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PCR-Based Detection of *Yersinia ruckeri* Infection in Rainbow Trout Fish

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Abstract: The aim of this study was designing a diagnostic kit for yersiniosis in the trout fish in Iran. Colonies of *Yersinia ruckeri* were collected from culture medium and a suspension was prepared in a lysing solution. DNA was extracted through a boiling and phenol chloroform method. Two primer sets targeting bacterial 16S rRNA and trout 18S rRNA. Polymerase chain reaction products were separated by gel electrophoresis. Among 20 suspected samples tested two samples were positive for both host and bacterial PCRs indicating the positive *Y. ruckeri* infection and remaining 18 samples were negative for pathogen. The performance of PCR reactions in negative samples were confirmed from amplification of internal control reactions targeting host. A PCR based diagnostic kit with an internal control was prepared for detection of *Yersinia ruckeri* in rainbow trout fish.

Key words: Rainbow trout, *Yersinia ruckeri*, PCR diagnostic kit

INTRODUCTION

One of the most common and serious bacterial diseases in salmonid farms is yersiniosis or enteric redmouth disease (ERM). This disease was first reported from a trout farm in Hagerman, Idaho, USA in the 1950s (Bullock *et al*., 1978; Rucker, 1966; Gibello *et al*., 1999; Tobback *et al*., 2007).

Yersinia is a non-spore-forming bacterium and gram negative coccobacillus belonging to the family of Enterobacteriaceae that often has flagella, actively growing cell are approximately 0.75 μm in diameter and 1.0-3.0 μm in length (Rucker, 1966; Tobback *et al*., 2007). It causes septicemia in addition to internal and superficial hemorrhage and leads to dramatic losses in mariculture industry. Clinically, yersiniosis resembles other gram negative septicemias, especially furunculosis and is characterized by bleeding in the base of fins, inside and around the mouth, bilateral exophthalmia with or without hemorrhage, peri ocular and peri oral hemorrhage. Internally, small hemorrhages are noted in liver, pancreas, large intestine, swim bladder and lateral muscles. In diseased fish, the spleen becomes enlarged and dark and the intestine is inflamed and filled with purulent material. Hemorrhagic inflammation in hindgut, accumulation of fluid in stomach and intestines, enlargement of hematopoietic organs, kidneys and splenomegaly are among the pathologic landmarks (Rucker, 1966; Gibello *et al*., 1999; Avci and Birincioglu, 2005; Tobback *et al*., 2007).

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Definitive diagnosis of the disease reduces empiric use of antibiotics and consequent development of drug resistance and limits the prevalence of the infection (Greisen *et al*., 1994; Falsey *et al*., 2007; Maltezou *et al*., 2008). Accurate and rapid diagnosis in asymptomatic fish is critical in preventing widespread outbreaks (Wilson *et al*., 2002; Wilson and Carson, 2003).

Since, traditional diagnostic methods for *Yersinia ruckeri* depending on clinical assessment, culture and biologic or serologic studies are time consuming and pathogen-dependent (Austin *et al*., 1986; Furones *et al*., 1993; Wilson and Carson, 2003). Development of molecular assays such as PCR has improved the turn around time, accuracy and sensitivity of diagnosis (Greisen *et al*., 1994; Argenton *et al*., 1996; Gibello *et al*., 1999; Lejeune and Rurangirwa, 2000; Wilson *et al*., 2002; Wilson and Carson, 2003).

PCR could be deeply affected by human error, environmental factors, reaction inhibitors, quality and quantity of substrates. Therefore, false negative results are common and constitute the major drawback for use of PCR in diagnosis (Greisen *et al*., 1994; Bleyen *et al*., 2007).

Various PCR methods have been suggested for diagnosis of *Yersinia* by researchers including PCR RFLP, simple PCR, multiplex PCR, PCR-ELISA (Argenton *et al*., 1996; Gibello *et al*., 1999; Altinok *et al*., 2001; Lejeune and Rurangirwa, 2000; Coquet *et al*., 2002; DelCerro *et al*., 2002; Wilson *et al*., 2002; Wilson and Carson, 2003; Olsson *et al*., 2004). Because, there was no a commercial kit available for rapid detection of yersiniosis in cultured trout in Iran, we designed a PCR -based kit with internal control for diagnosis of yersiniosis in trout fish.

