 3: 0.5 µL; Line 4: 0.1 µL; Line 5: negative amplified control (NAC), B) Amplification from second step with standardized conditions. Line 1: MWM; Line 2-4: Rs+ second step; Line 5: Negative extraction control (NEC) and Line 6: Negative amplified control (NAC).

Validation tests

Specificity

At specificity test it was determined that samples with DNA of *M. luteus* and *A. salmonicida*, did not amplified with primers to *R. salmononinarum* designed by Pascho *et al*. (1998), (Fig. 5)., in five replays, that's why calculated specificity was 100% (Tab. 3).

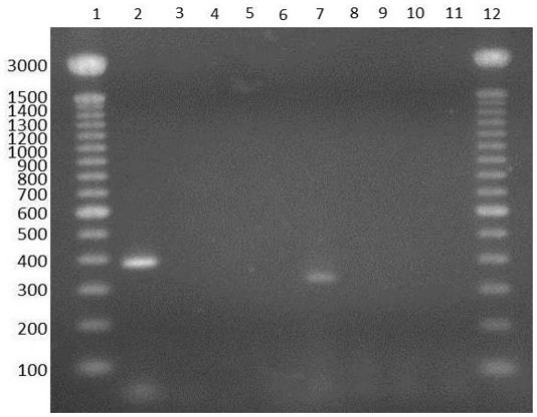

Fig. 5: Electrophoresis of agarose gel at 1.6%. Specificity test. Line 1 and 12: Molecular weight markers (MWM); Line 2: Positive control *Rs* first step; Line 3: *M. luteus* first step; Line 4: *A. salmonicida* first step; Line 5: Negative extraction control (NEC); Line 6: Negative amplified control (NAC); Line 7: Positive control *Rs* second step; Line 8: *A. salmonicida* second step; Line 9: *M. luteus* second step; Line 10: negative extraction control (NEC) and Line 11: negative amplified control (NAC).

Tab. 3: Results of the specificity test.

Tests	with disease	without disease	Totals
Positive	5	0	5
Negative	0	5	5
Totals	5	5	10

Specificity = 100%
Positive predictive value = 100%
Negative predictive value = 100%

Sensibility or detection limit

Regarding to PCR sensibility based on P3 and M21 primers from first step, the characteristic amplified product of 384 bp to *R. salmoninarum* was appreciated until second dilution (0.121 ng μL^{-1}, in the first step and 1.21×10^{-4} ng μL^{-1} at second step, based on initial concentration of 12.1 ng μL^{-1} (Fig. 6).

At 26.1 ng μL^{-1}concentration can be detected at first step a one tenth third dilution which correspond to 2.61×10^{-3} ng μL^{-1} concentration, while at second step the 320 bp can be observed a visible band until one tenth dilution that correspond to 2.61×10^{-10} ng μL^{-1} concentration (Fig. 7).

