Entwicklung von Testsystemen auf der Basis der "Loop Mediated Isothermal Amplification (LAMP)" Methode zum Nachweis von *Yersinia ruckeri*, dem Erreger der Rotmaulseuche (ERM) und von *Renibacterium salmoninarum*, dem Erreger der bakteriellen Nierenkrankheit (BKD) der Salmoniden

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Meinen Eltern, meinem Mann und meinen Kindern;
Sarah, Rani und Yosef in Liebe und Dankbarkeit

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1 Introduction

Worldwide harvest of fishery products has steadily increased to meet the growing global demand for seafood. Salmon and trout are among the most intensively cultured fish for both direct harvest and for release into the natural environment (Rhodes et al. 2008). Fish diseases are a common problem in fish culture. Like all animals, fish are subject to a variety of diseases. They can suffer from infectious diseases caused by bacteria, viruses, or parasites. Some of the most persistent diseases observed among cultured salmon are enteric redmouth disease (ERM) and bacterial kidney disease (BKD). *Renibacterium salmoninarum* is the causative agent of bacterial kidney disease and a significant threat to healthy and sustainable production of salmonid fish worldwide. This gram-positive bacterium causes morbidity and mortality in both farmed and wild fish in nearly all regions of the world where salmonids are found (Wiens 2006). BKD is also the greatest cause of infectious disease related mortality in restoration and conservation programs for several endangered species (Flagg et al. 1995, Hoffnagle et al. 2002).The spread of BKD has followed the rapid expansion of salmonid culture, and to date, most recorded outbreaks of BKD have occurred in fish culture facilities; the losses have been as high as 80% in stocks of Pacific salmon and 40% in stocks of Atlantic salmon (*Salmo salar*) (Evenden et al. 1993). *Renibacterium salmoninarum* is difficult to culture in vitro, genetic manipulation is challenging, and current therapies and preventative strategies are only marginally effective in disease control (Wiens et al. 2008).

Enteric redmouth disease (ERM) caused by *Yersinia ruckeri* is also a serious bacterial septicaemia affecting salmonids and other fish species of commercial importance worldwide (Furones et al. 1993). This microorganism has been consistently causing economic losses in the aquaculture industry since its first description. *Y. ruckeri* was initially isolated from rainbow trout, *Oncorhynchus mykiss*, in the Hagerman valley of Idaho, USA, in the 1950s (Rucker 1966) and is now widely found in fish populations throughout North America, Australia, South Africa and Europe (Tobback et al. 2007) . Outbreaks of ERM usually begin with low mortalities which slowly escalate and may result in high losses. The problem may become large-scale if chronically infected fish are exposed to stressful conditions such as high stocking densities and poor water quality (Horne & Barnes 1999).

INTRODUCTION

Traditionally the diagnosis of the disease is carried out by culturing bacteria on agar plates followed by phenotypic and serological properties of the pathogen or histological examination (Smith et al. 1987, Austin & Austin 1993). Some attempts have been made using biochemical tests, DNA homology and protease variability but these techniques have some disadvantages such as need for previous isolation of the pathogen and insufficient sensitivity to detect low levels of pathogen (Altinok et al. 2008). Molecular techniques such as polymerase chain reaction (PCR) can be used to solve that type of problems and increase sensitivity and specificity of pathogen detection. Individual PCR assays have been developed for detection and identification of the fish pathogens (Brown et al. 1994, Chase & Pascho 1998, Gibello et al. 1999, Altinok et al. 2001, Temprano et al. 2001, Suzuki & Sakai 2007). Despite the availability of numerous diagnostic methods, there is no single rapid, sensitive, inexpensive and less laborious method for field diagnosis of those diseases. Although PCR techniques have significantly increased our ability to detect BKD and ERM infection, their requirement for a high-precision thermal cycler has prevented these powerful methods from being widely used in the field or by private clinics as a routine diagnostic tool.

Alternate isothermal nucleic acid amplification methods, which require only a simple heating device, have been developed to offer feasible platforms for rapid and sensitive detection of a target nucleic acid. These include nucleic acid-based amplification (NASBA), loop-mediated isothermal amplification (LAMP) and ramification amplification (Notomi et al. 2000, Compton 1991, Zhang et al. 2001).

Loop-mediated isothermal amplification (LAMP) is an outstanding gene amplification procedure, in which the reaction can be processed at a constant temperature by one type of enzyme, and its rapid and simple features make it clearly different from the existing genetic tests (Notomi et al. 2000). The LAMP method is able to amplify a few copies of DNA to a tremendous amount in less than an hour with no special reagents required (Tomita et al. 2008). This technique is characterized by the use of 4–6 different primers specifically designed to recognize 6-8 distinct regions on the target gene; the reaction process proceeds at a constant temperature (60–65 °C) and is completed within 60 min using the strand displacement reaction (Notomi et al. 2000, Nagamina et al. 2001; 2002). Furthermore, in a LAMP assay, all steps from amplification to detection are conducted within one reaction tube under isothermal conditions.

These advantages can be used to prevent contamination, which can occur in PCR during the transfer of samples containing amplicons from tubes to gels for electrophoretic confirmation and preclude the need for complicated temperature control, as required for PCR (Okamura et al. 2008). Therefore, the LAMP assay does not require well-equipped laboratories to be performed, and the procedure may be easily standardized among different laboratories.

The aim of this work was to improve the diagnosis of both BKD and ERM by development of a simple, rapid, specific and sensitive molecular diagnostic assay using the loop mediated isothermal amplification technique.

2 LITERATURE REVIEW

2.1. Loop-mediated Isothermal Amplification (LAMP)

Conventional disease diagnosis is based mainly on clinical signs; isolation and identification of the aetiological agent bacteriologically, virologically, histopathologically or the uses of immunological technique such as enzyme-linked immunosorbent assay (ELISA). Nucleic acid amplification techniques, of which the polymerase chain reaction (PCR) is most common, are increasingly being used to identify infectious agents through analysis of small quantities of pathogen DNA or RNA (Mullis & Faloona 1987, Notomi et al. 2000, Gill & Ghaemi 2008). Although these are accurate and sensitive techniques, they often require sophisticated instrumentation and trained personnel which makes them difficult to use directly in fish farms and hatcheries.

Novel developments in molecular diagnostic tools have demonstrated the possibility of DNA amplification under isothermal conditions, i.e. without thermal cycling. A recently developed method termed loop-mediated isothermal amplification (LAMP) can amplify DNA with high specificity, efficiency and rapidity under isothermal condition (Notomi et al. 2000). Unlike PCR, a denatured template is not required (Nagamine et al. 2001) and DNA is generated in large amounts in a short time and positive LAMP reactions can be visualized with the naked eye (Mori et al. 2001, Iwasaki et al. 2003). The main advantage of this technique is its simplicity; only a water bath or heating block is needed to provide a constant temperature as the amplification proceeds under isothermal conditions.

The LAMP method employs a DNA polymerase and a set of four specially constructed primers that recognize six distinct sequences on the target DNA. An inner primer with sequences of sense and anti-sense strands of the target initiates LAMP. A pair of ‘outer’ primers then displaces the amplified strand with the help of *Bst* DNA polymerase which has a high displacement activity, to release a single stranded DNA, which then forms a hairpin to initiate the starting loop for cyclic amplification. Amplification proceeds in cyclical order, each strand being displaced during elongation with the addition of new loops with every cycle.

The final products are stem loop DNAs with several inverted repeats of the target and cauliflower-like structures with multiple loops due to hybridization between alternately inverted repeats in the same strand (Notomi et al. 2000). The reaction can be accelerated by using two extra loop primers (Nagamine et al. 2002).

2.1.1. Principals of LAMP primers design

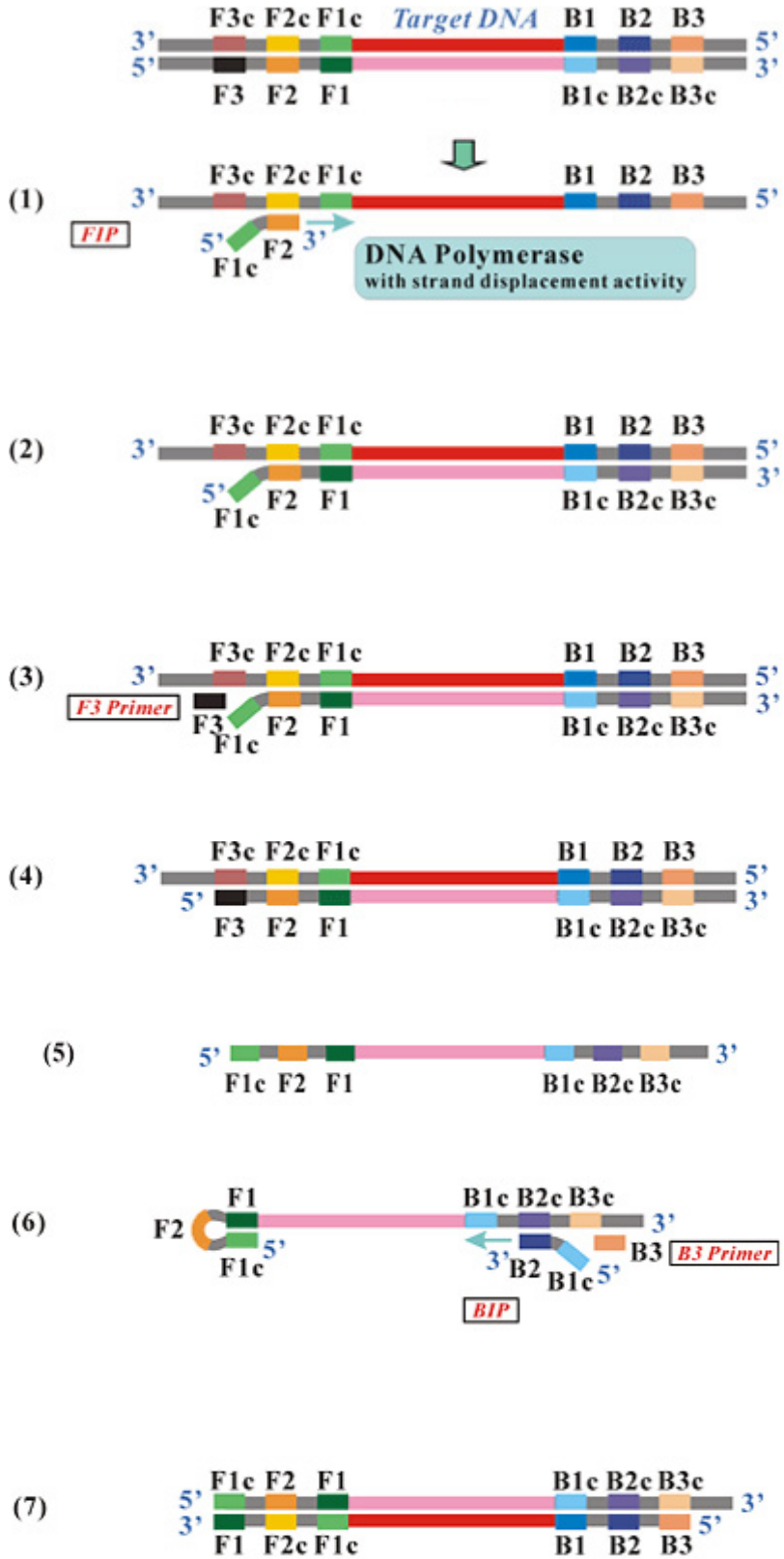
A set of two inner and two outer primers is required for LAMP. All four primers are used in the initial steps of the reaction, but in the later cycling steps only the inner primers are used for strand displacement synthesis. The outer primers are known as F3 and B3 while the inner primers are forward inner primer (FIB) and backward inner primer (BIP). Both FIP and BIP contains two distinct sequences corresponding to the sense and antisense sequences of the target DNA, one for priming in the first stage and the other for self-priming in later stages (Notomi et al. 2000). By using an additional set of two loop primers, forward loop primer (LF) and backward loop primer (LB), the LAMP reaction time can be further reduced. The size and sequence of the primers were chosen so that their melting temperature (T_m) is between 60-65 °C, the optimal temperature for *Bst* polymerase. The F1c and B1c T_m values should be a little higher than those of F2 and B2 to form the looped out structure. The T_m values of the outer primers F3 and B3 have to be lower than those of F2 and B2 to assure that the inner primers start synthesis earlier than the outer primers. Additionally, the concentrations of the inner primers are higher than the concentrations of the outer primers (Notomi et al. 2000).

Furthermore, it is critical for LAMP to form a stem-loop DNA from a dumb-bell structure. Various sizes of loop between F2c and F1c and between B2c and B1c were examined and best results are given when loops of 40 nucleotides (40nt) or longer are used (Notomi et al. 2000). The size of target DNA is an important factor that LAMP efficiency depends on, because the rate limiting step for amplification is strand displacement DNA synthesis. Various target sizes were tested and the best results were obtained with 130-200 bp DNAs.

2.1.2 Mechanism of LAMP reaction

LAMP relies on auto-cycling strand displacement DNA synthesis which is carried out at 60-65 °C for 45-60min in the presence of *Bst* DNA polymerase, dNTPs, specific primers and the target DNA template. The mechanism of the LAMP amplification reaction as illustrated in Figure 1 includes three steps: production of starting material, cycling amplification and elongation, and recycling (Notomi et al. 2000). To produce the starting material, inner primer FIB hybridizes to F2c in the target DNA and initiates complementary strand synthesis. Outer primer F3 hybridizes to F3c in the target and initiates strand displacement DNA synthesis, releasing a FIP-linked complementary strand, which forms a looped-out structure at one end. This single stranded DNA serves as template for BIP-initiated DNA synthesis and subsequent B3-primed strand displacement DNA synthesis leading to the production of a dumb-bell form DNA which is quickly converted to a stem –loop DNA. This then serves as the starting material for LAMP cycling, the second stage of the LAMP reaction.

During cycling amplification, FIP hybridizes to the loop in the stem-loop DNA and primes strand displacement DNA synthesis, generating as an intermediate one gapped stem loop DNA with an additional inverted copy of the target sequence in the stem, and a loop formed at the opposite end via the BIP sequence. Subsequent self-primed strand displacement DNA synthesis yields one complementary structure of the original stem-loop DNA and one gap repaired stem-loop DNA with a stem elongated to twice as long and a loop at the opposite end. Both of these products then serve as templates for BIP-primed strand displacement in the subsequent cycles, the elongation and recycling step. The final product is a mixture of stem-loop DNA with various stem length and cauliflower-like structures with multiple loops formed by annealing between alternately inverted repeats of the target sequence in the same strand (Notomi et al. 2000) see fig 1 (1-11).


F3c F2c F1c
Target DNA
B1 B2 B3
3'
5'
5'
3'
F3 F2 F1
B1c B2c B3c
(1)
FIP
F1c
F2
DNA Polymerase
with strand displacement activity
(2)
(3)
F3 Primer
F3
(4)
(5)
(6)
F1
F2
B1c B2c B3c
B3
B3 Primer
B2
B1c
BIP
(7)

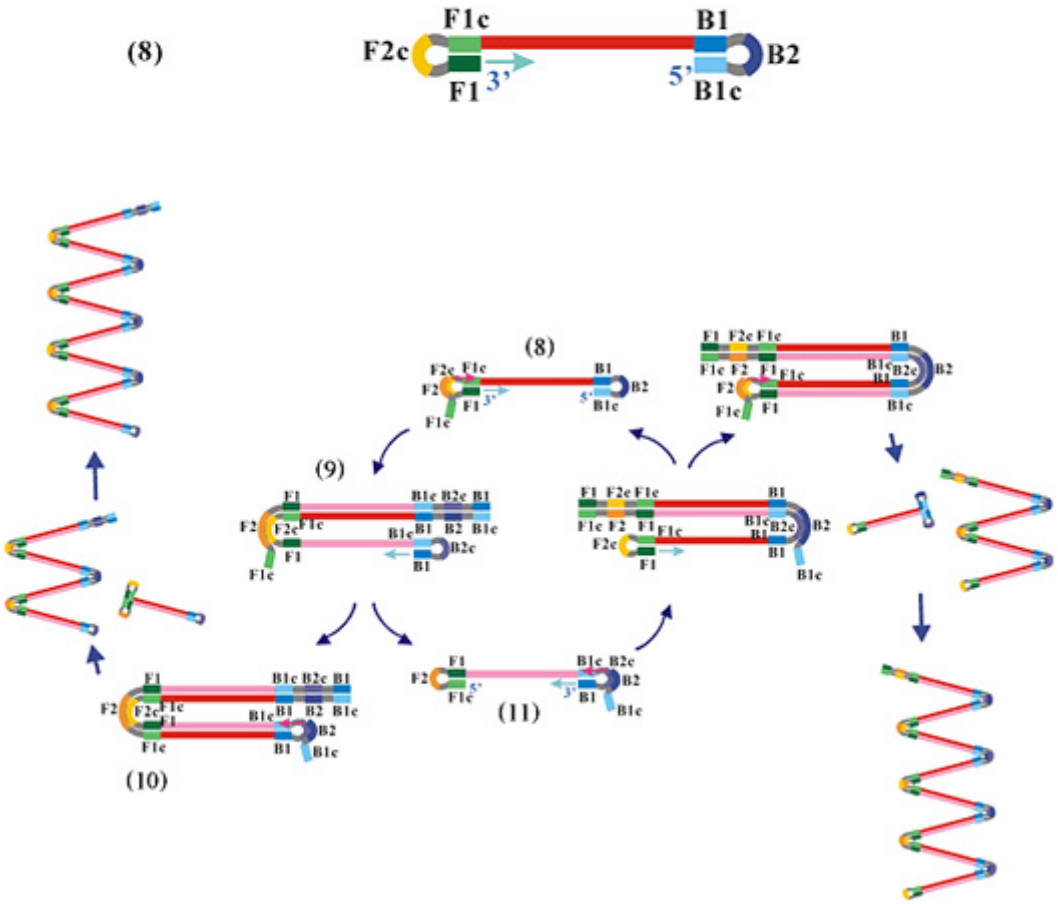


Fig.1. Mechanism of loop-mediated isothermal amplification (Eiken chemical Co. Ltd.)

2.1.3. Visualisation of LAMP amplification products

Several methods can be used to detect positive LAMP reactions. The most common is agarose gel electrophoresis, with the gel stained by an intercalating agent such as ethidium bromide. Under UV illumination, the gel shows a ladder like structure from the minimum length of target DNA up to the loading well, which are the various length stem-loop products of the LAMP reaction. Alternatively, given the large amount of LAMP product generated, products can be directly visualised in the reaction tube after incorporation of SYBR Green I stain which has high binding affinity to DNA (Karlsen et al. 1995).