MATERIALS AND METHODS

This study was conducted since March 2007 to September 2008 in Tehran, Iran. *Yersinia ruckeri* was provided by Iranian Veterinary Organization and cultured in TSA medium (pancreatic digest of casein 1.5%, soy peptone 0.05%, sodium chloride 0.05%, agar 1.5%) (Atlas, 2006). Infected trout samples were collected from farms around Iran and transferred to the laboratory in 20% ethylic alcohol or in frozen form.

DNA Extraction

Bacterial DNA was extracted as described by Lejeune and Rurangirwa (2000). Briefly, bacterial colonies were collected from culture medium and a suspension was prepared in a lysing solution (320 mM sucrose, 10 mM Tris, 5 mM $MgCl_2$, 1% SDS). The reaction tube was incubated at 37°C for 2 h. Subsequently, DNA was extracted through boiling and phenol chloroform method. DNA was precipitated by ethylic alcohol and fish tissue was extracted according to instructions provided by Roche Applied Science Company (MagNA Pure LC DNA Isolation Kit II, cat number 03186229001).

Primers

We selected two pairs of primers, one pair based on 16S rRNA sequence of *Yersinia ruckeri* as described earlier by DelCerro *et al*. (2002) and one pair was designed using CLC Bio software based on sequence of trout 18S rRNA of *Oncorhynchus mykiss* (GenBank accession AF308735) as internal control of PCR reaction.

The primers: Yer F 5'- CGAGGAGGAAGGGTTAAGT-3 and Yer R 5'- AAGGCACCAAG G CAT CTC T-3' amplifies 573 nucleotides from *Yersinia ruckeri* 16S rRNA gene. Primers: Omny F 5'- CTGTGGCAATTCTAGAGC-3' and Omny R 5'- CTGCCCTCTTAATCATGG-3' amplifies 752 nucleotides from trout 18S rRNA gene sequence.

PCR Reaction

PCR reaction contained 1 μg DNA (host DNA or host and bacterial DNA), 1X PCR buffer, 20 pico mol of each primer, 1.5 mM $MgCl_2$, 0.15 mM dNTPs, 1.25 units of Taq DNA polymerase

enzyme (CinnaGen, Iran) and distilled water up to 20 μL final volume. The reaction took place in the following settings: initial denaturation for 2 min at 94°C. Thirty cycles of denaturation for 30 sec at 94°C, annealing for 30 sec at 55°C and extension for 30 sec at 72°C and finally the reaction lasted 5 min at 72°C (McPherson *et al*., 2000).

Electrophoresis

Upon completion of the reaction, 10 μL of the PCR products was electrophoresed (Bio Rad Company, USA) along with a DNA ladder marker (Fermentas, Lithuani) on 1.5% agarose gel containing ethidium bromide and viewed using 260 nm ultraviolet wave in an UV transilluminator (Boffy, 1984).

RESULTS

DNA was extracted from infected trout tissues and multiplex PCR reactions were done by specific primers of *Yersinia ruckeri* 16S rRNA and *Oncorhynchus mykiss* 18S rRNA genes. Our results demonstrated that *Yersinia ruckeri* was detected in positive samples. Figure 1 shows the 1.5% agaros gel electrophoresis of positive samples (752 and 573 bp as PCR product of *Oncorhynchus mykiss* 18S rRNA genes as *Yersinia ruckeri* 16S rRNA, respectively) and negative samples (only 752 bp of PCR product of *Oncorhynchus mykiss* 18S rRNA gene as internal control of PCR reaction is seen).

An internal control was designed in this kit as a 752 bp DNA fragment from the trout 18S rRNA gene. Detection of the host PCR reflects the quality and proportion of PCR substrates and lack of a reaction indicates an error in the system. If the host PCR product is detected, the existence or lack of a pathogen PCR product is then verified; however, if a host PCR product were not detected, the existence of the pathogen is questionable and one should search for possible errors. We tested 20 suspected samples by this kit. There were two positive and 18 negative samples. As it showed in Fig. 1, there are only host PCR product in negative samples, but there are both host and pathogen PCR product in positive samples. The host PCR product serves as an internal control for PCR reaction and increase credit of designed kit for diagnosis of bacteria in samples.