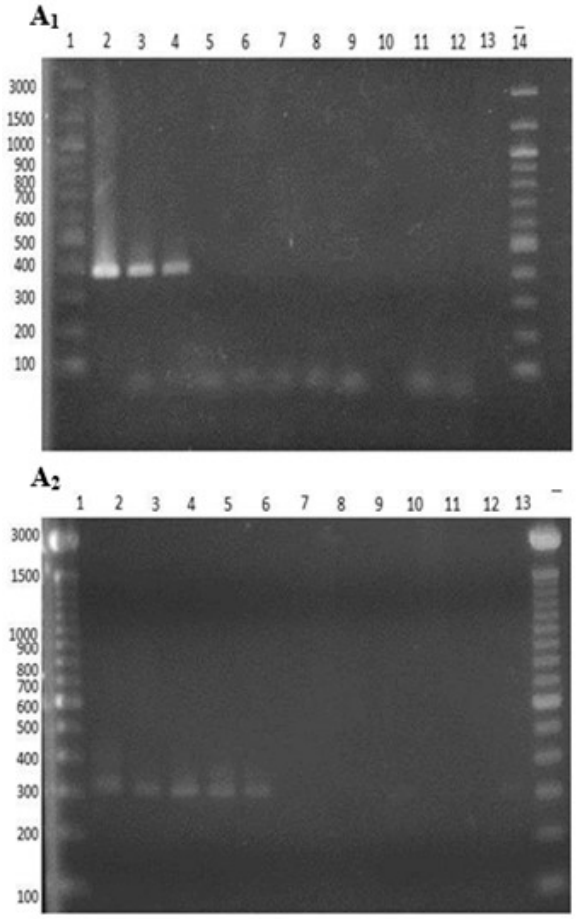

Fig. 6: Electrophoresis in agarose gel 1.6%, A_1) First step product of 384 bp and A_2) Second step of 320 bp. Line 1 and 14: Molecular Weight marker (MWM); Line 2: Positive *R. salmoninarum* (12.1 ng µL); Line 3: (0.121 x 10^{-1} ng µL^{-1}); Line 4: (1.21 x 10^{-2} ng µL^{-1}); Line 5: (1.21 x 10^{-3} ng µL^{-1}); Line 6: (1.21 x 10^{-4} ng µL^{-1}); Line 7: (1.21 × 10^{-5} ng µL^{-1}); Line 8: (1.21 × 10^{-6} ng µL^{-1}); Line 9: (1.21 × 10^{-7} ng µL^{-1}); Line 10: (1.21 × 10^{-8} ng µL^{-1}); Line 11: (1.21 × 10^{-9} ng µL^{-1}); Line 12: (1.21 × 10^{-10} ng µL^{-1}); Line 13: negative amplified control (NEC).

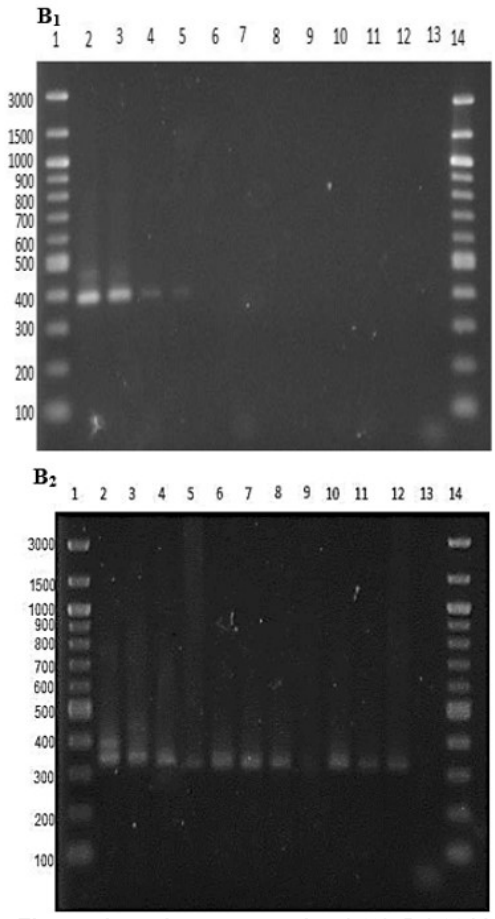

Fig. 7: Electrophoresis agarose gel at 1.6%. B_1) 384 bp first and B_2) 320 bp second step. Line 1 and 14: molecular weight marker (MWM); Line 2: Positive control *R. salmoninarum*; Line 3: 10^{-1} (0.261 ng µL^{-1}); Line 4: 10^{-2} (2.61 x 10^{-2} ng µL^{-1}); Line 5: 10^{-3} (2.61 x 10^{-3} ng µL^{-1}); Line 6: 10^{-4} (2.61 x 10^{-4} ng µL^{-1}); Line 7: 10^{-5}(2.61 × 10^{-5} ng µL^{-1}); Line 8: 10^{-6} (2.61 × 10^{-6} ng µL^{-1}); Line 9: 10^{-7} (2.61 × 10^{-7} ng µL^{-1}); Line 10: 10^{-8} (2.61 × 10^{-8} ng µL^{-1}); Line 11: 10^{-9} (2.61 × 10^{-9} ng µL^{-1}); Line 12: 10^{-10} (2.61 × 10^{-10} ng µL^{-1}); Line 13: negative amplified control (NAC).

Discussion

In recent years, several techniques were developed for *R. salmoninarum* detection. One of them is nested PCR, different to conventional technique, because of its best sensibility and specificity (Barbash 2004; OIE 2016). However, it is important to standardize the technique, according to their specific laboratory conditions, because it can exist variations, determined by equipment differences, reagents and personal. In this case, the variation shows at second step amplification. Due that, it was considered the criteria to optimize the nested PCR of Dieffenbach and Dveksler (2003), and Eguiarte (2007), which also was suggested by Innis *et al*. (1990) and Henegariu (1997). It was mentioned that reaction components, cycles and temperature alignment were no optimal, because it can show inhibition reaction or the appearance of non-expected products. Due that, parameters and conditions were modified, using different concentrations of magnesium chloride, enzymes (Taq, polymerase), primers, and cycles number and temperature primer alignment.