Addition of a fluorescent detection reagent (FDR) to the LAMP reaction mixture before starting the amplification allows the product to be directly visualised under UV illumination and reduces contamination. Calcein in the FDR combines initially with manganese ions and remains quenched. As pyrophosphate ions are produced as a by-product of the LAMP reaction, they bind with and remove manganese from the calcein, which results in detectable fluorescence which indicates the presence of the target genes (Imai et al. 2007, Yoda et al. 2007). Alternatively, a low molecular weight PEI can be added to the LAMP product after centrifugation for 10s at 6000 rpm to form an insoluble PEI-product complex containing the hybridized fluorescently labelled probe. Reaction tubes can then be visualized with a conventional UV illuminator or by fluorescence microscopy (Mori et al. 2006).

Another method for detection of positive LAMP reactions is to monitor the increased turbidity in the reaction mixture in real-time with a turbidimeter. The turbidity is derived from precipitation of magnesium pyrophosphate generated as a by-product and this correlates with the amount of DNA amplified.

In Aquaculture, LAMP was successfully applied to detect several micro-organisms (bacteria, viruses and metazoan parasites).

2.1.4. Detection of aquatic bacterial pathogens

Several bacterial pathogens affecting fish and shellfish have been detected successfully by LAMP. The first report of LAMP use in aquaculture was for edwardsiellosis (Savan et al. 2004). The detection of *Edwardsiella tarda* was achieved through targeting its haemolysin gene, and the LAMP assay proved to be more sensitive than the PCR assay. LAMP primers that targeted the eip18 gene were tested for detection of *Edwardsiella ictaluri*. The LAMP assay amplified six different strains of *E. ictaluri* and there were no other unspecific amplifications when tested with 12 related bacterial strains and this assay showed a higher sensitivity than real-time PCR as it could detect as few as 20 CFU (Yeh et al. 2005). Nocardioses was also detected with a LAMP assay which employed a set of four primers targeting the 16S-23S ribosomal RNA internal transcribed spacer region of *Nocardia seriolae.* LAMP was found to be more sensitive than the PCR assay (Itano et al.2005).

2.1.5. Detection of viral aquatic pathogens

Several LAMP assays have been developed to detect different fish and shellfish viruses, including: koi herpes virus (KHV) (Gunimaladevi et al. 2004, Soliman & El-Matbouli 2005); white spot syndrome virus (WSSV) in kuruma shrimp (*Marsupenaeus japonicus*) where the sensitivity of the LAMP assay was 10 fold higher than that of the nested PCR (Kono et al 2004); and red see bream iridovirus (RSIV) where the authors show that the turbidity was correlating to the number of the DNA copies which allowed quantification of the virus in fish (Caipang et al. 2004). Reverse transcription RT-LAMP for detection of RNA viruses has also often reported. A rapid RT- LAMP diagnostic assay for viral hemorrhagic septicaemia (VHS) was developed and the sensitivity of the assay was found to be similar to the RT-PCR (Soliman & El-Matbouli 2006). RT-LAMP was also used for detection of infectious haematopoietic necrosis virus (IHNV) (Gunimaladevi et al. 2005). This assay targeted the G-protein of the virus and was 10-fold more sensitive than nested PCR.

2.1.6. Detection of aquatic parasitic pathogens

In fish and shellfish, El-Matbouli & Soliman (2005a) developed a LAMP assay to detect *Tetracapsuloides bryosalmonae*, the causative agent of proliferative kidney disease, in salmonid fish by amplifying the small-subunit ribosomal RNA gene. This assay was 100-fold more sensitive than a PCR assay. El-Matbouli & Soliman (2005b) used LAMP also for rapid detection of *Myxobolus cerebralis* in fish and oligochaetes targeting the 18S rDNA. Parasite DNA was detected from infected oligochaetes, and from the anal, caudal and dorsal fins and operculum of clinically infected fish, with equivalent sensitivity as PCR. A rapid LAMP assay was developed to detect *Thelohania contejeani* (Microsporidia), the aetiological agent of porcelain disease in crayfish by targeting the small-subunit ribosomal RNA gene (El-Matbouli & Soliman 2006) with 100 times more sensitivity than PCR.

2.1.7. Advantages of LAMP

The primary characteristic of LAMP is its ability to amplify nucleic acid under isothermal conditions allowing the use of simple cost effective reaction equipments (Parida et al. 2008).

Both amplification and detection of nucleic acid sequences can be completed in single step by incubating the mixture of sample, primers, *Bst* DNA polymerase at a constant temperature (Notomi et al. 2000). In addition the amplification efficiency of LAMP is very high and the reaction proceeds rapidly as there is no need for initial heat denaturation of the template DNA, and it does not require thermal cycling which makes its application as a diagnostic tool easier and more rapid in molecular medicine (Nagamine et al. 2001).

One of the most important advantages of LAMP is that large amounts of DNA are generated in a short time increasing the concentration of pyrophosphate ions. The produced turbidity observed as a white precipitate enables visual detection of positive LAMP reactions (Mori et al. 2002, Iwasaki et al. 2003) and reduces time lost in post amplification analysis. Another important advantage of the isothermal amplification techniques is their tolerance to some inhibitory materials such as a culture medium and some biological substances that can affect the efficiency of PCR (Kaneko et al. 2007). As LAMP is less affected by the various components of clinical samples than PCR, there is no need for DNA purification (Nagamine et al. 2001).

2.2. Enteric Redmouth Disease

Yersinia ruckeri (*Y. ruckeri*) is the etiological agent of yersiniosis, or enteric redmouth disease (ERM), which causes significant economic losses in salmonid farming industries in many countries. *Y. ruckeri* was first isolated from rainbow trout *Oncorhynchus mykiss* in the Hagerman valley in Idaho, USA 1950s, and was previously called Hagerman redmouth (Rucker 1966, Bullock et al. 1978). It is now widespread in fish populations throughout North America (Busch 1982), Australia (Bullock et al. 1978, Llewellyn 1980), South Africa (Bragg & Henton 1986) and many European countries (De la Cruz et al. 1986, Davies & Frerichs 1989, Furones et al. 1993). Although ERM disease has been reported in other fish species, salmonids especially rainbow trout are most susceptible to infection (Furones et al. 1993). The host range has also expanded to include Atlantic salmon, Pacific salmon, and non-salmonids such as emerald shiners, *Notropis atherinoides*, fathead minnows, *Pimephales promelas* (Michel et al. 1986), goldfish, *Carassius auratus* (McArdle & DooleyMartyn 1985), and farmed whitefish, *Coregonus* spp. (Rintamaki et al. 1986). Additionally, ERM infections have occurred in several farmed marine species such as turbot, *Scophthalmus maximus*; seabass, *Dicentrarchus labrax*; and seabream, *Sparus auratus* (Vigneulle 1984). *Y. ruckeri* has also been isolated from other animals including birds and otters (Willmusen 1989). Enteric redmouth disease is manifested by a hemorrhagic inflammation of the perioral subcutis on fish. Acute death without clinical signs is common in young fish. As with the other enteric *Yersinia* pathogens, fish tend to be asymptomatic when harbouring *Y. ruckeri* until external stresses cause clinical disease. Outbreaks of ERM disease are most frequently observed in intensive farming situations where stress factors are increased. The significant environmental factors associated with stress are poor water quality, including an increased load of organic materials often from overcrowding, and increased water temperatures, which results in lowered oxygen content. Hence, systemic infection and significant mortality can occur in fish farms and hatcheries (Rodgers 1992, Evelyn 1996).

2.2.1. Characteristics of *Yersinia ruckeri*

Yersinia ruckeri is a member of the family *Enterobacteriaceae.* They are Gram-negative rod-shaped organisms with rounded ends. The cells are approximately 0.75 µm in diameter and between 1.0 and 3.0µm in length. *Y. ruckeri* is non-spore-forming bacterium which does not posses a capsule, but often has a flagellum (Ross et al. 1966). *Y. ruckeri* strains show variable motility as they don't all possess flagella (Davis & Frerichs 1989). Like the other members of the *Enterobacteriaceae*, *Y. ruckeri* is glucose-fermentative, oxidase-negative and nitrate-reductive (Ross et al. 1966). As *Y. ruckeri* are fairly homogenous in biochemical reactions, these tests can be used to distinguish *Y. ruckeri* from other species.

2.2.2. Typing of *Yersinia ruckeri* strains

Initially, *Y. ruckeri* was considered a homogeneous species (Ewing et al. 1978), but is now known to be heterogeneous. Serotypic differences of *Y. ruckeri* were originally associated with the ability of isolates to ferment sorbitol. For several years after the original isolation of *Y. ruckeri* from salmonids in the Hagerman Valley, Idaho, all isolates were serologically similar and did not ferment sorbitol (Ross et al. 1966; Busch 1982). These were called Type I. Even today, most *Y. ruckeri* that do not ferment sorbitol form a single and distinct serotype (Pyle & Schill 1985, Pyle et al. 1987). O'Leary (1977) described another serotype of *Y. ruckeri*, isolated from Pacific salmon (*Oncorhynchus* spp.), that fermented sorbitol. This was called Type II. Later research has shown that collections of *Y. ruckeri* that ferment sorbitol can be differentiated into as many as five distinct serotypes (Pyle & Schill 1985, Pyle et al. 1987, Stevenson & Airdrie 1984). Thus, the species has been subdivided into six serovars (Stevenson & Airdrie 1984), five O-serotypes (Davies 1990) or four O-serotypes with different subgroups (Romalde et al. 1993) by using different serotyping systems. Recently, *Y. ruckeri* strains have been grouped into clonal types on the basis of biotype, serotype and outer membrane protein (OMP) profiles (Davies 1991a). Strains of serovars I and II (Stevenson & Airdrie 1984), equivalent to serotypes O1a and O2b, respectively (Romalde et al. 1993), cause most epizootic outbreaks in cultured salmonids, serovar I being predominant in rainbow trout (Stevenson 1997). Within serovar I, six clonal OMP types have been recognized, but only two are associated with major disease outbreaks: clonal group 5, which includes the so-called Hagerman-strain and clonal group 2 (Davies 1991a & b).

Clonal group 5 comprises the majority of isolates, all of them motile and with a widespread distribution (Europe, North America and South Africa). Clonal group 2 includes only non-motile strains isolated in the UK.

2.2.3. Pathogenesis

Stressed or nutrient-starved cells of *Y. ruckeri* may enter a dormant state and remain inactive and survive outside the host for a long period of time (Romalde et al. 1994). The bacterium can survive for 4 months in un-supplemented water with salinities of 0-20 ppt, but survival is less at higher salinities (Thorsen et al. 1992). The ability to survive for long periods at low salinities explains why it is difficult to control ERM in freshwater salmonids (Altinok 2004). *Y. ruckeri* has been shown to form biofilms on both solid supports and in interactions with host fish and these biofilms represent an important survival strategy in the environment (Coquet et al. 2002). The injection of extracellular products (ECPs) of *Y. ruckeri* into fish leads to the characteristic signs of yersiniosis, such as haemorrhage in mouth and intestine (Romalde & Toranzo 1993). Environmental factors such as temperature and salinity can influence speed and severity of *Y. ruckeri* infections (Altinok 2004). The production of specific proteins which contribute to the virulence of the bacterium are involved in the colonization and invasion of different tissues, is regulated by temperature as it was noticed that the expression of some proteins are repressed at temperature higher than 28°C (Fernandez et al. 2004).

2.2.4. Clinical signs and histological observations

Affected fish are typically lethargic, anorexic and found in areas of low flow. They exhibit haemorrhages in and around the oral cavity (Fig. 2a) which leads to the name ‘redmouth disease’. Haemorrhages are also common on the body surface and at the base of the fins and along the lateral line, as well as the head region. Petechial haemorrhages on the surface of the liver, pancreas, pyloric caeca, swim bladder (Fig. 2b) and in the lateral musculature may occur.

The spleen is often enlarged and may have a black colouration. The intestine is inflamed, and filled with a thick, opaque and purulent fluid. As a result of fluid accumulation, the abdomen is often distended. Exophthalmia occurs, accompanied by orbital haemorrhaging which can appear as haemorrhagic rings around the eyes (Rucker 1966, Horne & Barnes 1999). Enteric redmouth disease commonly causes sustained, low level mortality, eventually resulting in high losses. Large scale, acute epizootics sometimes occur if chronically infected fish are stressed. Severity of *Y. ruckeri* infection depends mainly on the virulence of the strain and the degree of the environmental stress (Tobback et al. 2007).

Histological examination of tissues from infected trout shows an acute bacteraemia and attendant inflammatory response in virtually all tissues. Bacteria are especially conspicuous in vascular tissue and in areas of petechial haemorrhage (Rucker 1966). Bacterial colonization occurs in the capillaries of well vascularized tissue and is followed by dilation of small blood vessels; petechial haemorrhages; erythrocyte congestion; and oedema of the kidneys, liver, spleen, heart, and gills. Focal necrosis may occur in the liver, and marked accumulations of mononuclear cells in periportal areas. Haemorrhages develop in outer portions of the digestive tract (Busch 1982).

Fig. 2: Clinical signs of Enteric Redmouth Disease. 2a) Haemorrhages in the mouth area 2b) Spleen swollen, musculature and gills pale, haemorrhages in abdominal cavity and intestine (El-Matbouli et al. 2009)

2.2.5. Diagnosis

Diagnosis of ERM is typically based on clinical signs, isolation and identification of the causative agent. Serological characterisation such as a direct or indirect fluorescent antibody test, and monoclonal based, enzyme linked immunosorbent assay, may be used for serotypes I and II (Austin et al. 1986). However, three additional serotypes have been described and if these serological tests are negative, ERM cannot be ruled out. Antisera for these additional serotypes are not generally available. Confirmatory diagnosis of ERM requires isolation and identification of the causative agent, which is a gram negative, motile, rod-shaped bacterium with distinctive biochemical characteristics. It is cytochrome oxidase negative, produces acid but usually no gas in glucose, produces an alkaline slant and acid butt in triple sugar iron agar and reacts positively with ornithine and lysine decarboxylase. The isolate should also be negative with esculin and salicin to separate it from certain isolates of *Serratia liquifaciens* that do not ferment sucrose. The isolation of *Y. ruckeri* on the classic agar media tryptic soy agar is commonly used as the bacterium grows fairly rapidly (Sousa & Silva-Souza 2001, Austin et al 2003). *Y. ruckeri* can also grow on Columbia blood agar plates (Bomo et al. 2004), and McConkey agar (Gibello et al. 1999). Several molecular techniques are also used for detection of *Y. ruckeri* such as restriction fragment length polymorphism (RFLP) (Garcia et al 1998), Polymerase chain reaction (PCR) (Gibello et al. 1999, Altinok et al. 2001, Temprano et al. 2001). PCR assays have the advantage that they can detect low levels of *Y. ruckeri* and may possibly detect carrier fish, which is very important for prevention and control of ERM transmission. However, this approach relies on precision thermo cycling, requiring instrumentation which can be prohibitively expensive and that will require decontamination when transferred from one site to another in fish farms (Dukes it al. 2006).

2.2.6. Prevention

The transfer of carriers to hatcheries previously free of ERM has been well documented as the primary way this disease has been spread. Thorough inspections of hatchery fish populations prior to the shipment of fish to other hatcheries can prevent the inadvertent introduction of ERM.

Persistent monitoring of mortalities for cause of death coupled with annual fish health inspections is invaluable in developing a reliable history of the absence of ERM disease at a facility. These measures are necessary because the detection of *Y. ruckeri* in apparently healthy carrier fish can be difficult, especially when the fish are being reared in good environmental conditions. This fact, however, demonstrates the effectiveness of good fish cultural conditions in reducing the overall impact of ERM in hatcheries where it has been troublesome in the past (Busch & Lingg 1975).

2.2.7. Vaccines

Immunization of cultured trout against ERM can also be beneficial for prevention and control of ERM disease (Busch 1978). Commercial vaccines are now available which improve the ability of fish to ward off the disease. While immunization does not provide total protection against ERM, it apparently contributes sufficiently to the well-being of the fish to be worthwhile. Care should be taken to starve the fish for 24-72 h prior to handling and prophylactic treatments should be given to rid the fish of sub-clinical cases of bacterial gill disease or external parasites. If precautionary measures are neglected, stresses associated with the immunization process can elicit outbreaks of other diseases (Busch 1978). Enteric redmouth disease is the first fish disease for which a practical, commercially available bacterin was developed. The first successful experimental bacterin, reported by Klontz (1963), was intended for oral delivery and was improved upon by later investigators (Ross & Klontz 1965, Anderson & Ross 1972). Anderson & Nelson (1974) then showed that injection of a bacterin was superior to oral administration. However, injection is not practical for immunizing large numbers of small fish. However, disease outbreaks do occur from time to time in spite of the vaccination because of carrier fish (Stevenson 1997). Novel vaccines are being based on subunit or DNA. Effective protection against yersiniosis was achieved through active immunization using the *YrpI* toxoid injected intramuscularly (Fernandez et al. 2003). Future vaccination strategies may consist of polyspecific vaccines based on a mixture of antigens or DNA-encoding antigens from different pathogens that would protect against several diseases (Fernandez et al. 2003).

There is growing interest in the use of live, attenuated vaccines against bacterial diseases because they provide higher immunity than use of dead organisms, possibly due to the induced expression of stress proteins (Temprano et al. 2005). The gene *aroA,* which is involved in biosynthesis of aromatic amino acids, is widely being studies for use with vaccines for various fish diseases. Induced dysfunction of the aroA gene through introduction of certain mutations leads to auxotrophy of the bacterium for different metabolites. For *Y. ruckeri*, a highly attenuated mutant can be constructed by insertion of a DNA fragment containing a kanamycin resistance domain into the *aroA* gene. The bacterium is hence unable to grow in fish tissues where metabolites are unavailable. It was reported that vaccination of rainbow trout using a *Y. ruckeri aroA* vaccine has been providing higher protection than the currently used vaccines. Use of live vaccines has the disadvantage that it may facilitate the spread of bacteria into the aquatic environment (Vivas et al. 2004).

2.2.8. Probiotics

Probiotics have been administrated to cultured trout to control bacterial fish diseases such as ERM. Probiotics are dietary supplements which contain potentially beneficial bacteria or yeasts. They are live microorganisms which, when administered in adequate amounts confer a health benefit on the host by production of inhibitory compounds, immune modulation and stimulation, and improving microbial balance. Improved resistance of rainbow trout against *Y. ruckeri* after oral administration of *Bacillus subtilis* and *B. licheniformis* was demonstrated by Raida et al (2003).

Carnobacterium maltaromaticum B26 and *Carnobacterium divergens* B33 were selected as being potentially useful as probiotics against *Y. ruckeri* (Kim & Austin 2006). Specifically, fish fed with B26 demonstrated significantly increased phagocytic activity of the head kidney macrophages, whereas the use of B33 led to significant increases in respiratory burst and serum lysozyme activity. Also, the gut mucosal lysozyme activity for fish fed with both cultures was statistically higher than the controls (Raida et al. 2003, Kim & Austin 2006).