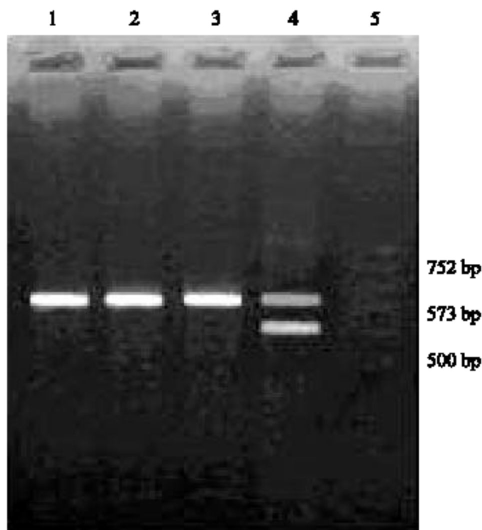

Fig. 1: Electrophoresis of PCR product on 1.5% agarose gel. Lane 1-3: PCR product of negative samples, where only a host (trout) PCR product is observed, lane 4: PCR product of positive sample with both host and pathogen PCR product and lane 5: 100 bp DNA ladder

DISCUSSION

It seemed that yersiniosis has a worldwide distribution and is considered endemic in most trout producing countries and in natural hosts in marine habitats. According to British Trout Association in 1998, annual loss related to yersiniosis assuming costs related to loss, decreased growth, reduced food conversion ratio, increased use of antibiotic and delayed harvest equals 10% of all the costs in the industry.

Regarding the importance of fish production in country's agriculture and the diversity of natural and artificial habitats for salmonid and natural hosts of *Yersinia*, definitive diagnosis of yersiniosis is of significant importance. It is currently possible to diagnose the disease by replication of the organism's DNA and use of its specific primer (Gibello *et al*., 1999; Wilson and Carson, 2003). There was not a commercial kit available for diagnosis of *Yersinia ruckeri* in trout with an internal control for PCR reaction in Iran. In this study, we provide a kit for detection of *Yersina ruckeri* in rainbow trout fish in farms.

Polymerase chain reaction results could be profoundly influenced by human error, environmental factors like different thermocyclers, varying efficacy of primers and presence of inhibitors such as polysaccharide complexes and therefore negative results are not rare in this method (Bleyen *et al*., 2007). As a result, inclusion of an internal control in each microtube and assessment of execution of the reaction seems a reasonable solution for the above mentioned limitation. In present study, we used primers designed according to gene sequences of bacterial 16S rRNA (EU401667) and trout 18S rRNA (AF308735) for diagnosis and control of the PCR reaction in a multiplex PCR format. In every reaction, the presence of product of the host PCR shows a correct reaction and lack of such products points to an error. In other words, host PCR product must be seen in all samples even the negative ones.

There are some studies to detection of *Yersinia ruckeri* by PCR method in trout fish (Gibello *et al*., 1999; Altinok *et al*., 2001; DelCerro *et al*., 2002), but there is no internal control for confirmation of PCR reaction in negative samples for any of them. There is an advantageous for our kit, because it has an internal control for confirmation of PCR reaction in negative samples.

CONCLUSION

We designed a diagnostic kit based on PCR reaction with an internal control for rapid and sensitive diagnosis of *Yeasinia ruckeri* in trout fish. It proposed for devepement in future as multiplex PCR reaction for diagnosis of other trout bacterial disease in one reaction tube.

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REFERENCES

Altinok, I., J.M. Grizzle and Z. Liu, 2001. Detection of *Yersinia ruckeri* in rainbow trout blood by use of the polymerase chain reaction. Dis. Aquat. Organ., 44: 29-34.

Argenton, F., S. De Mas, C. Malocco, L. Dalla Valle and G. Giorgetti, 1996. Use of random DNA amplification to generate specific molecular probes for hybridization tests and PCR-based diagnosis of *Yersinia ruckeri*. Dis. Aquat. Organ., 24: 121-127.

Atlas, R.M., 2006. The Handbook of Microbiological Media for the Examination of Food. 2nd Edn., CRC Press, New Yrok, ISBN: 0849335612, pp: 359.

Austin, B., I. Bishop, C. Gray, B. Watt and J. Dawes, 1986. Monoclonal antibody-based enzyme-linked immunosorbent assays for the rapid diagnosis of clinical cases of enteric redmouth and furunculosis in fish farms. J. Fish Dis., 9: 469-474.