Don *et al.* (1991), mentioned that it can occur spurious alignment in the recognition of the two hybridization sites, only partially with respect true ones, where was generated little products, that were amplified more efficiently, until they were more predominant that desired product. With respect to validation tests of PCR technique, the evaluated specificity with primers designed by Pascho *et al*. (1998), did not show cross reaction evidence with the

phylogenetically associated bacteria samples like: *A. salmonicida*, *M. luteus* to genome of *R. salmoninarum*, in comparison with Mc Intosh *et al*. (1996) results, that obtained cross reaction with *Yersinia rukeri* genome with simple PCR with primers forward G6481 and revers G6480. Hariharan *et al*. (1995), observed the same cross reaction with *M. luteus* using immunodiagnostic method.

According with results obtained by nested PCR, which was able to detect at first step, a 2.61×10^{-3} ng μL^{-1}concentration, which was equivalent to 652.5 cells of *Rs* and for second step 2.61×10^{-10} ng μL^{-1} concentration, which was equivalent of 0.00006525 cells of *Rs*, considering the estimation made by Miriam *et al*. (1997), that exist 4 fg of DNA per cell of *R. salmoninarum*. At the same time, considering an initial amplification of 12.1 ngμL^{-1}it was detected at first step 0.121 ng μL^{-1}equivalent to 30,250 cells and 1.21×10^{-4} ng μL^{-1}at second step, equivalent to 30.25 cells.

Nested PCR increase the sensibility, because it could be observed in the results, the amplified band intensity was gradually decreasing as the dilution concentrations increase, until it was imperceptible. It can be observed that with nested PCR, the positive weak of simple reaction it was perceptible and read as positive, improvement one tenth of sensibility. This situation is very useful in detection material, because it can show as negative, a sample which had only low DNA concentrations at first step of amplification, originating negative false results. In other way, DNA concentrations near 26.1 ngμL^{-1}, increase the probability to detect pathogenic agent.

Pascho *et al*. (1998), mentioned that nested PCR it was sufficiently sensitivity, because it could detect approximately 10 cells of *R. salmoninarum* and was included in the comparison to confirm that *R. salmoninarum* cells, were present in samples with negative results with positive ELISA, and by MF-FAT. As well, being reported that nested PCR could detect *R. salmoninarum* in ovary fluid samples, that were recorded as negative by ELISA. That's why this technique was low sensitivity.

Elliot *et al*. (2013), mentioned that sensitivity ranges between log10 1.0 ± 0.4 CFU mL^{-1} (0) in ovary fluid, log10 1.9 ± 0.5 CFU mL^{-1}in kidney, using bacteriological culture mediums, regarding to nested PCR in ovary fluid, it was detected log10 3.8 ± 1.5 CFU $mL^{-1}$and log10 3.6 ± 2.2 CFU mL^{-1}in kidney, reasons why this nested PCR technique was recommended, because it increase sensitivity.

Minimum detected level must be stablished in the laboratory where PCR technique was developed, it depends from several factors, like: training and skill analyst, the good equipment's operation and calibration, DNA quality, primer quality, installation, etc. Of course, there are other inherent sample factors, like: no obtainment of target tissue and/or adequate fraction that contain the pathogenic agent, and that the sample contains inhibitors of PCR reaction (Borst *et al*., 2004; Cáceres and Vásquez, 2013).

With respect to the presence of study pathogen in Mexico, the only reported background is not clear, and it is cited in manuscript of Salgado *et al*. (2010), which made bacterial isolation in federal farmers of rainbow trout production. However, in case of *R. salmoninarum* they employed specialized culture medium KMD-2 to their possible isolation for six weeks period at 15°C without obtaining growth, because is a bacterium which show difficulties to growth. This result show that this method is impractical for their use in situations where a fast diagnosis was needed, because their incubation period was long (12 to 19 weeks), and this time is needed to see visible bacteria colonies (Benediktsdóttir *et al*., 1991; Elliot *et al*. 2013).