2.2.9. Therapy

Antimicrobial compounds are widely used in the therapy of fish infected with bacterial pathogens. For *Y. ruckeri*, treatment involves application of sulphamethazine for 5 days followed by the administration of chloramphenicol or oxytetracycline for 3 days (Rucker 1966). The use of oxalic acid for prophylaxis and therapy of ERM in rainbow trout (Rodgers & Austin 1983), and potentiated sulphonamides has shown potential in the treatment of both natural and experimental *Y. ruckeri* infections (Bullock et al. 1983). Several antibiotics, including oxytetracycline, erythromycin, quinolones, are effective in controlling ERM (Ceschia et al. 1987). Although the antibacterial tiamulin reportedly controlled ERM (Bosse & Post 1983), it failed to control experimental *Y. ruckeri* infection in rainbow trout (Bullock & Herman 1988).

Some American isolates of *Y. ruckeri* have shown complete resistance to therapeutic levels of both oxytetracyclines and sulphonamids (Post 1987). Regardless of the effectiveness of antibacterial drugs, they alone cannot be relied upon for the control of this disease. Adverse environmental factors and excessive handling stresses must be eliminated or the disease may recur shortly after drugs are withdrawn. Acquired resistance of *Y. ruckeri* to both tetracyclines and sulphonamids has been described (de Grandis & Stevenson 1985). Other experiments have clearly shown the potential for decreased susceptibility of *Y. ruckeri* to oxolinic acid, oxytetracycline, and potentiated sulphonamide under in vitro conditions. Therefore, it is important that the emergence of antimicrobial resistance among bacterial fish pathogens is minimized by continual monitoring, careful drug use and optimisation of the treatment and the cycled use of chemotherapeutants (Rodgers 2000).

2.3. Bacterial Kidney Disease

Bacterial Kidney Disease (BKD) is a serious systemic infection of salmonids. The development of the disease is normally slowly progressive and frequently fatal. It seldom shows up in fish until they are 6-12 months old (Fryer & Sanders 1981). *Renibacterium salmoninarum*, the pathogen that causes BKD, is a current threat to salmonids and cultured broodstocks of endangered salmon species. Mortality due to BKD is commonly sporadic and occurs over an extended period of time, although subacute outbreaks with 25 to 50% mortality occurring within a few weeks have been reported. Juvenile salmonids show the greatest susceptibility to infection with the level of susceptibility being highly variable between species. Latent carriers of the disease are common, as infected fish may carry the pathogen for years without showing any clinical signs. The disease may only be manifested clinically when such carrier fish are stressed (Wood & Yasutake, 1956, Bullock & Herman 1988). The first reports of a new disease in salmon (*Salmo salar)* from the rivers Aberdeenshire Dee and Spey in Scotland (Smith 1964) and from hatchery reared brook trout (*Salvelinus fontinalis*), brown trout (*Salmo trutta*) and rainbow trout (*Oncorhynchus mykiss*) in the Western United States (Belding & Merrill 1935) in the early 1930s describe a complex disease mainly affecting the kidneys. Lesions indicative of metabolic disturbances and of reactions against an infectious agent were described. The disease was referred to as Dee disease, "white boil disease" or as Kidney disease. Comparison of the clinical presentation, pathologic lesions and characterization of the infective bacteria finally confirmed the identical aetiology of the diseases (Earp et al. 1953, Smith 1964). The disease is today known as Bacterial Kidney Disease (BKD) and the aetiological agent is *Renibacterium salmoninarum* (Sanders & Fryer 1980). BKD is one of the few bacterial diseases that can be spread both horizontally (Mitchum et al. 1979) and vertically: *R. salmoninarum* can be transmitted in eggs (Bullock et al. 1978). The disease, initially recorded in freshwater situations, is now recognised as a costly problem in ocean-farmed salmonids. Limited survival of *R. salmoninarum* in water, for up to a few weeks, indicates that the bacterium is an obligate pathogen of fish, either in clinically diseased fish or in latent carrier fish (Evendan et al. 1993). BKD has only been described in salmonid fish although the propagation of *R. salmoninarum* has been demonstrated in other fish species such as cyprinids (Sakai et al. 1989) and in sablefish *Anoplopoma fimbria* (Bell et al. 1990). The disease is reported from

North and South America, Europe (Denmark, England, Finland, France, Germany, Iceland, Norway, Scotland and Sweden) and in Japan (Kinkelin 1974, Fryer & Sanders 1981, Hoffmann et al. 1984, Kimura & Awakura 1977, Fryer & Lannan, 1993).

2.3.1. *Renibacterium salmoninarum*

The gram-positive diplobacillus, *Renibacterium salmoninarum*, has been identified as the causative pathogen of BKD in cultivated and wild salmonids. The bacterium is aerobic and fastidious in its growth requirements (Sanders & Fryer 1980). It is a small (0.5x1.0μm), non-acid-fast, periodic-acid-Schiff positive, non-sporulating, non-motile rod that grows best at 15-18°C and not >25°C. The mechanical strength of the cell wall is indicative of the presence of peptidoglycan, which is common in most Gram-positive bacteria, together with polysaccharides and teichoic acids. The rigidity of the cell wall means that it is not possible to lyse the cell with a lysozyme. The cell surface is hydrophobic and has haemagglutinating properties (Daly & Stevenson 1987). The hemagglutinin was demonstrated to be identical with the soluble heat stable p57 antigen (Daly & Stevenson 1990) which is the target antigen in several immunological diagnostic assays (Daly 1989). Based on its morphological appearance of pleomorphic short rods which frequently occur in pairs, the kidney disease bacterium was initially considered to be a species of the genus *Corynebacterium* (Ordal & Earp 1956). A taxonomic study of 25 isolates by Austin and Rodgers (1980) showed diversity among strains. One group of 6 strains was related to *Corynebacterium pyogenes,* a second group of 12 isolates represented a new taxon, and 7 strains did not fall into either group. A new genus was proposed after studies of the cell wall composition and the guanine/cytosine (G/C) content (53 %) of DNA (Sanders & Fryer 1980).

This bacterium lives both extracellularly and intracellularly in the salmonid host and has been shown to survive and even multiply within macrophages (Young & Chapman 1978, Gutenberger et al. 1997). This permits the bacterium to spread readily throughout the host body. The intracellular location and the widespread distribution of *R. salmoninarum* within the body make the infection difficult to treat because antibiotics may not reach all of the locations where the organism resides.

2.3.2. Biochemical characteristics

A strong catalase positive, a negative oxidase test and proteolytic activity has been described for *R. salmoninarum* (Ordal & Earp 1956, Goodfellow et al. 1985). The API-ZYM test is suitable for identification, as this is an enzymatic test which does not require propagation of the bacterium (Goodfellow et al. 1985, Austin & Austin 1987). The organism is β- hemolytic on media supplemented with blood (Bruno & Munro 1986) and it can liquefy gelatin, degrade Tween, and hydrolyze casein. The bacterium is negative for esculin hydrolysis, DNase, urease, nitrate reduction, phosphatase, methyl red, indole test and carbohydrate utilization test.

2.3.3. Cultivation

Several different agar-media were tested for sub-cultivation. Growth occurred on Dorset's medium which is used to culture *Mycobacterium tuberculosis.* Modifications of Dorset's medium, mainly by the addition of L-cysteine, greatly increased growth of the bacterium. Although several modifications of different agar media for *R. salmoninarum* have been developed, such as L-cysteine supplemented Mueller Hinton medium (Wolf & Dunbar, 1959), Kidney Disease Medium (KDM, Evelyn 1977) and Charcoal agar (KDM-C, Daly & Stevenson 1985), the bacterium grows slowly and secondary bacterial infections are a serious problem in diagnostics. Selective kidney disease medium (SKDM) containing cycloheximide, cycloserine, polymyxin B sulphate and oxolinic acid has been found useful to reduce contamination by other bacteria and fungi (Austin et al. 1983). Incubation times for isolation of *R. salmoninarum* on SKDM were studied and the initial growth of the organism could be detected as long as 19 weeks post-inoculation of the kidney sample (Benediktsdóttir et al. 1991). Fish tissues have been shown to have an inhibitory effect on the growth of *R. salmoninarum* (Evelyn et al. 1981). Growth of *R. salmoninarum* is also possible in serum-free broth, consisting of 1% peptone, 1% yeast extract and 0.1% L-cysteine, suitable for growing large batches of bacteria (Daly & Stevenson 1993). The cultivation of *R. salmoninarum* in cell cultures, using fish cell lines EPC, CHSE and RTG-2 have been successfully demonstrated by several studies (Mcintosh et al.1997, González et al. 1999).

2.3.4. Occurrence

Although BKD occurs mainly in freshwater, significant mortality also occurs in saltwater (Banner et al. 1983). A consequence of infection in juvenile anadromous salmonids is that they are unable to acclimatise to seawater and die. The mortality of infected smolts of Coho salmon was 17.2% in freshwater and 4% in saltwater (Fryer & Sanders 1981). Even if salmon are lightly infected when they enter saltwater, the disease continues to progress and deaths occur (Banner et al. 1986).

2.3.5. Susceptibility

In hatcheries rearing Pacific salmon, bacterial kidney disease is detected most frequently in spring Chinook, coho and sockeye (Sanders & Fryer 1980). A cross between pink and chum salmon (a "chumpy") was found to be extremely susceptible (Wood 1974). According to Winter et al. (1980), some strains of steelhead trout are resistant to BKD. Fewer problems were reported with BKD in the culture of salmon than in trout. Among trout and char, the brook trout is probably the most susceptible species. Rainbow and brown trout would rank next in susceptibility while steelhead trout are the most resistant (Mitchum & Sherman 1981). The Japanese scallop (*Patinopecten yessoensis)* has been suggested as a possible vector for *R. salmoninarum* (Sakai & Kobayashi 1992). The role of salmon lice (*Lepeophteirus salmonis*) - like other bloodsucking ectoparasites - as vectors for *R. salmoninarum* has been investigated. The lice can occasionally harbour the pathogen (Frerichs & Roberts 1989) but until now there is no evidence for active BKD transmission caused by salmon lice recorded in the literature. Although the isolation of *R. salmoninarum* from the kidneys of the sea lamprey *Petromyzon marinus* has been reported, the role played by sea lamprey in the epidemiology of BKD requiers further investigation (Eissa et al. 2006).

2.3.6. Pathogenesis

Fish to fish (horizontal) transmission of BKD has been reported both in hatcheries and in the wild. Hatchery-reared Atlantic salmon fingerlings contracted BKD from naturally infected wild fish in the hatchery water supply (Frantsi et al. 1975).

Mitchum et al. (1979) showed that wild trout could transmit the disease to stocked hatchery trout that had been previously free of the disease. Feeding of *R. salmoninarum* infected flesh and viscera to juvenile Chinook salmon resulted in transmission of BKD (Fryer & Sanders 1981), indicating uptake through the intestinal wall. Subsequently, pasteurisation of fish feed has been used to halt this route of transmission.

The main reservoirs of infection are subclinically infected or latent carrier salmonids (Fryer & Sanders 1981, Bullock & Herman 1988). The pathogen has been reported to survive in faeces and pond sediments for up to 21 days (Bullock & Herman 1988).

Bacterial kidney disease is easily transmitted from parent to progeny in the eggs. The bacterium is so intimately related to the egg or to the developing embryo that egg disinfection is ineffective (Bullock et al. 1978).

According to Evelyn (1993), infection of eggs usually originates from the *Renibacterium*-infected coelomic fluid of the female brood fish. It has been shown, however, that intra-ovum and progeny infections may also occur prior to ovulation, directly from ovarian tissue (Evelyn 1993). This seriously complicates containment of BKD and explains why egg and fish exchanges have played a significant role in the spread of the disease.

Pathogenesis and mortalities in connection with BKD can be affected by environmental conditions such as temperature (Sanders et al. 1978), salinity (Fryer & Sanders 1981) and stress (Mesa et al. 1998). It was found that BKD is most severe in hatcheries whose supply water was relatively soft (Warren 1963). Many interrelated factors, including mineral metabolism, must be considered. It was shown at one hatchery, that the lowest incidence of BKD occurred in yearling Atlantic salmon fed a diet with increased levels of trace minerals (Fe, Cu, Mn, Co, I, and F) and low levels of calcium (Paterson et al. 1981).

2.3.7. Clinical signs

Infected fish may appear normal. In rainbow and brown trout, there may be a “buckshot” appearance due to the presence of numerous small, open ulcers in the skin that expose the underlying musculature (Fryer & Sanders 1981, Bullock & Hermann 1988, Evendan et al. 1993). As indicated by its name, BKD severely affects the kidneys, and, to a lesser extent, the spleen and liver.

The kidneys are usually swollen, convex, and have a corrugated or lumpy surface (Fig. 3a) in sharp contrast to the smooth, concave surface of healthy kidneys. Creamy, soft, off-white cysts represent massive colonies of the causative organism. Such cysts are common in the posterior kidney and may vary in size and number. They should not be confused with normal stanneous bodies located in the mid-kidney or with nephrocalcinosis (kidney stones) that may fill excretory tubules of the kidney. A bloody, turbid, or yellow-brown fluid often accumulates in the abdominal cavity and around the heart. Other internal organs and visceral fat may appear normal or appear unusually white. The intestinal tract may contain a white or yellow viscous fluid (Bullock and Herman 1988). The presence of a fibrous capsule is variable and lack of encapsulation is often associated with more aggressive infections or more susceptible species.

These latter lesions can be well encapsulated and may even be resolved in species such as Atlantic salmon (*Salmo salar*), which appear to be fairly resistant to BKD. In the more susceptible Pacific salmon (*Oncorhynchus*), however, the granulomas are rarely encapsulated. Other common signs of BKD include pale gills due to anaemia, abdominal distension due to the accumulation of ascitic fluid, and exophthalmia due to osmo-regulatory disruption (Bullock and Herman 1988) as well as losses of one or both eyes (Fig. 3b) (Hoffmann 2005).

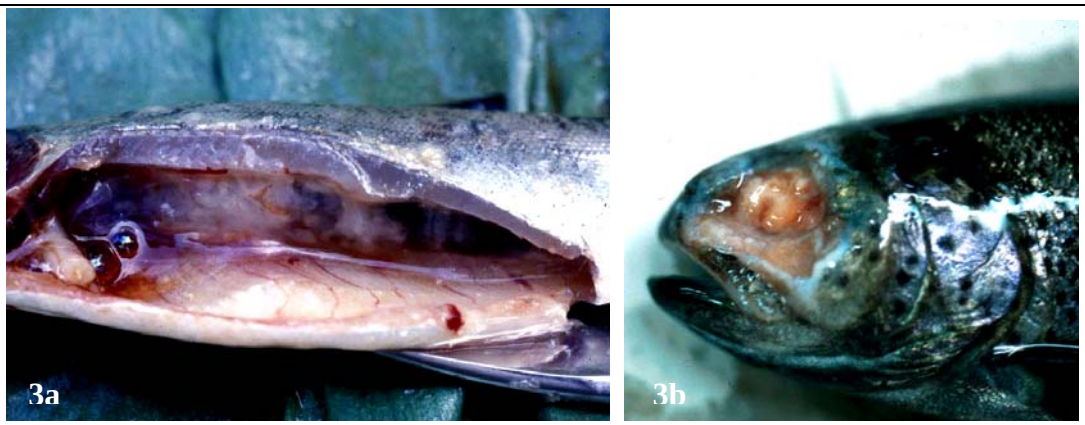


Fig. 3: Clinical signs of Bacterial Kidney Disease. 3a) BKD infected rainbow trout with swollen and convex kidney with corrugated surface and Granulomes 3b) Eye loses in BKD infected Rainbow trout (Hoffmann 2005)

2.3.8. Diagnosis

Isolation and bacteriological identification of the slowly replicating and fastidious *R. salmoninarum* can require 6 to 19 weeks (Benediktsóttir et al. 1991) and is usually impractical for routine diagnosis. Several immunological techniques have been developed; such as enzyme-linked immunosorbent assay (ELISA), dot blot and Western blot for detection of *R. salmoninarum* antigens in internal organs (Pascho & Mulcahy 1987, Sakai et al. 1987, Griffiths et al. 1991, Hsu & Bowser 1991, Rockey et al. 1991, Gudmundsdóttir et al. 1993, Olea et al. 1993, Jansson et al. 1996). It is common to use antigen-antibody affinity to detect and visualise the presence of the antigens. The strength of the antibody-epitope interaction and the stability of the antibody-antigen complex are crucial for the efficiency of the method. The sensitivity and specificity depend on the quality of the antibodies employed. Monoclonal antibodies, produced by a single B-cell line, used to be superior in specificity since only a single epitope is identified and all antibodies are identical. The polyclonal antibodies, obtained from several B-cell lines, react with various epitopes on the antigen and consequently have several targets for binding, resulting in a higher potential for recognition.

ELISA is mostly used for assessing the prevalence and the severity of *R. salmoninarum* in wild salmonids (Magnússon et al. 1994, Maule et al. 1996, Elliott et al. 1997). Jansson and Ljungberg (1998) screened naturally infected populations and experimentally challenged rainbow trout and Atlantic salmon for *R. salmoninarum* using both ELISA and SKDM culture. Four weeks after experimental infection, both methods detected all fish as positive. However, 12 weeks after challenge more experimentally infected fish were considered positive by ELISA than by culture. In contrast, culture appeared to be more sensitive than ELISA for screening naturally infected fish. Additionally, detection of *R. salmoninarum* in kidney samples by ELISA and isolation on selective medium was compared and described (Gudmundsdóttir et al. 1993). The study comprised 1239 kidney samples and showed that the ELISA test gave significantly higher numbers of positive samples. Unfortunately ELISA does not work very well on ovarian fluid (Pascho et al. 1998).

Successful detection by an immunohistochemical method depends on availability of antiserum/monoclonal antibodies (mAbs) required giving a reaction of high sensitivity and specificity (Heines & Chelack 1991). Polyclonal antisera can lead to cross-reactivity as observed with other bacteria (Rockey et al. 1991). Cross reactions have been reported with

Carnobacterium piscicola, *Corynebacterium aquaticum* (Dixon 1985, Bandin et al. 1993, Toranzo et al. 1993, Leon et al. 1994, Wood et al. 1995), *Brevibacterium linens* (Magnússon et al. 1994), *Lactobacillus* sp., (Teska et al 1995) *Pseudomonas* spp. (Brown et al. 1995, Yoshimizu et al. 1987), *Rothia dentocariosa*, *Bacillus sphaericus* (Dixon 1987) and *Arthrobacter globiformis* (Jansson et al. 1996). The most promising results were obtained using mAbs 4D3 and 2G5 – both specific for the 57-58-kD outer membrane protein p57 which is unique for *R. salmoninarum* (Wiens & Kaattari 1989, 1991).
Antigens, however, can be masked depending on the pH and temperature of the fixation solutions (Takamyia et al. 1978). Microwave (MW) treatment has successfully been used to unmask antigenic determinants concealed during fixation. Immunohistochemical detection of *R. salmoninarum* can be considered as a highly specific method to identify this bacterium, with the advantages that it is rapid and can be applied to stored tissue samples, and is therefore independent of sampling time.