Avci, H. and S.S. Birincioglu, 2005. Pathological findings in rainbow trout (*Oncorhynchus mykiss* Walbaum, 1792) experimentally infected with *Yersinia ruckeri*. Turk. J. Vet. Anim. Sci., 29: 1321-1328.

Bleyen, N., K. De Gussem, J. De Gussem and B. Goddeeris, 2007. Specific detection of *Histomonas meleagridis* in turkeys by a PCR assay with an internal amplification control. Vet. Parasitol., 143: 206-213.

Boffy, S.A., 1984. Agarose Gel Electrophoresis of DNA. In: Methods in Molecular Biology, Nucleic Acids, John Walker, M. (Ed.). Homana Press, USA., pp: 43-50.

Bullock, G.L., H.M. Stuckey and E.B. Shotts, 1978. Enteric red mouth bacterium: Comparison of isolates from different geographic areas. J. Fish Dis., 1: 351-356.

Coquet, L., P. Cosette, L. Quillet, F. Petit and G.A. Junter, 2002. Occurrence and phenotypic characterization of *Yersinia ruckeri* strains with biofilm-forming capacity in a rainbow trout farm. Applied Environ. Microbiol., 68: 470-475.

DelCerro, A., I. Marquez and J.A. Guijarro, 2002. Simultaneous detection of *Aeromonas salmonicida*, *Flavobacterium psychrophilum* and *Yersinia ruckeri*, three major fish pathogens, by multiplex PCR. Applied Environ. Microbiol., 68: 5177-5180.

Falsey, A., Y. Murata and E. Walsh, 2007. Impact of rapid diagnosis on management of adults hospitalized with influenza. Arch. Intern. Med., 167: 354-360.

Furones, M.D., M.L. Gilpin and C.B. Munn, 1993. Culture media for the differentiation of isolates of *Yersinia ruckeri* base on detection of a virulence factor. J. Applied Bacteriol., 74: 360-366.

Gibello, A., M.M. Blanco, M.A. Moreno, M.T. Cutuli and A. Domenech *et al*., 1999. Development of a PCR assay for detection of *Yersinia ruckeri* in tissues of inoculated and naturally infected trout. Applied Environ. Microbiol., 65: 346-350.

Greisen, K., M. Loeffelholz, A. Purohit and D. Leong, 1994. PCR primers and probes for the 16SrRNA gene of most species of pathogenic bacteria, including bacteria found in cerebrospinal fluid. J. Clin. Microbiol., 32: 335-351.

Lejeune, J. and F. Rurangirwa, 2000. Polymerase chain reaction for definitive identification of *Yersinia ruckeri*. J. Vet. Diagn. Invest., 12: 558-561.

Maltezou, H., V. Tsagris, A. Antoniadou, L. Galani and C. Douros *et al*., 2008. Evaluation of a rapid antigen detection test in the diagnosis of streptococcal pharyngitis in children and its impact on antibiotic prescription. J. Antimicrob. Chemother., 62: 1407-1412.

McPherson, M.J., S.G. Moller, R. Beynon and C. Howe, 2000. PCR: The Basics from Background to Bench. 1st Edn., BIOS Scientific Publishing Ltd., USA., pp: 9-21.

Olsson, C., S. Ahrné, B. Pettersson and G. Molin, 2004. DNA based classification of food associated Enterobacteriacea previously identified by biolog GN microplates. Syst. Applied Microbiol., 27: 219-228.

Rucker, R., 1966. Red mouth disease of rainbow trout (*Salmo gairdneri*). Bull. Int. Epizoot., 65: 825-830.

Tobback, E., A. Decostere, K. Hermans, F. Haesebrouck and K. Chiers, 2007. *Yersinia ruckeri* infections in salmonid fish. J. Fish. Dis., 30: 257-268.

Wilson, T., J. Carson and J. Bowman, 2002. Optimisation of one-tube PCR-ELISA to detect femtogram amounts of genomic DNA. J. Microbiol. Methods, 51: 163-170.

Wilson, T. and J. Carson, 2003. Development of sensitive, high-throughput one-tube RT-PCR-enzyme hybridization assays to detect selected bacterial fish pathogens. Dis. Aquat. Organ., 54: 127-134.