It is important to not forget that infectious disease diagnosis is a process and not a technique, where it is need additional tests to make a complete integration, like microbiological culture and pathologic analysis, because their hasty application and valorization of obtained results by PCR can result incorrect, that induce to conclude that the causative agent of the disease is present te same tense (Cáceres and Vásquez, 2013).

Finally, it must be highlight that in the Official Federation Diary (2012), it is reported that kidney bacterial disease is present, but without none confirmed report and the method to obtain their detection, so it is considered that is important to have the sensitive and fast methods like molecular, for this and other aquatic organism's potential diseases.

Conclusion

PCR primers P3, M21 and P4, M38 under described conditions is considered as sensitive and specific method against employed bacteria strain and can be recommended to determine the disease causal agent of kidney, produced by positive Gram bacteria *R. salmoninarum*. However, the technique by itself does not replace the disease diagnosis process.

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References

- ✓ Abidi R., Fariya N. and Chauhan U.K. (2015) Development and standardization of PCR technique to detect myxozoan parasites and its use in identification of two exotic *Myxobolus* species from Indian catfish *Clariasbatrachus* (Linn). International Journal of Fisheries and Aquatic Studies, 2: 374-377.
- ✓ Barbash P. (2004) Corroborative Testing of *Renibacterium salmoninarum* by Polymerase Chain Reaction (PCR). Chapter 7. In: NWFHS Laboratory Procedures Manual, 2d ed. USFWS - Lamar Fish Health Center. U.S.A.:1-20.
- ✓ Benediktsdóttir E., Helgason S. and Gudmudsdóttir S. (1991) Incubation time for the cultivation of *Renibacterium salmoninarum* from Atlantic salmon, *Salmo salar* L., broodfish. Journal of fish diseases, 14: 97-102.
- ✓ Borst A., Box A.T. A. and Flit. (2004) False-Positive Results and Contamination in Nucleic Acid Amplification Assays: Suggestions for Prevent and Destroy Strategy. European Journal of Clinical Microbiology and Infectious Diseases, 23: 289-299.
- ✓ Cáceres M.J. and Vásquez Y.R. (2013) Uso de la técnica de PCR en el proceso de diagnóstico de enfermedades infecciosas en organismos acuáticos, su validación y su interpretación. Ciencia Pesquera, 21: 57-65.
- ✓ Capielli G., Natale A., Nardelli S., Frangipane A. and Pietrobelli M. (2006) Validation of a commercially available cELISA test for canine neosporosis against an indirect fluorescent antibody test (IFAT). Preventive Veterinary Medicine, 73: 315-320.
- ✓ Chase D.M., Elliott D.G. and Pasho R.J. (2006) Detection and quantification of *Renibacterium salmoninarum* DNA in salmonid tissues by real-time quantitative polymerase chain reaction analysis. Journal of Veterinary Diagnostic Investigation, 18: 375-380.
- ✓ Chase D. and Pascho R. J. (1998) Development of a nested polymerase chain reaction for amplification of a sequence of the p57 gene of *Renibacterium salmoninarum* that provides a highly sensitive method for detection of the bacterium in salmonid kidney. Diseases of Aquatic Organisms, 34: 223-229.
- ✓ Hariharan H., Qian B., Despres B., Kibenge F.S., Heaney S.B. and Rainnie D.J. (1995) Development of a specific biotinylated DNA probe for the detection of *Renibacterium salmoninarum*. Canadian Journal of Veterinary Research, 59: 306-310.
- ✓ Dieffenbach W.C. and Dvekseler G.S. (2003) PCR Primer a Laboratory Manual. 2d ed. Cold Spring Harbor Laboratory Press, NY, U.S.A.
- ✓ Don R.H., Cox P.T., Wainwrigth B.J., Baker K. and Mattick J.S. (1991) "Touchdown" PCR to circumvent spurious priming during gene amplification. Nucleic Acid Research, 9: 4008.
- ✓ Eguiarte L.E., Souza V. and Aguirre X. (2007) Ecología molecular. Comisión nacional para el conocimiento y uso de la biodiversidad, CONABIO, México.
- ✓ Elliot D.G., Applegate L.J., Murray A.L., Purcell M.K. and McKibben, C.L. (2013) Bench-top validation testing of selected immunological and molecular *Renibacterium salmoninarum* diagnostic assays by comparison with quantitative bacteriological culture. Journal of Fish Diseases, 36: 779-809.
- ✓ Henegariu O., Heerema N.A., Dlouhy S.R., Vance G.H. and Vogt P.H. (1997) Multiplex PCR: critical parameters and step-by-step protocol, 23: 504-511.
- ✓ Innis M.A., Gelfand D.H., Sninsky J. and White, T.J. (1990) PCR protocols-a guide to methods and applications. Academic Press. Inc. Massachusetts, U.S.A.: 461-482.
- ✓ Janda M.J. and Abbott S.L. (2007) 16S rRNA Gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils and pitfalls. Journal of Clinical Microbiology, 2761-2764.
- ✓ Jiménez F. (2006) Introducción a la Sanidad Acuícola. En: Técnicas de Diagnóstico Presuntivo para Enfermedades de Peces. Universidad Autónoma de Nuevo León, México.: 1-20.
- ✓ Kenneth J.D., DVM and MSCE. (2009). Measures of accuracy and performance of diagnostic tests. Journal of Veterinary Cardiology, 11: 533-540.
- ✓ Mc Intosh D., Meaden O.G. and Austin B. (1996) A simplified PCR-based method for the detection of *Renibacterium salmoninarum* utilizing preparations of rainbow trout (*Oncorhynchus mykiss*, Walbum) lymphocytes. Applied and Environmental Microbiology, 62: 3929-3932.
- ✓ Miriam A., Griffiths S.G., Lovely J.E and Linch W. (1997) PCR probe-PCR Assays to monitor Broodstock Atlantic Salmon (*Salmo salar* L.), ovarian fluid and kidney tissue for presence of DNA of fish pathogen *Renibacterium salmoninarum*. Journal of Clinical Microbiology, 35:1322-1336.
- ✓ O.I.E, (2008) Validación y control de calidad de los métodos de la reacción en cadena de la polimerasa utilizados en el diagnóstico de las enfermedades infecciosas. Manual de la OIE sobre animales terrestres. Organización Mundial de la Salud Animal, Paris, Francia.:1-8.
- ✓ O.I.E, (2009) Manual de protocolos de diagnóstico para animales acuáticos. Sexta ed. Organización Mundial de la Salud Animal, Paris, Francia.1-60.
- ✓ Pascho R.J., Elliot D.G. and Chase D. M. (2002) Comparison of traditional and molecular methods for detection of *Renibacterium salmoninarum.* In: Cunninham, 2002 (eds) Molecular diagnosis of salmonid diseases, Kluwer Academic Publishers, U.S.A.: 157-209.
- ✓ Pascho R.J., Chase D. and Mc Kibben C.L. (1998) Comparison of the Membrane-Filtration Fluorescent Antibody Test, the Enzyme-Linked Immunoabsorbent Assay, and the Polymerase Chain Reaction to Detect *Renibacterium salmoninarum* in Salmonid Ovarian Fluid.