Molecular techniques, such as the dot-blot assay for detection of ribosomal RNA from *R. salmoninarum* (Mattsson et al. 1993) and PCR-assays for amplification and identification of unique sequences of bacterial nucleic acids have been developed (Brown et al. 1994, Magnússon et al. 1994, Miriam et al. 1997, Chase & Pascho 1998, Pascho et al. 1998). Brown et al. (1994) describe a PCR method for detection of *R. salmoninarum* in Coho (*Oncorhynchus kisutch*) and Chinook (*O. tshawytscha*) salmon eggs. The 501 base pair amplicon belongs to the gene coding for the antigenic p57 protein. The reaction can be performed from only 100 microliters of homogenized tissue and allows detection of as few as 2 bacterial cells per egg with a high degree of specificity. Magnússon et al. (1994) used a PCR method to detect *R. salmoninarum* in ovarian fluid samples and compared it with culture and ELISA methods from kidney. A nested PCR has been developed by Chase and Pascho (1998) to detect a 320 bp DNA segment of the gene encoding the p57 protein. The sensitivity of the method was increased a hundredfold compared to a conventional PCR method. The results of a comparison of methods to detect *R. salmoninarum* in ovarian fluid (Pascho et al. 1998), and those of Brown et al. (1994) suggested that the prevalence of *R. salmoninarum* among eggs of mating pairs from populations in which BKD is enzootic may be far greater than anticipated. Miriam et al. (1997) had cautioned, however, that PCR-positive samples may contain some proportion of dead *R. salmoninarum* with detectable levels of DNA.

Miriam et al. (1997) had also compared PCR, nested PCR and culture (SKDM) for detection of *R. salmoninarum* in ovarian fluid and kidney tissues in both Atlantic salmon broodstock and experimentally challenged salmon coming to the conclusion that PCR assays are valuable as complementary techniques for conformation of BKD, especially as only small amounts of sample are necessary for analysis and good sensitivity has been demonstrated.

2.3.9. Control and Prevention

Once established, BKD is an extremely difficult disease to manage hence, prevention remains the first and strongest line of defence (Fryer & Lannan 1993). Firm policies, careful planning, a good understanding of the aetiology of BKD, and a thorough monitoring program are essential to a successful program of prevention and control. If these efforts fail, a number of other steps can be taken to contain the spread and minimize the overall effect of BKD. A regular screening programme to prevent horizontal, as well as vertical transmission of BKD has been the most important measure (Elliott et al. 1989, Fryer & Lannan 1993). McCarthy et al. (1984) reported an attempt to vaccinate fish susceptible to BKD using two preparations of formalin-inactivated cells of *R. salmoninarum*. The bacterins were administered without adjuvant by Intra peritoneal injection, immersion, or two-step hyperosmotic infiltration. No significant protection was afforded by these methods. Furthermore, Kaattari & Holland (1990) treated salmonids with a number of potential immunogens in an attempt to confer immunity to fish susceptible to BKD infection. These immunogens included cell-wall fractions, fractured cells and extracellular products, and were administered by intraperitoneal injection, orally, and by immersion with and without adjuvant. None of these early preparations protected fish and some exacerbated the disease. Research on new vaccines is being carried out among several research groups worldwide. Recent investigations, however, have demonstrated that oral administration of *R. salmoninarum* expressing low levels of cell associated p57, resulted in an extension of the mean time to death after challenge (Piganelli et al. 1999). This indicates that the cell-mediated immune response is involved in recovery, due to the intracellular survival and the composition of inflammatory cells in connection with signs of regression (Munro & Bruno 1988). An alternate approach to vaccine development has been made through use of killed *R. salmoninarum* cells that are devoid of intact cell-surface-associated protein p57 (Christensen et al. 1999).

The vaccine may be enteric-coated for oral delivery, to protect the vaccine from proteases and from the relatively low pH levels of the stomach. This allows the vaccine to reach the hindgut and associated lymphoid tissue, which maximizes the effectiveness of the vaccine for protecting fish. This vaccine can be used in combination with immunostimulants, such as β-glucans. The immunostimulant may be incorporated into the formulation of the oral vaccine so that the immunostimulant is released following the administration. This is believed to prime the immune system. Alternatively, the BKD vaccine may be separately from the immunostimulant, for example the BKD vaccine might be orally administered and the immunostimulant administered by IP injection or by immersion, either prior to, simultaneously with, or after the administration of the vaccine. Rhodes et al. (2004) presented DNA adjuvants and whole bacterial cell vaccines against *R. salmoninarum* that were tested in Chinook salmon fingerlings. These authors concluded that whole cell vaccines of either a non-pathogenic *Arthrobacter* spp. or an attenuated *R. salmoninarum* strain produced limited protection against acute *i. p.* challenge with virulent *R. salmoninarum*. They also concluded that addition of either synthetic oligodeoxynucleotides or purified *R. salmoninarum* genomic DNA as adjuvant did not increase protection; however a combination of both whole cell vaccines significantly increased survival among fish naturally infected with *R. salmoninarum*. The surviving fish treated with this combination vaccine showed reduced levels of bacterial antigens in the kidney (Rhodes et al. 2004).

Broodstock segregation is a practical method for reducing the prevalence and levels of *R. salmoninarum* in hatchery- reared salmon (Pascho et al. 1991) and for increasing survival during their downriver migration and entry into seawater (Pascho et al. 1993, Elliot et al. 1995). This procedure aims to interrupt vertical transmission of *R. salmoninarum* by isolating or destroying eggs from brood fish that exhibit clinical signs of BKD or test positive against R. *salmoninarum* antigens.

Due to the complicated nature of BKD and its obvious threat to fisheries, Hoskins et al. (1976) recommended complete destruction of infected stocks and disinfection of the holding facilities to eradicate the disease. Eradication can be of value in single fish farms or hatcheries that receive their water supply from a specific pathogen free source. However, this procedure is considered by fisheries managers as impractical due to the widespread occurrence of *R. salmoninarum* (Sanders & Fryer 1980).

2.3.10. Treatment

Control of BKD with conventional methods such as chemotherapeutics remains problematic due to the intracellular nature of *R. salmoninarum* infection, and currently there is no practical treatment for the disease. Austin (1985) tested more than 70 antimicrobial compounds both *in vivo* and *in vitro*. He found that the antibiotics clindamycin, erythromycin, kitasamycin, penicillin G and spiramycin were useful for combating early clinical cases of BKD and that cephradine; lincomycin and rifampicin were effective prophylactically but were of limited use therapeutically.

There have been reports that injection of erythromycin phosphate into brood stock females prior to spawning significantly reduces the vertical transmission of BKD (Evelyn et al 1986 Sakai et al. 1986, Lee & Gordon 1987, Armstrong et al. 1989, Lee & Evelyn 1994). This might, however, increase the risk of selection for erythromycin-resistant bacteria (Evelyn et al. 1986). Brood stock injection does not eliminate *R. salmoninarum* infection in tissues and eggs but combined with good husbandry techniques it is possible to significantly reduce the incidence of BKD in hatcheries by this means (Lee & Evelyn 1994). Brood stock culling and the destruction of gametes from BKD positive parents have been demonstrated to reduce the prevalence of the disease (Gudmundsdottir et al. 2000).

The risk of BKD introduction can be lowered by paying special attention to prevent introduction of infected fish or gametes (Evelyn et al. 1984, Yoshimizu 1996). This can only be achieved through prior examination and quarantine. The main method to reduce the risk of spreading BKD is to introduce live fish and eggs only from sites which carry out well-regulated health screening programmes to confirm the absence of *R .salmoninarum*. Restricting imports to eggs will further reduce risks. Health screening programmes must be carried out over a prolonged period of time (2 years minimum) by the Competent Authorities using recognised techniques such as ELISA, PCR and standard bacteriological methods (OIE Diagnostic Manual 2000).

The intent of this study considering the impact of *Y. ruckeri* and *R. salmoninarum* on fish health was to develop and evaluate two easy to perform, cost effective, sensitive and rapid diagnostic assays applicable in the praxis and on the field. The LAMP protocols presented here meet these criteria and are proved to be a novel development in molecular diagnostics.

3 PUBLICATIONS

3.1. Publication 1

Saleh M, Soliman H, El-Matbouli M (2008): Loop-mediated isothermal amplification as an emerging technology for detection of *Yersinia ruckeri* the causative agent of enteric redmouth disease in fish. **BMC Veterinary Research 4: 31**

BMC Veterinary Research

Methodology article

Loop-mediated isothermal amplification as an emerging technology for detection of *Yersinia ruckeri* the causative agent of enteric red mouth disease in fish

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Abstract

Background: Enteric Redmouth (ERM) disease also known as Yersiniosis is a contagious disease affecting salmonids, mainly rainbow trout. The causative agent is the gram-negative bacterium *Yersinia ruckeri*. The disease can be diagnosed by isolation and identification of the causative agent, or detection of the *Pathogen* using fluorescent antibody tests, ELISA and PCR assays. These diagnostic methods are laborious, time consuming and need well trained personnel.

Results: A loop-mediated isothermal amplification (LAMP) assay was developed and evaluated for detection of *Y. ruckeri* the etiological agent of enteric red mouth (ERM) disease in salmonids. The assay was optimised to amplify the *yruI*/*yruR* gene, which encodes *Y. ruckeri* quorum sensing system, in the presence of a specific primer set and *Bst* DNA polymerase at an isothermal temperature of 63°C for one hour. Amplification products were detected by visual inspection, agarose gel electrophoresis and by real-time monitoring of turbidity resulted by formation of LAMP amplicons. Digestion with *Hph*I restriction enzyme demonstrated that the amplified product was unique. The specificity of the assay was verified by the absence of amplification products when tested against related bacteria. The assay had 10-fold higher sensitivity compared with conventional PCR and successfully detected *Y. ruckeri* not only in pure bacterial culture but also in tissue homogenates of infected fish.

Conclusion: The ERM-LAMP assay represents a practical alternative to the microbiological approach for rapid, sensitive and specific detection of *Y. ruckeri* in fish farms. The assay is carried out in one hour and needs only a heating block or water bath as laboratory furniture. The advantages of the ERM-LAMP assay make it a promising tool for molecular detection of enteric red mouth disease in fish farms.

Background

Yersiniosis or enteric red mouth disease (ERM) is a serious systemic bacterial infection of fishes which causes significant economic losses in salmonid aquaculture worldwide [1]. Although infection with this agent has been reported in other fish species, salmonids especially rainbow trout

Oncorhrynchus mykiss, are highly susceptible to ERM [2,3]. The disease was first described in the rainbow trout in the United State in 1958, from Hagerman Valley, Idaho by Rucker [4], and later the causative organism named *Yersinia ruckeri* [5]. The disease is endemic in North America [3] and widespread elsewhere. It was also described in 1981 in France, Germany and United Kingdom and has now been reported in most of Europe, Australia [6,7] and South Africa [8].

The causative agent, *Yersinia ruckeri*, is a gram-negative, non-spore-forming rod-shaped bacterium with rounded ends and like the other members of the Enterobacteriaceae family is glucose-fermentative, oxidase-negative and nitrate-reductive [9,10]. ERM outbreaks usually begin with low mortality, and then escalate to result in high losses. Characteristic symptoms of ERM are haemorrhages of the mouth and gills, though these are rarely seen in acute infections but may be present in chronic infections, diffuse haemorrhages within the swim bladder, petechial haemorrhage of the pyloric caecae, bilateral exophthalmia, abdominal distension as a result of fluid accumulation, general septicaemia with inflammation of the gut, the spleen is often enlarged and can be almost black in colour [4]. Transmission occurs by direct contact with carrier fish, other aquatic invertebrates and birds [4,11]. The ability of *Y. ruckeri* to survive and remain infective in the aquatic environment is considered to be a major factor in spread of the disease. Furthermore, *Y. ruckeri* is able to form biofilms and grow on surfaces and solid supports in fish tanks, like many bacteria in aquatic environments, which lead to recurrent infections in rainbow trout farms [12]. Although vaccination has for a decade been very successful in the control of infections caused by *Y. ruckeri* in trout farms [13], cases of yersiniosis have been reported in trout farms where vaccination didn't provide enough protection against the infection [14] and due to carrier state [13]. Different diagnostic methods have been developed for detection of *Y. ruckeri* including culturing, serological and molecular techniques. Isolation and identification using agar media and the organism's biochemical characteristics are considered the gold standard for *Y. ruckeri* diagnosis. Serological methods for detection of *Y. ruckeri* have also been developed and these include ELISA, agglutination, and the immunofluorescence antibody technique (IFAT) [15]. Molecular techniques are able to detect low levels of the bacterium and facilitate detection of asymptomatic carriers, which is very important for prevention of ERM transmission and spread [16]. Restriction fragmentation-length polymorphism [17] and PCR assays [18-20] are widely used for detection of low levels of *Y. ruckeri* in infected trout tissues and blood and also for detection of asymptomatic carriers. Although PCR has been shown to be a powerful and sensitive tool in detection of *Y. ruckeri*, its requirements for expensive equipments, a precision thermocycler and laboratory training limit its use in the field as a routine diagnostic tool.

Alternate isothermal nucleic acid amplification methods, which require only a simple heating device, have been developed to offer feasible platforms for rapid and sensitive detection of a target nucleic acid. These include nucleic acid-based amplification (NASBA), loop-mediated isothermal amplification (LAMP) and ramification amplification [21-23]. LAMP is a nucleic acid amplification method that synthesises large amounts of DNA in a short period of time with high specificity [22,24]. The strand displacement activity of *Bst* DNA polymerase impels auto-cyclic DNA synthesis with loop-forming primers to yield long-stem loop products under isothermal conditions: 60–65°C for about 60 min [22,25]. The LAMP reaction requires four or six primers that target six or eight separate DNA sequences on the target and give the assay very high specificity [22,25]. LAMP amplification products can be detected by gel electrophoresis, by real time monitoring of turbidity with a turbidimeter [24,26] or with the naked-eye. Visual detection can be accomplished using different methods such as detection of a white precipitate (magnesium pyrophosphate), use of an intercalating DNA dye such as SYBR Green I gel stain [27], use of florescent detection reagent, FDR, [28], or use of oligonucleotide probes labelled with different fluorescent dyes and low molecular weight cationic polymers such as polyethylenimine, PEI [29].

LAMP-based assays have been developed for numerous aquaculture animal pathogens, including white spot syndrome virus [30], yellow head virus [31], *Edwardsiella tarda* [32] and *Nocardia seriolae* [33], *Tetracapsuloides bryosalmonae*, *Myxobolus cerebralis*, *Thelohania contejeani* [34-36], Koi herpes virus (CyHV-3) and viral hemorrhagic septicaemia (VHS) [27,37]. The objective of this study was to develop and evaluate LAMP, as a simple, rapid and sensitive diagnostic tool for ERM disease.

Methods

Bacteria

The bacterial strains used in this study were listed in (table 1). *Y. ruckeri* strains were cultured on trypticase-soy-agar [3]. The purity of the cultures was tested with Gram stain and confirmed biochemically with the API 20E rapid identification system.

Each strain from other bacterial strains was propagated on its specific medium and then tested by Gram stain and biochemically.

DNA extraction

DNA was extracted from bacterial cultures using QIAamp® DNA mini kit (QIAGEN, Hilden, Germany). Bacterial

Table 1: Bacterial species assayed in ERM-LAMP experiments

Bacterial Strains	Source
Y. ruckeri	DSMZ[1] 18506 (ATCC 29473)
Y. ruckeri	CECT[2] 955
Y. ruckeri	CECT 956
Y. ruckeri	Dr. Joachim Nils[3]
Y. aldovae	DSMZ 18303 (ATCC 35236)
Y. enterocolitica	DSMZ 4780 (ATCC 9610)
Y. frederiksenii	DSMZ 18490 (ATCC 33641)
Y. intermedia	DSMZ 18517 (ATCC 29909)
Y. kristensenii	DSMZ 18543(ATCC 33638)
Aeromonas salmonicida	Clinic for Fish and Reptiles
Aeromonas sorbia	Clinic for Fish and Reptiles
Renibacterium salmoninarum	Clinic for Fish and Reptiles
Flavobacterium columnare	Clinic for Fish and Reptiles
Pseudomonas aeroginosa	Clinic for Fish and Reptiles

1) DSMZ: Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German Collection of Micro-organisms and Cell Cultures) Braunschweig, Germany.
2) CECT: Colección Española de Cultivos Tipo (Spanish Type Culture Collection) Valencia, Spain.
3) Fischgesundheitsdienst im Staatlichen Untersuchungsamt, Veterinäruntersuchungsamt Mittelhessen, Giessen, Germany.

cells were harvested in a microcentrifuge tube by centrifugation at 5000 × *g* for 10 min. Cell pellets were re-suspended in 180 μl lysis buffer (20 mg/ml lysozym; 20 mM Tris-HCl, pH 8.0; 2 mM EDETA; 1.2% Triton) and incubated at 37°C for 30 min. Proteinase K and Buffer AL were then added and mixed by vortexing. After 30 min incubation at 56°C, ethanol was added and thoroughly mixed to yield a homogenous solution. DNA was then extracted as per manufacturer's instructions. DNA was extracted from tissue samples (liver, kidney, spleen) by QIAamp® DNA mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions following the animal tissues protocol.

Oligonucleotide primers

ERM-LAMP primers were designed according to the published sequence of *yruI*/*yruR* (GenBank accession number AF274748, [20]) using Primer Explorer version 4 (Net Laboratory, Tokyo, Japan). Five primers were constructed; two outer primers F3 and B3, two inner primers: forward inner primer (FIP) backward inner primer (BIP) and loop forward primer (LF). FIP comprised the F1c sequence complementary to F1, a TTTT linker, and F2 sequence. BIP consisted of the B1c sequence complementary to B1, a TTTT Linker and B2 sequence. After modification of the 3' end with Rox, the loop forward primer LF was used as an Oligo DNA Probe (ODP). PCR specific primers IF-2 and IR-2 were used to amplify 1000 bp of *yruI*/*yruR* genes of *Y. ruckeri* [20]. Details of the LAMP and PCR primers are given in (Table 2).

Optimization of ERM- LAMP condition

ERM-LAMP reactions were carried out in a Loopamp real-time turbidimeter (LA-200, Teramecs Co., Ltd., Kyoto, Japan) at 60, 63 and 65°C, for 30, 45 and 60 min, followed by 80°C for 2 min to terminate the reaction. The reaction mixture contained 40 pmol each of inner primers FIP and BIP, 5 pmol each of outer primers F3 and B3, 20 pmol of LF (forward loop primer), 1.4 mM of dNTP mix, 1.6 M betaine (Sigma-Aldrich, GmbH, Schnelldorf, Germany), 4.5 mM $MgSO_4$, 8 U of *Bst* DNA polymerase (New England Biolabs GmbH, Frankfurt, Germany), 1× of the supplied Thermopol buffer, and a specified amount of template DNA in a final volume of 25 μl. Reaction mix without DNA template was included as a negative control.

PCR amplification

Amplification was performed in a 50 μl reaction volume with 2× ready mix PCR Master mix (Thermo Scientific, Hamburg, Germany) which contained (75 mM Tris-HCl (pH 8.8), 20 mM $(NH_4)_2 SO_4$, 1.5 mM $MgCl_2$, 0.01% Tween-20, 0.2 mM each nucleotide triphosphate, 1.25 U thermoprime plus DNA polymerase, and red dye for electrophoresis), 1.5 μl of DNA template and 20 pmol each of forward and reverse primers. The amplification was carried out in Mastercycler Gradient thermocycler, Eppendorf, with the following cycling profile: 94°C for 2 min, then 40 PCR cycles of 92°C for 1 min (DNA denaturation), 65°C for 1 min (primer annealing) and 72°C for 1.5 min (DNA extension), with a terminal extension step of 72°C for 5 min.

Table 2: Details of oligonucleotide primers used for ERM-LAMP assay and PCR assay.

Primer name	Length	Sequence (5'-3')
F3	20-mer	TCGATATAGTTACCTTCCGG
B3	18-mer	ATGGGCAGTGAACTGTAG
FIP	46-mer	TGTTCGTTTATTGAACTTCACCGATTTTCGTCGAACTGAGCGTTAA
BIP	50-mer	AAGCTGATTTCCATAAATTCCGAGTTTTTAATGACATGGAGTTTGATGAG
Loop Forward(LF)	25-mer	AGGTATCGTGTGTTAGGATTATCGT
ODP	25-mer	AGGTATCGTGTGTTAGGATTATCGT-Rox
IF-2	24-mer	GAGCGCTACGACAGTCCCAGATAT
IR-2	24-mer	CATACCTTTAACGCTCAGTTCGAC

Detection of the amplification products

Three detection methods were used: real-time turbidity detection, agarose gel analysis and visual detection. Changes in absorbance at 650 nm were measured for real-time turbidity detection with a Loopamp real-time turbidimeter (LA-200). A cut off value was determined based on the mean of the negative detection control optical density. Specimens with an optical density of less than 0.1 were determined to be negative for *Y. ruckeri* bacterial DNA. LAMP and PCR amplification products were analysed by gel electrophoresis stained with GelRed™ Nucleic Acid Gel Stain, 10,000× in water (BIOTREND Chemikalien GmbH, Köln, Germany) and then visualised under UV light. A TrackIt™ 100 bp DNA ladder (Invitrogen GmbH, Karlsruhe, Germany) was used as molecular weight marker. Visual detection of the LAMP products was carried out either by using 1 μl of Fluorescent Detection Reagent, FDR, (Eiken Chemical Co., Ltd) added before incubation of the reaction mixture at 63°C, or by addition of 1 μl of 1:10 diluted SYBR Green I nucleic acid gel stain 10,000 × concentration in DMSO (Cambrex BioSceince, Rockland, Inc., ME, USA) to the LAMP product after termination of the reaction. Any colour changes of the reaction mixture were noted. For detection with Rox- labelled probe, 0.2 μmol of low molecular weight PEI (Wako chemical GmbH, Neuss, Germany) was added to the LAMP product after centrifugation for 10 s at 6000 rpm to form insoluble PEI-amplicon complex, containing the Rox- labelled probe, which was precipitated by additional centrifugation at 6000 rpm for 10 s. Reaction tubes were then visualised under a conventional UV illuminator or by fluorescence microscopy.

Restriction analysis digestion of the ERM- LAMP products

To confirm the structure of the LAMP amplicons, it was purified using a High pure PCR purification kit (Roche Molecular Biochemicals, Mannheim, Germany) and then subjected to digestion with restriction enzyme *Hph*I (New England BioLabs GmbH, Frankfurt, Germany). Fragment sizes were analyzed by 2% agarose gels electrophoresis stained with GelRed™ Nucleic Acid Gel Stain, 10,000× in water (BIOTREND Chemikalien GmbH, Köln, Germany) and then visualised under UV light.

ERM- LAMP assay specificity

DNAs from *Y. ruckeri strains* and from other bacterial strains (*Y. aldovae, Y. enterocolitica, Y. frederiksenii, Y. intermedia, Y. kristensenii, Aeromonas salmonicida, Aeromonas sorbia, Pseudomonas aeruginosa, Renibacterium salmoninarum and Flavobacterium columnare*) were tested by ERM-LAMP assay to assess the specificity of the constructed primers. DNA from non-infected fish tissues and a negative LAMP reaction control were used to detect any non-specific amplification.

Sensitivity of the ERM-LAMP assay

One microgram genomic *Y. ruckeri* DNA was 10-fold serially diluted to assess the lower detection limit of the LAMP assay compared with conventional PCR. The products were analysed visually and by 2% agarose gel electrophoresis.

Feasibility of the ERM- LAMP assay

The use of the ERM-LAMP assay to detect *Y. ruckeri* DNA in clinical specimens was evaluated by testing 15 rainbow trout samples infected with ERM submitted to our clinic and 4 control fish samples. These fish were suffering from diffuse haemorrhages in the swim bladder and enlarged black spleen. The samples were tested by both ERM-LAMP assay and PCR assay.

Results

Optimal amplification of the *Y. ruckeri yruI/yruR* gene by ERM-LAMP assay was obtained at 63°C for 60 min, as shown by both agarose gel electrophoresis and real time turbidity measurements. Amplified products exhibited a ladder-like pattern on the gel (Fig. 1). Specificity of the amplification was confirmed by digestion of the LAMP products using *Hph*I restriction enzyme (Fig. 1), the sizes of the resultant digestion products were as predicted (87 bp and 108 bp). Results obtained with the visual detection methods correlated with agarose gel electrophoresis results. When FDR used, a strong green fluorescence was emitted by LAMP positive reactions (F ig. 2, Tube No.3) when exposed to UV light and no fluorescence was evident for a negative reaction (Fig. 2, Tube No. 4). Likewise, after addition of SYBR Green I dye, the ERM-LAMP products appeared green (Fig. 2, Tube No. 5), while in the negative control tube the original orange colour of SYBR Green I did not change (Fig. 2, Tube No. 6). With Rox-labelled probe, a pellet formed emitted a red fluorescence for a positive reaction (Fig. 2, Tube No. 2), but there was neither pellet nor fluorescence observed in the negative control tube (Fig. 2, Tube No. 1).

The specificity of ERM-LAMP primers was confirmed by amplification of *yruI/yruR* gene from all *Y. ruckeri* tested strains while there are no amplification products detected from the other bacterial species, non-infected fish tissues or negative (no template) LAMP reaction control (Fig. 3). Both agarose gel electrophoresis and visual detection methods showed that, the lower detection limit of the ERM- LAMP method is 10^{-6} dilution, which equal to 1 pg of the *Y. ruckeri* genomic DNA (Fig. 4), while PCR showed no amplification after a dilution of 10^{-5} which equal to 10 pg *Y. ruckeri* genomic DNA (Fig. 5). The LAMP assay detected *Y. ruckeri* DNA from 15 infected fish samples, which were also positive by PCR (Fig. 6 &7). Samples from all 4 control fish were negative in both assays.

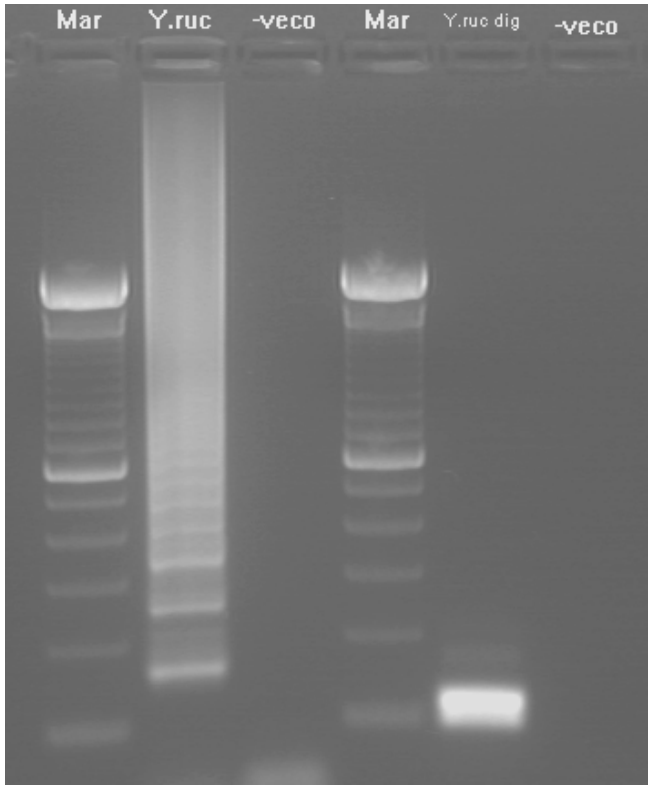

Figure 1
ERM-LAMP. *Yersinia ruckeri* loop-mediated isothermal amplification (ERM-LAMP) products and restriction analysis of ERM- LAMP product with *Hph*I enzyme. Lane Mar = 100-base-pair DNA ladder, lane Y. ruc = Amplified *Y. ruckeri* LAMP product shows a ladder-like pattern, lane Y. ruc dig = Digested *Y. ruckeri* LAMP product with *Hph*I with production of 87 bp and 108 bp bands, lane – veco = Negative (No template) control.

Discussion

Efficient, rapid and timely diagnosis is critical for successful management of diseases in aquaculture. For field diagnosis, the optimal detection system should be economical, quick, and easy to operate, moreover should meet the requirements of specificity and sensitivity [38]. ERM disease is a serious infection that causes sever economic losses in salmonid aquaculture. It usually occurs as an acute condition with high morbidity and mortality rates, which necessitates rapid and accurate methods for detection of its causative agent, *Y. ruckeri* [18]. A traditional microbiological approach for isolation and identification usually takes 2 to 3 days, and given that different numerical profiles for *Y. ruckeri* can be obtained with commercial multi-substrate identification systems, particularly the API 20E system, they must be interpreted with caution [3]. Although PCR assays are more accurate, specific, and faster than the microbiological approach [18-20], they require precision equipments which are beyond the capacity of most diagnostic sites to purchase, maintain and operate, and the complexity of the assay procedures obviates the possibility of point-of-care use.

In this study, a rapid and sensitive diagnostic system based on LAMP technology was developed to detect *Y. ruckeri*. The ERM-LAMP assay requires only a simple water bath or heating block to incubate the reaction mixture at 63°C for 1 hr before the reaction products are visualised. The assay utilizes a single DNA polymerase that is active at relatively high isothermal amplification temperatures, which diminishes the probability of non-specific priming [39]. The *yruI/yruR* quorum sensing system encoding gene of *Y. ruckeri* was chosen as a suitable target, as it controls virulence gene expression through cell to cell communication and has great potential for rapid and specific identification of this fish pathogen [20]. Although there is a serotypic diversity among *Y. ruckeri* strains [40,41], *yruI/yruR* gene was amplified from all *Y. ruckeri* tested strains by PCR and produced one RFLP pattern which demonstrate a high degree of genotypic homogeneity among *Y. ruckeri* strains regarding this gene [20].

A LAMP assay requires at least 4 highly specific primers to distinguish six distinct regions on the target DNA [42]. In developing the ERM-LAMP assay, several primer sets were appraised, with the most effective set presented here. The assay was optimized to amplify *Y. ruckeri* at 63°C using a set of 4 or 5 primers. In initial trials of the assay, a characteristic ladder-like pattern of LAMP amplification is demonstrated upon gel electrophoresis [43] and confirmed the identity of the product by *Hph*I digestion. The ERM-LAMP assay was able to amplify the target *yruI/yruR* gene from all *Y. ruckeri* tested strains while it did not show any cross-reactivity with a panel of DNAs from other Yersinia species or from other related bacterial species, which confirm its specificity. Due to the isothermal nature of the LAMP assay, there is no time lost in temperature cycling, which leads to extremely high efficiency compared with regular PCR [22,44]. Another advantage of LAMP is that real-time monitoring of the reaction is possible [24] and this decreases the time needed to get results and reduces the risk of carry-over contamination in the post-PCR process [45]. Alternatively, LAMP reaction products can be visualized using SYBR Green I nucleic gel stain which has high binding affinity to double stranded DNA and hence turns from orange to green as the LAMP amplicons are produced [46,47]. LAMP product can also be monitored by placing a reaction tube directly on a UV transilluminator; when the FDR added into the reaction mixture. The calcein in FDR is initially combined with manganese ions and is quenched, but as amplification generates by-product pyrophosphate ions, these bind to and remove manganese from the calcein, resulting in fluorescence which is intensified further as calcein combines with magnesium

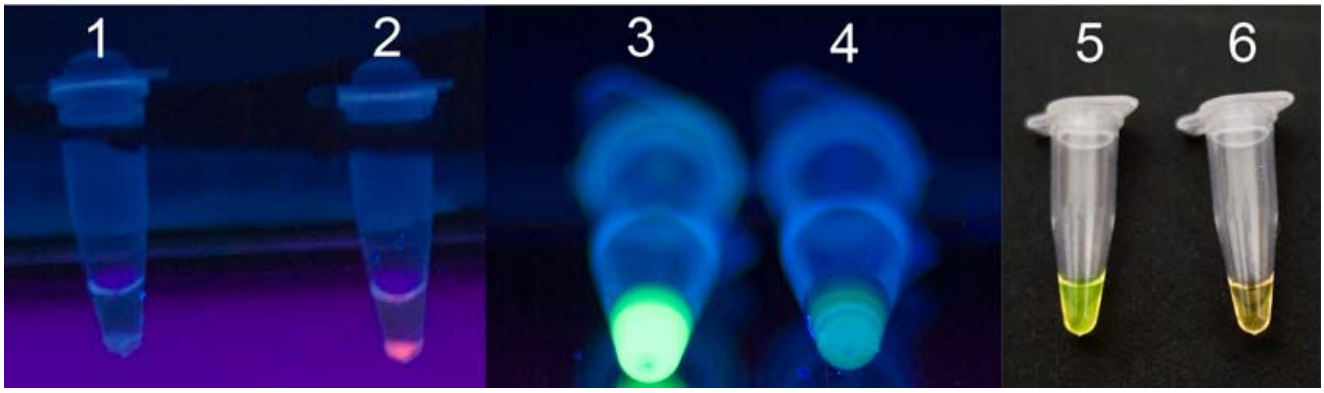

Figure 2
Visual detection of ERM-LAMP product. Using different naked eye detection methods: 1 = Negative control of ERM-LAMP reaction using Rox- labelled probe, there is neither pellet nor red fluorescence; 2 = Positive ERM-LAMP reaction using Rox- labelled probe, the pellet emitted red fluorescence; 3 = positive sample by using FDR, emitted strong green fluorescence when exposed to UV light; 4 = negative sample by using FDR, did not emitted strong green fluorescence under UV light; 5 = positive sample with green colour by using SYBR green I stain; 6 = negative sample with orange colour by using SYBR green I stain.

ions [28,45]. On the other hand, if low molecular weight PEI is used, this forms an insoluble complex with high molecular weight DNAs, like LAMP products, which then captures the hybridized Rox-labelled probe into a pellet which fluoresces red under UV light [29]. All of our data confirmed that visual detection of assay results was compatible with the real-time turbidity measurement and agarose gel electrophoresis. Hence simple visual detection facilitates use of the assay in basic laboratories and in fish farms.

Compared with biochemical, microbial culture methods and PCR assay (24–48 hrs, 3 hrs respectively); the ERM-LAMP is convenient, rapid, and sensitive. The ERM-LAMP

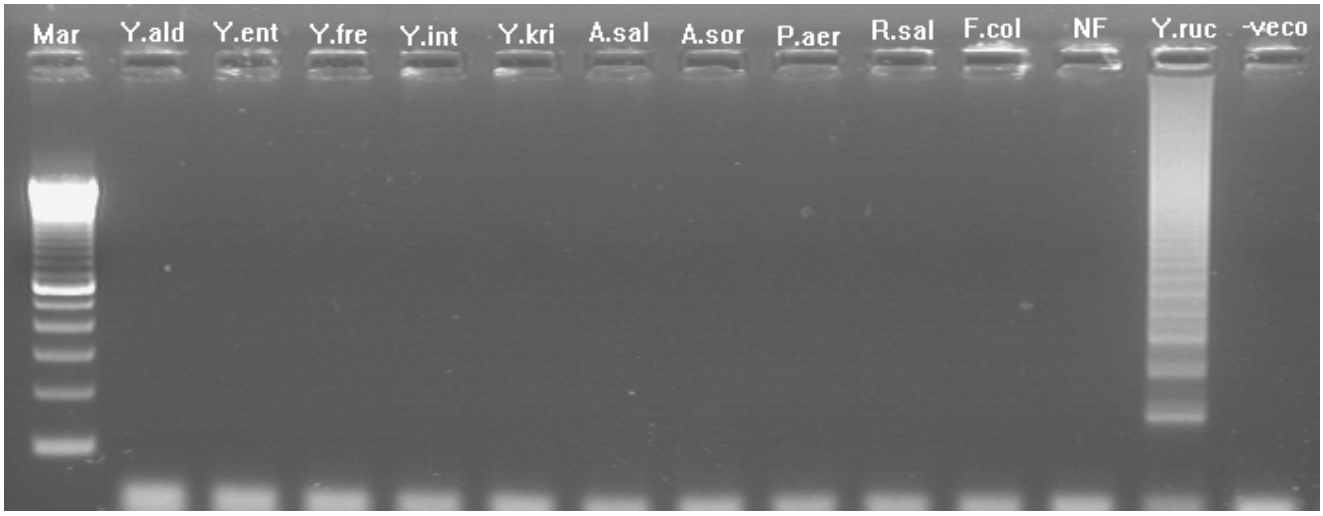

Figure 3
Specificity of ERM-LAMP primers for detection of *Y. ruckeri* DNA. Lane Mar = 100-base-pair DNA ladder, lane Y. ald = DNA from *Yersinia aldovae*, lane Y. ent = DNA from *Yersinia enterocolitica*, lane Y. fre = DNA from *Yersinia frederiksenii*, lane Y. int = DNA from *Yersinia intermedia*, lane Y. kri = DNA from *Yersinia kristensenii*, lane A. sal = DNA from *Aeromonas salmonicida*, lane A. sor = DNA from *Aeromonas sorbia*, lane P. aer = DNA from *Pseudomonas aeruginosa*, lane R. sal = DNA from *Renibacterium salmoninarum*, lane F. col = DNA from *Flavobacterium columnare*, lane NF = DNA from non-infected Fish tissues, lane Y. ruc = DNA from *Yersinia ruckeri*, lane – veco = Negative control.