Journal of Veterinary Diagnostic Investigation,10: 60-66.

- ✓ Powell M., Overturf K., Hogge C. and Johnson K. (2005) Detection of *Renibacterium salmoninarum* in Chinook salmon, *Oncorhynchus tshawytscha* (Walbaum), using quantitative PCR. Journal of Fish Diseases, 28: 615-622.
- ✓ Salgado M., Palomares E., Jurado M., Marín A., Vega F. and Soriano V.E. (2010) Isolation and distribution of bacterial flora in farmed rainbow trout from México. Journal of Aquatic Animal Health, 22: 244-274.
- ✓ Sosa L.F., Colín M.A.R., Ingle M.G., Torres R.L.M. and Hernández M.M. (2000) Cultivo de trucha. En: Estado de salud de la acuacultura. SEMARNAP, INAPESCA y Dirección General de Investigación en Acuacultura. México (eds), D.F.: 109-146.
- ✓ Trullols E., Rui S.I. and Rius F.X. (2004) Validation of qualitative analytical methods. Trends in Analytical Chemistry, 23: 137-145.
- ✓ Wetsgard J.O. (2008). Basic Method Validation, 3rded. Blackwell Science Ltd., Oxford, UK. In: Elliot D.G., Applegate L.J.; Murray A.L., Purcell M.K. and Mc Kibben C.L. (2013) Bench-top validation testing of selected immunological and molecular *Renibacterium salmoninarum* diagnostic assays by comparison with quantitative bacteriological culture. Journal of Fish Diseases, 36: 779-809.