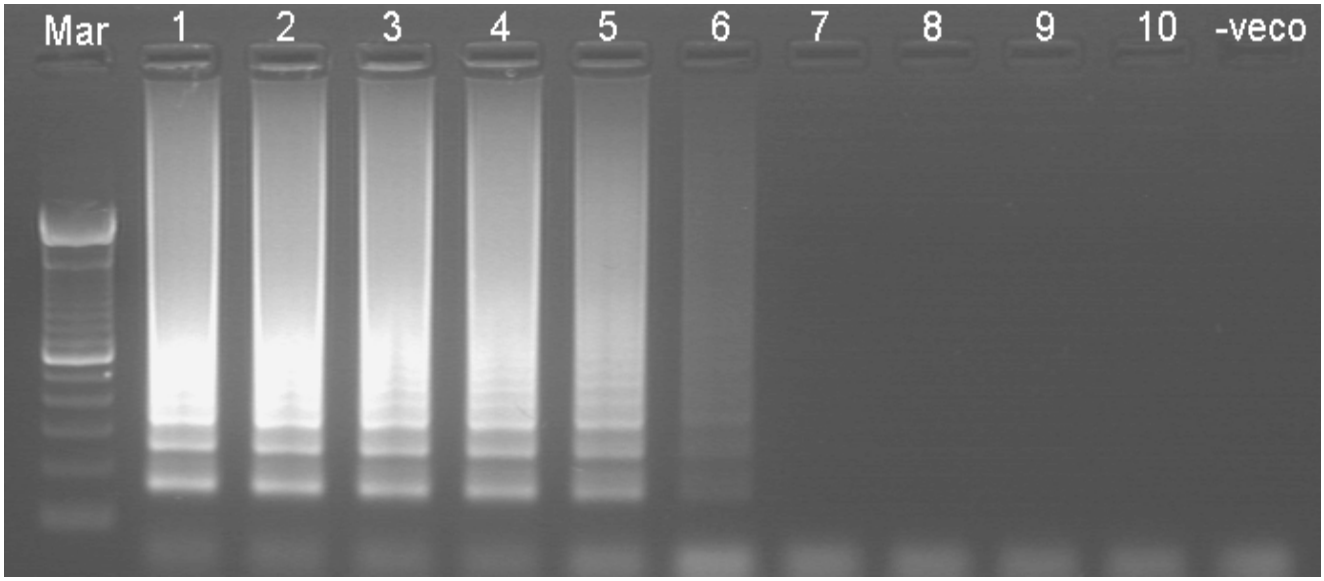

Figure 4
Sensitivity of ERM-LAMP assay. Lower detection limit of the *Yersinia ruckeri* DNA by LAMP assay. Lane Mar = 100-base-pair DNA ladder, lane 1–10 = 10-fold serial dilution of 1 μg *Yersinia ruckeri* DNA from 10^{-1}-10^{-10}; lane – veco = No template control.

assay is 10-fold more sensitive than regular PCR as it detected a very low concentration of *Y. ruckeri* genomic DNA (1 pg), while the PCR can detect only till 10 pg *Y. ruckeri* genomic DNA. The assay successfully detected *Y. ruckeri* DNA in infected fish samples and hence appears suitable for use with clinical specimens.

Conclusion

Loop mediated isothermal amplification assay as a new diagnostic tool for diagnosis of ERM disease in salmonids was developed and evaluated. The ERM-LAMP assay is rapid, as its result appeared after one hour, and sensitive than the conventional diagnostic method of ERM disease. The ERM-LAMP assay requires only a regular laboratory

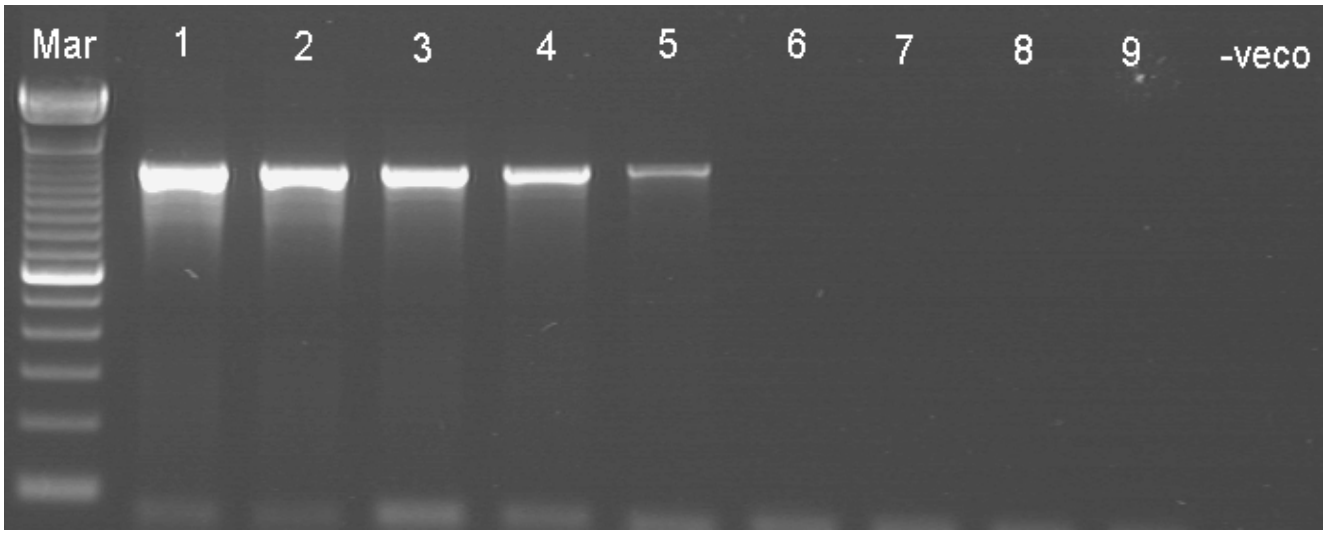

Figure 5
Sensitivity of ERM-PCR assay. Lower detection limit of (1000 bp fragment) *Yersinia ruckeri* DNA by PCR. Lane Mar = 100-base-pair DNA ladder, lane 1–9 = 10-fold serial dilution of 1 μg *Yersinia ruckeri* DNA from 10^{-1}-10^{-9}; lane – veco = No template control.

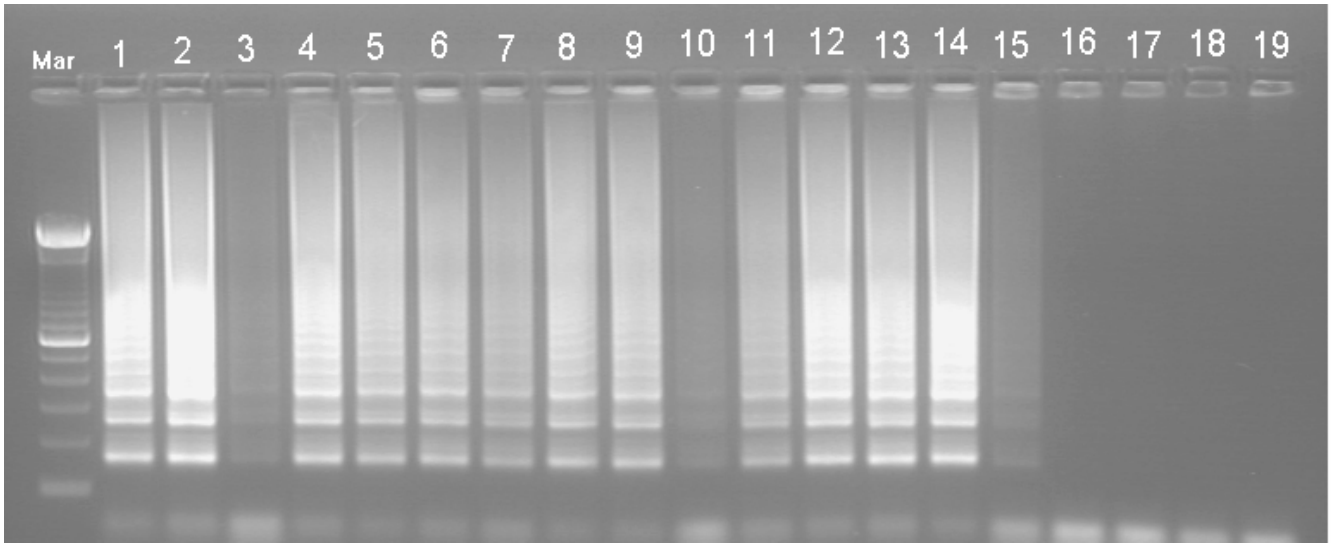

Figure 6
Feasibility of ERM-LAMP assay. Detection of *Yersinia ruckeri* DNA from 15 infected kidney samples by ERM-LAMP while there is no amplifications appeared with the non-infected kidney samples. Lane Mar = 100-base-pair DNA ladder, lanes 1–15 = DNA from infected kidney samples, lanes 16–19 = DNA from non-infected kidney samples.

water bath and is hence suitable as a routine diagnostic tool in private clinics and field applications where equipment such as thermal cycling machines and electrophoresis apparatus are not available.

Authors' contributions

MS carried out all the experimental work, data acquisition and drafted the manuscript. HS participated in the design of the study, analysis and interpretation of the data and helped to draft the manuscript. ME–M conceived and supervised the study, and revised the manuscript critically

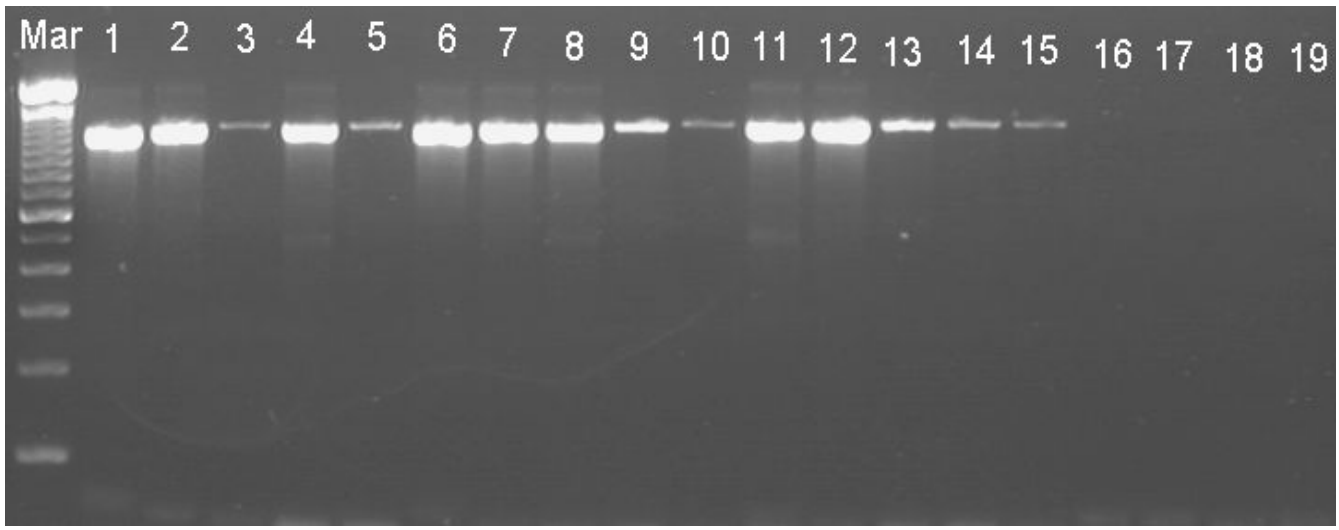

Figure 7
Feasibility of ERM-PCR assay. Detection of *Yersinia ruckeri* DNA from 15 infected kidney samples by ERM-PCR while there is no amplifications appeared with the non – infected kidney samples. Lane Mar = 100-base-pair DNA ladder, lanes 1–15 = DNA from infected kidney samples, lanes 16–19 = DNA from non-infected kidney samples.

for important intellectual content. All authors read and approved the final manuscript.

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3.2. Publication 2

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Loop-mediated isothermal amplification (LAMP) for rapid detection of *Renibacterium salmoninarum*, the causative agent of bacterial kidney disease

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ABSTRACT: A loop-mediated isothermal amplification (LAMP) assay was developed for rapid, specific and sensitive detection of *Renibacterium salmoninarum* in 1 h without thermal cycling. A fragment of *R. salmoninarum* p57 gene was amplified at 63°C in the presence of *Bst* polymerase and a specially designed primer mixture. The specificity of the BKD-LAMP assay was demonstrated by the absence of any cross reaction with other bacterial strains, followed by restriction digestion of the amplified products. Detections of BKD-LAMP amplicons by visual inspection, agrose gel electrophoresis, and real-time monitoring using a turbidimeter were equivalently sensitive. The BKD-LAMP assay has the sensitivity of the nested PCR method, and 10 times the sensitivity of one-round PCR assay. The lower detection limit of BKD-LAMP and nested PCR is 1 pg genomic *R. salmoninarum* DNA, compared to 10 pg genomic *R. salmoninarum* DNA for one-round PCR assay. In comparison to other available diagnostic methods, the BKD-LAMP assay is rapid, simple, sensitive, specific, and cost effective with a high potential for field application.

KEY WORDS: *Renibacterium salmoninarum* · BKD · LAMP · Diagnosis

INTRODUCTION

Bacterial kidney disease (BKD) is a systemic disease of fresh and salt water salmonids worldwide; the disease is caused by *Renibacterium salmoninarum* (Fryer & Sanders 1981, Evenden et al. 1993, Bruno 2004). It is generally a chronic, granulomatous and often fatal infection, although acute disease may occur (Miriam et al. 1997). It causes mortality in all host age groups and poor growth rates in chronically infected fish (Bruno 2004). *R. salmoninarum* can be transmitted both horizontally among cohorts and vertically by intra-ovum inclusion (Evelyn et al. 1984, Balfry et al. 1996). BKD was first described in Atlantic salmon *Salmo salar* in Scotland (Mackie et al. 1933), then in rainbow trout *Oncorhynchus mykiss* from Massachusetts, USA (Belding & Merril 1935). In Germany, BKD was first recorded in farmed salmon and trout by Hoffmann et al. (1984) and has since been reported elsewhere in Europe, Japan, South America and many states of the USA. *R. salmoninarum* is a small, Gram-positive, non-motile diplobacillus that has fastidious nutritional requirements (Austin et al. 1983, Daly & Stevenson 1993, Teska 1994, Starliper et al. 1998). Acute BKD is characterized by dark colouration of the fish, bloody ascites, exophathalmia, and granulomatous lesions of internal organs such as the kidney, whereas asymptomatic carriers can complete an entire life cycle and successfully spawn (Fryer & Lannan 1993). Control measures have been investigated to limit the spread of the disease; however, most have had limited success (Elliott et al. 1989, Moffitt 1992). To facilitate successful control of BKD, there is a need for a series of diagnostic tests that can detect the bacterium during the different phases of the infection (White et al. 1995).

Bacteriological culture is the benchmark method for conventional diagnosis of BKD. However, due to the long incubation times (6 to 19 wk at 15°C) and the tedious process required for primary isolation of *Reni-*

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bacterium salmoninarum, culture is often impractical for routine diagnosis (Benediktsdottir et al. 1991). Alternate techniques have been developed for detection of the bacterium including direct and indirect fluorescent antibody assay (Bullock & Stuckey 1975, Austin & Austin 1993), and the enzyme-linked immunosorbent assay (ELISA) (Pascho et al. 1987, Jansson et al. 1996). These techniques also have some drawbacks as they are not sensitive enough to detect low levels of the pathogen in asymptomatic fish and, in the case of the fluorescent antibody assay, may give false positive reactions (Austin et al. 1985, Armstrong et al. 1989). Conversely, inconsistent results may arise with ELISA due to the variable quality of antibody lots and cross reactivity with other bacterial species (Scott & Johnson 2001, Powell et al. 2005). To overcome the drawbacks of these methods, several polymerase chain reaction (PCR) assays have been developed for sensitive and rapid detection of BKD in infected fish tissues and eggs (Brown et al. 1994, Leon et al. 1994, Magnusson et al. 1994, Chase & Pascho 1998, Powell et al. 2005, Chase et al. 2006, Rhodes et al 2006, Suzuki & Sakai 2007).

Although PCR assays are powerful and sensitive tools for diagnosis of BKD, they require expensive equipment, precision thermocycling and laboratory training, which limits their use as routine diagnostic tools in the field.

Loop-mediated isothermal amplification (LAMP) is a technique developed recently to amplify nucleic acid under isothermal conditions. It offers a rapid, inexpensive and accurate tool for all life sciences, including diagnosis of pathogens and detection of genetic disorders (Notomi et al. 2000). Unlike PCR, LAMP does not require a denatured template, but depends on the high strand displacement activity of *Bst* polymerase (Nagamine et al. 2001). The technique employs a set of 4 specific primers that recognize 6 distinct nucleotide sequences of the target DNA. LAMP is initiated by an inner primer, which amplifies the sense and anti-sense strands of the target, then an outer primer displaces the amplified strand to give a single stranded DNA. This single-stranded DNA serves as a template for further DNA synthesis primed by the second inner and outer primers that hybridize to the ends of the target to produce a stem loop DNA structure (Notomi et al. 2000). Amplification proceeds in a cyclical order, each strand being displaced during elongation with the addition of new loops in each cycle. The final products are stem loop DNAs with several inverted repeats of the target and a cauliflower-like structure of multiple loops that arise from hybridization between alternately inverted repeats in the same strand. An additional set of 2 primers can accelerate the reaction (Nagamine et al. 2002).

Several means for visually detecting LAMP amplicons without agarose gel electrophoresis have been developed. One of these is the visual detection of magnesium pyrophosphate, a white precipitate that is produced during DNA amplification and which can be easily detected by the naked eye or by real time monitoring of turbidity in the reaction tube with a turbidimeter (Mori et al. 2001, 2004). Alternatively, LAMP products can be monitored by a colour change resulting from addition of an intercalating DNA dye such as SYBR Green I gel stain (Soliman & El-Matbouli 2005) or fluorescent detection reagent (FDR) (Yoda et al. 2007). Fluorescently labelled probe and cationic polymers such as low molecular weight polyethylenimine (PEI) have also been used for visual detection of LAMP amplicons (Mori et al. 2006). In aquaculture, LAMP assays have been developed for several fish and shellfish pathogens including white spot syndrome virus (Kono et al. 2004), *Edwardsiella tarda* (Savan et al. 2004), *E. ictaluri* (Yeh et al. 2005), *Flavobacterium columnare* (Yeh et al 2006), yellow head virus (Mekata et al. 2006), iridovirus (Caipang et al. 2004), infectious hematopoietic necrosis virus (Gunimaladevi et al. 2004), koi herpes virus (Gunimaladevi et al. 2005) and *Nocardia seriolae* (Itano et al. 2005). In our laboratory, we have designed several LAMP assays to detect the pathogens *Tetracapsuloides bryosalmonae*, *Myxobolus cerebralis*, koi herpes virus (CyHV-3), viral hemorrhagic septicaemia (VHS), *Thelohania contejeani* (El-Matbouli & Soliman 2005a,b, 2006, Soliman & El- Matbouli 2005, 2006).

The aim of the current work was to develop an accelerated, cost effective, specific and sensitive LAMP assay with high potential for field diagnosis of BKD in salmonids.

MATERIAL AND METHODS

Bacterial strains. *Renibacterium salmoninarum* was kindly provided by S. Braune, Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit, Veterinärinstitut Hannover, Germany. The bacteria was cultured on selective kidney disease medium (SKDM) agar (Austin et al. 1983). The purity of the culture was tested by Gram stain and confirmed by biochemical tests.

The other bacterial strains, viz. *Aeromonas salmonicida*, *A. sobria*, *Yersinia ruckeri, Flavobacterium columnare*, and *Pseudomonas aeroginosa* were from our Clinic of Fish and Reptiles (formerly Institute of Zoology, Fish Biology and Fish Diseases), University of Munich, Germany. Each strain was propagated on its specific medium and then tested by Gram stain and biochemically for confirmation of identity.

DNA extraction. Bacterial genomic DNA was extracted using a QIAamp® DNA mini kit (Qiagen). Bacterial cells were harvested in a micro-centrifuge tube by centrifugation at 5000 × *g* for 10 min. Cell pellets were re-suspended in 180 µl lysis buffer (20 mg ml^{-1} lysozyme, 20 mM Tris-HCl, pH 8.0, 2 mM EDTA, 1.2% Triton) and incubated at 37°C for 30 min. Proteinase K and buffer AL were then added and mixed by vortexing. After 30 min incubation at 56°C, ethanol was added and thoroughly mixed to yield a homogenous solution. DNA was then extracted as per manufacturer's instructions. DNA was extracted from kidney tissue samples using a QIAamp® DNA mini kit. Kidney tissues were incubated with the lysozyme buffer (80 mg ml^{-1} lysozyme, 80 mM Tris-HCl, pH 8.0, 8 mM EDTA, 4.8% Triton) at 37°C for 1 h after the initial lysis step. DNA was then extracted according to the manufacturer's instructions following the animal tissues protocol.

Oligonucleotide primers. LAMP primers and fluorescently labelled probe were designed based on the major soluble antigen protein p57 encoding gene of *Renibacterium salmoninarum* (GenBank accession number AF123890) using LAMP primer design software (PrimerExplorer Ver.4). Five primers were used for LAMP assay: 2 outer primers (F3 and B3), 2 inner primers (Forward Inner Primer [FIP] and Backward Inner Primer [BIP]) and loop forward primer (LF) (Table 1). The FIP comprised an F1c sequence complementary to F1, a TTTT linker, and an F2 sequence. The BIP consisted of a B1c sequence complementary to B1, a TTTT Linker and a B2 sequence. After modification of the 3′ end with fluorescein isocyanate, the loop forward primer LF was also used as Oligo DNA Probe (ODP).

Table 1. Details of oligonucleotide primers used for BKD-LAMP assay, PCR and nested PCR assay

Primer name	Length	Sequence (5′-3′)
F3	20-mer	GCCCGGTAGAGGTTAAAGTC
B3	18-mer	CGGAACCAGCATTTGGCT
FIB	43-mer	GGAGTTGCTCCATCTGGTGCA TTTT CCGCAACAGCA ACTGACA
BIP	45-mer	CTGGTAAATGGTGGTCTGGCGA TTTT CCGCAACAGC AACTGACA
F Loop	21-mer	GTGTTGGTCACTACCCACGTA
ODP	21-mer	GTGTTGGTCACTACCCACGTA-Fluorescein isocyanate
FL7	20-mer	CGCAGGAGGACCAGTTGCAG
RL5	20-mer	TCCGTTCCCGGTTTGTCTCC
P3	19-mer	AGCTTCGCAAGGTGAAGGG
P4	25-mer	ATTCTTCCACTTCAACAGTACAAGG
M21	21-mer	GCAACAGGTTTATTTGCCGGG
M38	22-mer	CATTATCGTTACACCCGAAACC

Specific primers FL7 and RL5 for one-round PCR and P3, P4, M21, M38 for nested PCR (Table 1) were used to amplify 372bp and 383bp DNA fragments of the major soluble antigen p57 encoding gene of *Renibacterium salmoninarum*, respectively, following Miriam et al. (1997) and Pascho et al. (1998).

BKD-LAMP assay. To optimise the LAMP assay, different concentrations of the primers, $MgSO_4$ and *Bst* DNA polymerase, different incubation temperatures and different times were evaluated. The optimized BKD-LAMP assay was carried out in a 25 µl reaction volume contained: 1× Thermopol buffer (20 mM Tris-HCl pH 8.8, 10 mM KCl, 4.5 mM $MgSO_4$, 10 mM $(NH_4)_2SO_4$, 0.1% Triton X-100) (New England BioLabs), 1.6 M betaine (Sigma-Aldrich), 1.4 mM of each dNTPs (Sigma-Aldrich), 60 pmol each of inner primers FIP and BIP, 5 pmol each of outer primers F3 and B3, 30 pmol of LF primer, 8U *Bst* DNA polymerase (New England BioLabs), 2 µl of DNA template and PCR grade water to 25 µl. The mixture was incubated at 63°C in a Loopamp real-time turbidimeter (LA-200, Teramecs) for 60 min and then heated to 85°C for 2 min to terminate the reaction. Reaction mix without DNA template was included as a negative control.

PCR amplification. One-round PCR amplification was performed in a 50 µl reaction volume which comprised 46.5 µl of 1.1× ready mix PCR Master mix (ABgene) (containing: 75 mM Tris-HCl (pH 8.8), 20 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 0.01% Tween-20, 0.2 mM each of nucleotide triphosphate, 1.25 U Thermoprime Plus DNA Polymerase, red dye for electrophoresis), 20 pmol of each forward and reverse primers and 1.5 µl of DNA template. The reaction mixture was subjected to the following cycling profile: 94°C for 2 min, followed by 5 cycles of 94°C for 15 s (denaturating), 63°C for 2 min (annealing), and 72°C for 15 s (extending) and then 35 cycles of 94°C for 15 s, 63°C for 15 s, and 72°C for 15 s and a final extension step at 72°C for 1 min. DNA template was omitted from a reaction mix and used as a negative control.

Nested PCR amplification. In the first round, amplification was carried out in a 50 µl reaction volume which comprised 43 µl of 1.1× ready mix PCR Master mix (ABgene) (containing: 75 mM Tris-HCl (pH 8.8), 20 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 0.01% Tween-20, 0.2 mM each of nucleotide triphosphate, 1.25 U Thermoprime Plus DNA Polymerase, red dye for electrophoresis), 0.2 mM of each P3 and M21 primers and 5 µl DNA template. In the second round, amplification was performed in a 50 µl reaction volume, which contained 47 µl of 1.1× ready mix PCR Master mix, 0.2 mM of each P4 and M38 primers and 1 µl of the first round PCR product as a DNA template. Both reaction mixtures were subjected to the following cycling profile: 94°C for 5 min, followed by 30 cycles of 94°C for 30 s,

60°C for 30 s, and 72°C for 1 min and a final extension step at 72°C for 10 min. DNA template was omitted from a reaction mix and used as a negative reaction control.

Detection of the amplification products. LAMP products were visually detected either by using 1 µl of Fluorescent Detection Reagent (FDR, Eiken Chemical) added to the reaction mixture before incubation at 63°C, or by addition of 1 µl of 1:10 diluted SYBR Green I nucleic acid gel stain at 10 000 × concentration in DMSO (Cambrex BioScience) to the mixture after reaction termination and observation of the colour changes of the reaction mixture. For detection with the fluorescently labelled probe, 0.2 µmol of low molecular weight (MW 600) polyethylenimine (PEI) (Wako Chemical) was added to the reaction mixture after centrifugation for 10 s at 6000 rpm to form an insoluble PEI-amplicon complex containing the fluorescently labelled probe, which was precipitated by additional centrifugation at 6000 rpm for 10 s. Reaction tubes were then visualised under a conventional UV illuminator or by fluorescence microscopy. Alternatively, increased turbidity derived from magnesium pyrophosphate byproduct was monitored using a real-time turbidimeter (LA-200, Teramecs). An assay was regarded as positive when turbidity reached the threshold value fixed at 0.1, which is double the average turbidity value of several replicate negative controls. For electrophoretic analysis, LAMP, PCR and nested PCR amplification products were analysed by gel electrophoresis on 2% agarose in Tris acetate–EDTA buffer, TAE, (0.04M Tris acetate, 1 mM EDTA), stained with GelRed™ Nucleic Acid Gel Stain, 10 000× in water (BIOTREND Chemikalien) and then visualised under UV light. A TrackIt™ 100 bp DNA ladder (Invitrogen) was used as molecular weight marker.

Restriction analysis of the LAMP products. To confirm the structure of the LAMP amplicons, some of the reaction products were purified using a High Pure PCR purification kit (Roche Molecular Biochemicals) and then subjected to digestion with *Eco*RV restriction enzyme (New England BioLabs). Fragment sizes were analyzed by electrophoresis in 2% agarose gels followed by staining with GelRed™ Nucleic Acid Gel Stain 10 000× in water (BIOTREND Chemikalien). A TrackIt™ 100 bp DNA ladder (Invitrogen) was used as molecular weight marker.

BKD-LAMP assay specificity. The specificity of the BKD-LAMP assay for *Renibacterium salmoninarum* DNA was evaluated by testing it against DNA from a suite of bacterial strains, viz. *Aeromonas salmonicida, Aeromonas sobria, Pseudomonas aeruginosa, Yersinia ruckeri* and *Flavobacterium columnare*. DNA from non-infected fish tissues was used to determine any non-specific amplification, while a no template control was used as a negative reaction control.

Sensitivity of the BKD-LAMP assay. The sensitivity of the assay was assessed by testing 10-fold serial dilutions of 1 µg genomic *Renibacterium salmoninarum* DNA in comparisons with one-round and nested PCR assays. Reaction mix without DNA template was included as a negative reaction control. BKD-LAMP amplification products were analysed visually and by agarose gel electrophoresis.

Applicability of the BKD-LAMP assay. The feasibility of using the BKD-LAMP assay to detect the *Renibacterium salmoninarum* DNA in clinical specimens was evaluated by testing 20 rainbow trout kidney samples infected with BKD and 6 uninfected kidney samples from our clinic's diagnostic material. The samples were tested by both BKD-LAMP assay and PCR assay. Reaction mix without DNA template was included as a negative control.

RESULTS

The optimized BKD-LAMP assay successfully amplified the target sequence of the *Renibacterium salmoninarum* major soluble antigen p57 gene as demonstrated by agrose gel electrophoresis and real time monitoring of turbidity. The amplified products were observed as a ladder-like pattern on the gel (Fig. 1). The specificity of the LAMP products was confirmed by restriction endonuclease digestion with *Eco*RV, which produced 90 and 120 bp bands instead of the ladder-like pattern that disappeared (Fig. 1). No amplification product was detected in the negative controls.

The BKD-LAMP products appeared green after addition of SYBR Green I dye, whereas the original orange colour of SYBR Green I did not change in the negative control tubes (Fig. 2A). Positive LAMP reactions using FDR emitted strong green fluorescence when exposed to UV light, while negative controls were unchanged (Fig. 2B); colour change was also observable with the naked eye under normal visible light. When performed with a fluorescently labelled probe, the pellets formed with positive reactions emitted green fluorescence, while neither pellets nor fluorescence was observed in the negative control tubes (Fig. 2C). The BKD-LAMP assay specifically amplified DNA extracted from *Renibacterium salmoninarum*. No amplification products were detected with the DNA from other tested bacterial strains, non infected fish tissues or no-template control. The detection limit of the BKD-LAMP and nested PCR assays for *R. salmoninarum* major soluble antigen protein p57 encoding gene was about 1 pg per reaction (dilution 10^{-6}), while the detection limit of the one-round PCR assay was

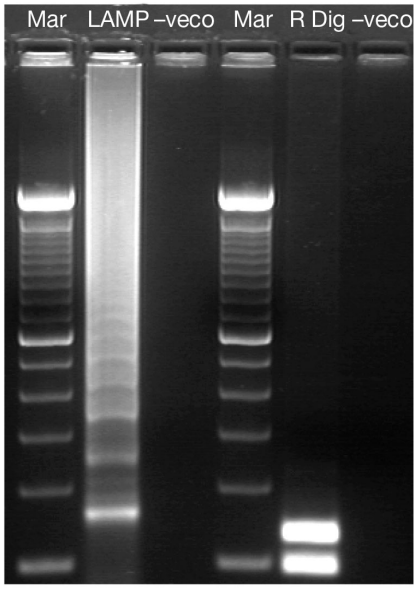

Fig. 1. *Renibacterium salmoninarum.* Loop-mediated isothermal amplification (LAMP) products and restriction analysis of *R. salmoninarum* LAMP product with *EcoR*V enzyme. Lane Mar: 100-base-pair DNA ladder; lane LAMP: amplified *R. salmoninarum* LAMP product showing a ladder-like pattern; lane R Dig: *R. salmoninarum* LAMP product digested with *EcoR*V with production of 90 bp and 120 bp bands; lane –veco: no template control

about 10 pg per reaction (dilution 10^{-5}) (Fig. 3). The BKD-LAMP assay successfully detected *R. salmoninarum* DNA from 20 infected kidney samples, which were also shown positive by PCR and nested PCR. Kidney samples from all 6 uninfected fish and the no template control were negative (Fig. 4).

DISCUSSION

Rapid detection of *Renibacterium salmoninarum* is fundamental to control measures for preventing the spread of the BKD. Although PCR assays are powerful, sensitive and efficient tools for diagnosis of BKD (Pascho et al. 2002), the requirement of a thermalcycler, an expensive and sophisticated instrument, has limited their application for field diagnostic tests.

In this study a one-step, real-time LAMP assay was developed for rapid diagnosis of BKD. The amplification is performed in a single tube and requires only a simple water bath or heating block to incubate the reaction mixture. Design of appropriate primers for LAMP is key for optimization of the assay because it requires 4 primers that recognize 6 distinct regions on the target DNA (Enosawa et al. 2003). For detection of *Renibacterium salmoninarum*, p57 protein is a good marker for active infection as it is the predominant cell

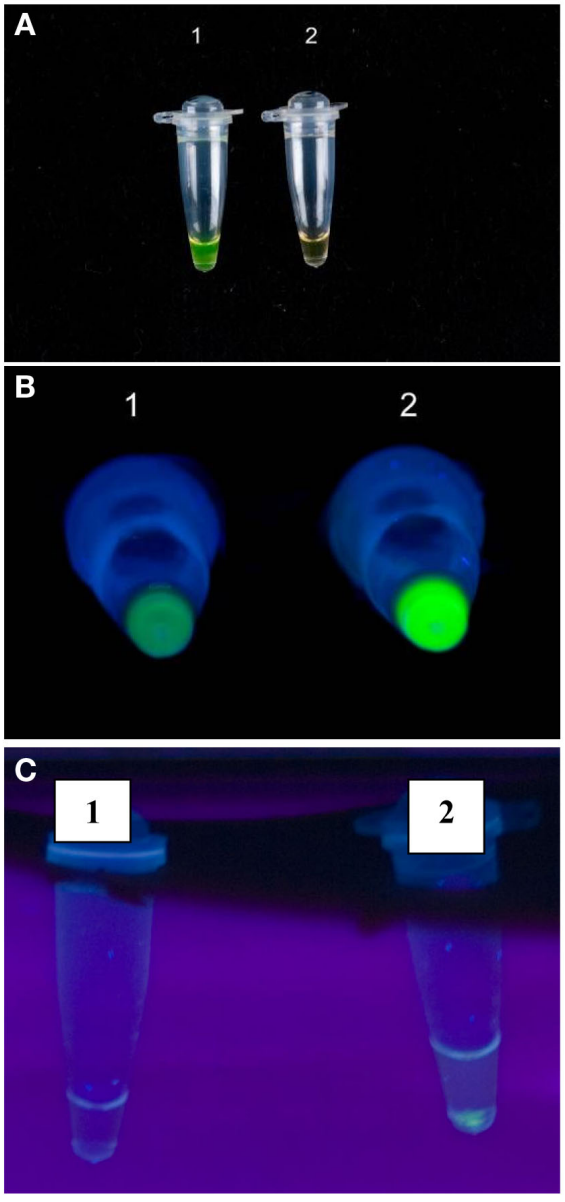

Fig. 2. Visual detection of BKD-LAMP product by using different naked eye detection methods: (A) Positive sample (1) with green colour using SYBR green I stain, and (2) negative sample with orange colour. (B) Negative sample (1) using FDR (no strong green fluorescence) and (2) positive sample with strong green fluorescence. (C) Negative reaction (1) using fluoresceinisocyanate-labelled probe (no pellet, no green fluorescence) and (2) positive reaction (pellet is fluorescing green)

surface and secreted protein produced by the bacterium (Getchell et al. 1985, Wiens & Kaattari 1989, Grayson et al. 1999). Consequently, most molecular diagnostic assays for *R. salmoninarum* are based on detection of the gene which codes for p57 (Brown et al. 1995, Miriam et al. 1997, Chase & Pascho 1998, Cook & Lynch 1999). We designed multiple LAMP primers

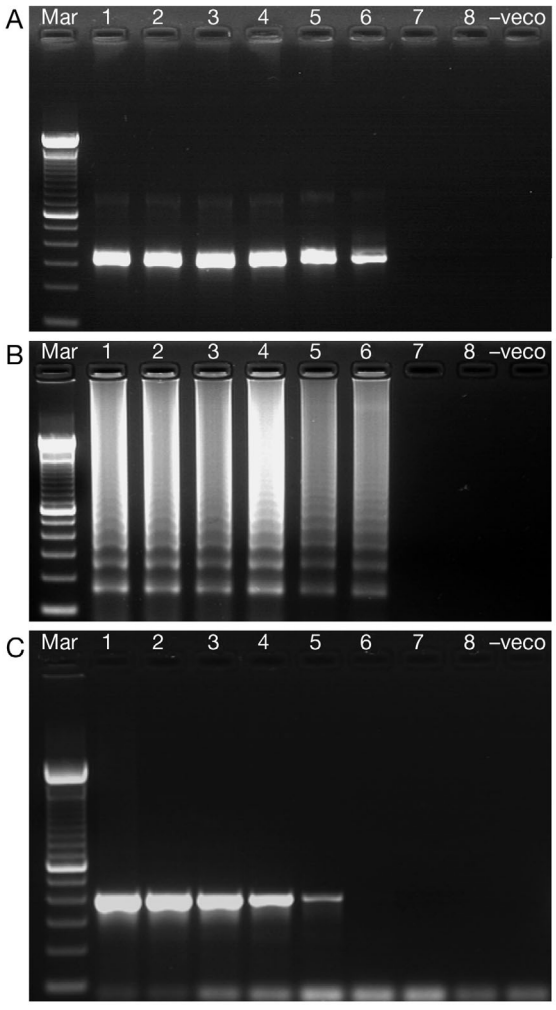

Fig. 3. (A) Sensitivity of nested PCR assay in detecting 383 bp *Renibacterium salmoninarum* DNA fragment. (B) Sensitivity of BKD-LAMP primers detecting *R. salmoninarum* DNA. (C) Sensitivity of one-round PCR assay detecting 372 bp *R. salmoninarum* DNA fragment. Lanes Mar: 100-base-pair DNA ladder; lanes 1–8: 10-fold serial dilutions of 1 µg *R. salmoninarum* DNA from 10^{-1}–10^{-8}; lanes –veco: no template control

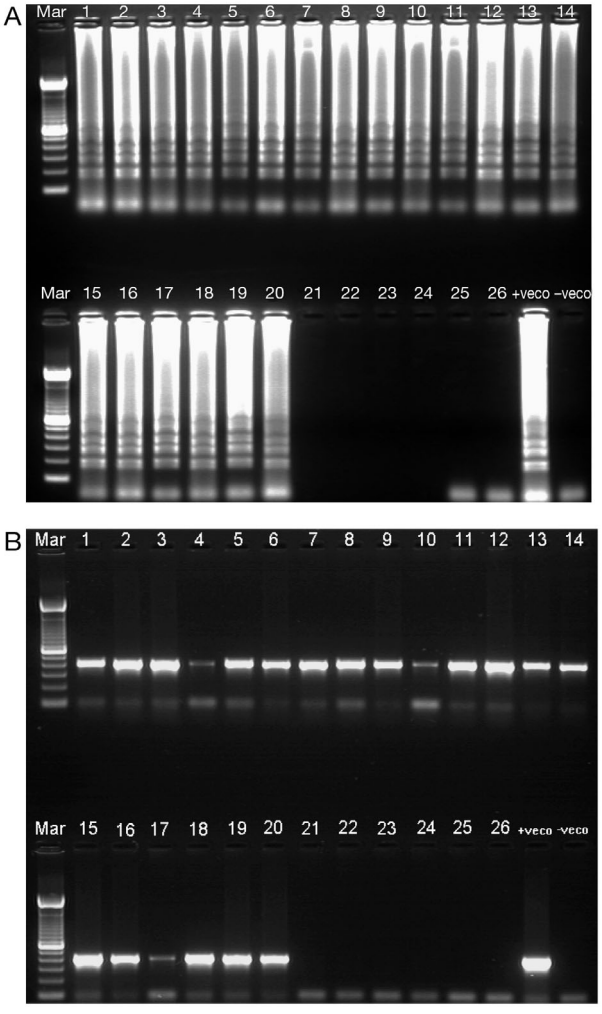

Fig. 4. (A) Feasibility of BKD-LAMP assay for detection of *Renibacterium salmoninarum* DNA from 20 infected kidney samples, with no amplification products from uninfected kidney samples. (B) BKD-PCR assay demonstrating the 372 bp fragment of *R. salmoninarum* DNA from 20 infected kidney samples, with no amplifications products from uninfected kidney samples. Lanes Mar: 100-base-pair DNA ladder; lanes 1–20: DNA from infected kidney samples; lanes 21–26: DNA from uninfected kidney samples; lanes +veco: DNA from *R. salmoninarum* as a positive control; lanes –veco: no template control

based on the major soluble antigen gene encoding p57. All primer combinations were able to detect *R. salmoninarum* DNA, but the optimal LAMP primer set used in this assay had highest sensitivity and specificity for detection of the target sequence. The BKD-LAMP assay was optimized to amplify *R. salmoninarum* DNA in 1 h at 63°C using a set of 4 or 5 primers. The amplification products when electrophoresed on a gel appeared in a ladder-like pattern, which arose from the formation of a mixture of stem loop DNAs of various stem lengths and cauliflower-like structures with multiple loops formed by annealing between alternately inverted repeats of the target sequence in the same strand (Thai et al. 2004). The identity of the amplicons was confirmed by *EcoR*V restriction enzyme digest.

The LAMP method was both highly specific and highly efficient, and, since it uses 4 primers that recognize 6 distinct sequences on the target DNA, its specificity is extremely high (Notomi et al. 2000). The specificity of the BKD-LAMP assay was confirmed by amplification of DNA from *Renibacterium salmoninarum* only and no amplification of DNA from a suite of other bacterial strains. The LAMP method also has an extremely high amplification efficiency due in part to its isothermal nature; there is no requirement for temperature changes to facilitate enzyme function or

inhibit the reaction during later stages of amplification, a typical problem with PCR (Notomi et al. 2000, Nagamine et al. 2001). The LAMP reaction produces a large amount of the byproduct magnesium pyrophosphate, which leads to turbidity in the reaction mixture. As the increase in turbidity correlates with the amount of DNA amplified, the LAMP reaction can be monitored in real-time with a turbidimeter (Mori et al. 2001). Also, the reaction can be monitored visually with SYBR Green I gel stain, which has high binding affinity to double stranded DNA, and changes from orange to green as the LAMP amplicons are produced (Karleson et al. 1995, Iwamoto et al. 2003). To avoid any contamination that may have arisen from opening the LAMP reaction tube to add SYBR Green, we tested a different visual indicator, FDR, which was added with the initial reagents. Calcein in the FDR combines with manganese and quenches it, but as pyrophosphate ions produced by the LAMP reaction preferentially bind with calcein and displace manganese, fluorescence occurs, indicating production of the target amplicons (Imai et al. 2007, Yoda et al. 2007). A third method of BKD-LAMP amplification product visualisation was the addition of cationic polymers to the reaction mixture. Low molecular weight PEI was used to form an insoluble PEI-LAMP product complex which contained the hybridized fluorescently labelled probe. PEI was selected because it is widely used as a nucleic acid precipitant for nucleic acid purification (Cordes et al. 1990). It also has the ability to form an insoluble complex with high molecular weight DNAs like LAMP amplification products, but does not form insoluble complexes with single-stranded anionic polymers of low molecular weight (Mori et al. 2006). All samples that assayed positive by visual inspection were also positive by gel electrophoresis.

The sensitivity of the BKD-LAMP assay was compared with one-round PCR and the nested-PCR assays recommended by OIE for diagnosis of BKD. BKD-LAMP assay had sensitivity equivalent to that of nested PCR and it was 10-times more sensitive than the one-round PCR assay. BKD-LAMP requires only a single tube (so there is negligible possibility of contamination), is complete within 1 h (compared to 5 h for nested PCR), needs only a simple water bath or heating block, does not need post-amplification processing by electrophoresis and is as sensitive as nested PCR assay. This higher sensitivity and superior performance should allow the BKD-LAMP assay to detect small amounts of *Renibacterium salmoninarum* DNA in infected samples, which will improve diagnosis of BKD in salmonids. We successfully detected *R. salmoninarum* DNA in samples of infected fish kidney and hence demonstrated the use of the BKD-LAMP assay on clinical specimens.

Since positive LAMP assay results can be seen by the unaided eye, rather than by electrophoresis, and denaturation of the template is not necessary, the LAMP reaction can be carried out in a simple water bath or heating block in the field. Additionally, the use of cheaper or disposable equipment for the assay would overcome difficulties in decontaminating instruments such as thermocyclers that would need to be transferred between premises for PCR assays (Dukes et al. 2006).

In conclusion, the BKD-LAMP assay represents a rapid, specific, sensitive and cost-effective technique with high potential for field deployment. The assay can be used in fish farms and small laboratories for more rapid detection of BKD, which would allow accelerated instigation of control measures.

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4 Discussion

Fish and shellfish diseases and emerging pathogens are a constant threat to the sustainability and economic viability of aquaculture. Growing economic importance of aquaculture worldwide has led to increasing interest in rapid, sensitive, specific and reliable methods for detection and identification of fish pathogens (Nilsson & Strom 2002). The timely detection of pathogens is necessary to enable appropriate measures to be taken to prevent and manage disease outbreaks (Teng et al. 2007).

Bacterial fish diseases and infections are very common in fish keeping and are one of the hardest health problems to effectively manage; they are troublesome to commercial producers as well as the recreational pond owner (Francis-Floyd 2005). As successful fish health management begins with prevention of disease rather than treatment, the key is early, accurate diagnosis of the pathogen (Teng et al. 2007). *Yersinia ruckeri*, the etiological agent of enteric redmouth disease (ERM) and *Renibacterium salmoninarum*, the agent of bacterial kidney disease (BKD), are highly contagious bacterial pathogens that cause severe economic losses in salmonid aquaculture worldwide (Austin & Austin 1993, Bruno 2004, Evendan et al. 1993, Fryer & Sanders 1981, Raida et. al. 2008). Diagnostic methods for ERM and BKD are well established and rely on basic techniques which include: isolation of bacteria on selective media, Gram-staining, biochemical characterization of the isolated bacteria, and confirmatory assays such as ELISA, immunofluorescence, restriction fragment length polymorphism (RFLP) and polymerase chain reaction (PCR) (Linde et al. 1999, Garcia et al. 1998, Gibello et al. 1999, Altinok et al. 2001, Temprano et al. 2001, Austin et al. 1983, Bullock & Stuckey 1975, Eliott & Barila 1987, Gudmundsdóttir et al. 1993, Jansson et al. 1996, Pascho et al. 2002).

However, these techniques have drawbacks which reduce their reliability and efficacy. There is a long incubation period before individual colonies can be observed on selective media (Benediktsdóttir et al. 1991). Conventional biochemical testing may fail to correctly identify some isolates (Ibrahim et al. 1993).

Complementary immunological techniques lack sensitivity and false positive serological reactions are reported (Austin et al. 1985, Armestrong et al. 1989). Molecular techniques such as PCR have the disadvantage of requiring laboratory equipment and trained personnel, and they are time consuming with a high risk of cross-contamination between samples, especially in nested PCR, and they are not well adapted for field based diagnosis (Belak & Ballagi-Pordany 1993). While real-time PCR assays have many advantages over conventional PCR methods including rapidity, quantitative measurement, lower contamination rate, higher sensitivity, higher specificity and easy standardisation (Mackay et al. 2002), they too require either precision instrumentation for DNA amplification or a complicated method for detection of amplified products (Parida et al. 2008). To overcome the disadvantages of these methods, considerable effort has been devoted to develop rapid sensitive, specific and reliable assays for diagnosis of ERM and BKD.

This project focussed on development and evaluation of two novel assays for detection of *Y. ruckeri* and *R. salmoninarum* based on loop-mediated isothermal amplification (LAMP). LAMP is a powerful, innovative gene amplification technique which is emerging as a simple, fast diagnostic tool for early detection and identification of microbial diseases (Parida et al. 2008). The developed LAMP assays were performed by incubation of the reaction mixtures at a constant temperature of 63°C in a regular water bath or heating block for 1hr.

Conventional *Taq* DNA polymerase is not suitable for LAMP as it is easily inactivated by tissue- and blood-derived inhibitors such as myoglobin, heme-blood protein complex and immunoglobulin G (Belec et al. 1998, Akane et al. 1994, Al-Soud et al. 2000, Johnson et al. 1995). Hence the use of *Bst* DNA polymerase, which has two distinct activities: linear target isothermal multimerisation and amplification, and cascade rolling-circle amplification (Hafner et al. 2001). There is no requirement for heat denaturation of the template DNA as this is achieved with high concentrations of betaine, a reagent that facilitates DNA strand separation through isostabilization (Baskaran et al. 1996, Nagamine et al. 2001). Betaine reduces base stacking and increase not only the overall rate of reaction but also target selectivity by significantly reducing amplification of irrelevant sequences (Rees et al. 1993, Baskaran et al. 1996, Rajendrakumar et al. 1997, Notomi et al. 2000). The mechanism of loop mediated isothermal amplification is similar to cascade rolling circle amplification, and is based on the principle of autocycling strand displacement DNA synthesis.

In the first step of the LAMP reaction, *Bst* polymerase synthesises new DNA between the F3 and B3 primers; this is the same reaction as standard PCR and requires homology between the primers and the template DNA. In the next step, the newly synthesised strands are recognised by the inner primers FIP and BIP to start loop mediated autocycling amplification (Kuboki et al. 2003) to produce stem-loop DNA structures with several inverted repeats of the target and cauliflower-like structures with multiple loops (Iwamoto et al. 2003). Occasionally, a different LAMP amplification pattern can appear as a result of linear target isothermal multimerisation and amplification, as LAMP primers and target DNA seem to randomly multimerize (Kuboki et al. 2003).

An appropriate target gene with a high degree of genotypic homology among the bacterial strains was selected for construction of the LAMP primers. The *Y. ruckeri* quorum sensing system encoding gene (*yruI / yruR*) was chosen, as it controls virulence gene expression through cell to cell communication. It was amplified by PCR from all *Y. ruckeri* strains and produced only one RFLP pattern which demonstrated a high degree of genotypic homogeneity across the *Y. ruckeri* strains (Temprano et al. 2001). For *R. salmoninarum*, the major soluble antigen protein (p57) coding gene was selected as the target. This antigen protein is a good marker for active infection as it is the predominant cell surface and secreted protein produced by the bacterium (Getchell et al. 1985, Wien& Kaattari 1989, Grayson et al. 1999).

Selection of highly sensitive and gene-specific primers is of the utmost importance for success of the LAMP reaction. Several primer sets were designed for both *Y. ruckeri* and *R. salmoninarum* but only the set that produce the best result was used for each LAMP assay. Two outer and two inner primers, and a fluorescently labelled probe were designed for both pathogens. These four primers and probe recognize seven different regions on the target sequence, which not only improves the specificity of the assay but also minimizes the probability of false positives (Notomi et al. 2000, Nagamine et al. 2002, Maeda et al. 2005). In contrast to the single band of PCR, LAMP assays generate a ladder-like pattern when electrophoresed on an agarose gel, due to the presence of cauliflower-like structures with multiple loops (see publication 1, Fig 4 and 5 & publication 2, Fig 3 and 4) formed by annealing of alternately inverted repeats of the target in the same strand (Notomi et al. 2000 ,Thai et al. 2004).

DISCUSSION

The progress of the LAMP reaction can be easily monitored in real-time with spectrophotometric analysis using real-time turbidimeter, which records turbidity as optical density (O. D.) at 400 nm every 6s. The increase in turbidity is a unique characteristic of the LAMP reaction and is due to formation of white magnesium pyrophosphate. Real-time turbidity measurement is not possible for regular PCR due to hydrolysis of pyrophosphate at the high temperatures used in the denaturation step (Mori et al. 2004, Parida et al. 2008). There is a linear correlation between the turbidity and the amount of amplified DNA. The turbidity is seen when DNA yield is ≥ 4µg, when pyrophosphate ion concentration is >0.5 ppm. The LAMP reaction typically produces a DNA yield of ≥ 10µg compared to 0.2µg in PCR in 25µl reaction scale (Nagamine et al. 2001, Parida et al. 2008). The point at which a LAMP assay can be judged as positive varies form pathogen to pathogen, depending on the primer set and nature of the selected template. The cut-off values of positivity for the *Y. ruckeri* and *R. salmoninarum* assays were determined by measuring the time at which turbidity increased above a threshold value (0.1), which was twice the average turbidity of the negative control in several replicates.

One of the most attractive characteristics of the LAMP assay is the potential for visual positive/negative assessment (see publication 1, Fig 2 publication 1 & publication 2, Fig 2). This eliminates the need for laborious and time consuming post amplification operations such hybridization or electrophoresis (Iwamoto et al. 2003). Turbidity can be qualitatively assessed after a short centrifugation to deposit the magnesium pyrophosphate in the bottom of the reaction tube (Mori et al. 2001). The amplified DNA can be visualized by addition of the intercalating dye SYBR Green I to the LAMP products. There is a colour change from orange to green in positive reactions (see publication 1 Fig 2, tube 5 and 6 & publication 2 Fig 2, A). Although SYBR Green I has a high binding activity to DNA (Karlsen et al. 1995), the colour change is discernable in LAMP assays but not in regular PCR due to the high DNA yields of LAMP (≥ 10µg compared to 0.2µg in PCR in 25µl reaction scale Nagamine et al. 2001, Parida et al. 2008). A third method of visualization is to precipitate the DNA directly with low molecular weight polyethylenimine (PEI) (Cordes et al. 1990). PEI forms an insoluble complex with high molecular weight DNA such as LAMP amplification products.

The PEI-LAMP product complex contains the hybridized fluorescently labelled probe (see publication 1 Fig 2 tubes 1 and 2 & publication 2 Fig 2, C). Since PEI strongly inhibits the LAMP reaction, PEI must be added to the reaction mixture after the LAMP reaction has taken place (Mori et al. 2006).

As an alternative to SYBR Green I stain and PEI visualization, fluorescence detection reagent (FDR) was used for determination of the ERM and BKD LAMP assay results. This was to avoid potential carry-over contamination which may arise by opening the LAMP reaction tubes to add SYBR Green I stain or PEI, as FDR is added to the reaction mixture prior to amplification. FDR contains calcein which remains quenched when bound with manganese ions. However, as the LAMP reaction progresses, pyrophosphate ions are produced which bind to and remove manganese from the calcein, which results in fluorescence. This emission is intensified as calcein combines with magnesium ions, which indicates DNA amplification (Imai et al. 2007, Yoda et al. 2007). This fluorescence can be observed on a UV transilluminator (see publication 1 Fig 2, tube 3 and 4 & publication 2 Fig 2, B).

The specificities of the ERM and BKD LAMP assays were confirmed by restriction enzyme analysis of the amplified product with *HphI* and *EcoRV* respectively. Both enzymes produced the expected patterns for amplification of the target genes. Specificity of the LAMP assays was further confirmed by use of different bacterial strains and clinical samples which showed no cross-reactivity.

The specificity and amplification efficiency of the LAMP assays are extremely high. LAMP proceeds more rapidly than regular PCR as there is no time required for thermal cycling, and inhibition reactions at later stages are less likely to occur (Notomi et al. 2000, Nagamine et al. 2001). The LAMP assays are also about 10-fold more sensitive than PCR. Moreover, they detected the target pathogens in the clinical fish specimens with high sensitivity, specificity, and rapidity compared to microbial, biochemical culture methods which required 2 days to 4 weeks.

In conclusion, the developed ERM and BKD LAMP assays are easy to perform, cost effective, sensitive and rapid diagnostic techniques for assessment of *Y. ruckeri* and *R. salmoninarum* infections. These assays should be immediately applicable for routine diagnostics in laboratories and fish farms and could potentially be used for preliminary field screening and surveillance of both *Y. ruckeri* and *R. salmoninarum*.

5 Summary

Development of Loop Mediated Isothermal Amplification (LAMP) assays for detection of *Yersinia ruckeri*, the causative agent of Enteric Redmouth Disease (ERM) and *Renibacterium salmoninarum*, the causative agent of Bacterial Kidney Disease (BKD) in Salmonids

Loop-mediated isothermal amplification (LAMP) is a powerful, innovative gene amplification technique which is emerging as an easy to perform and rapid diagnostic tool for detection and identification of microbial diseases.

Early and accurate detection is of paramount importance concerning the diagnosis of the highly contagious bacteria *Yersinia ruckeri* and *Renibacterium salmoninarum*. An easy to perform diagnostic technique is also required if assays should be carried out in field inquiries. The method provides a single step, reaction tube assay only requiring a temperature-controlled water bath. In the experiments of the presented study, LAMP assays were conducted for *Y. ruckeri* (the pathogen causing Enteric Redmouth Disease, ERM) and *R. salmoninarum* (the pathogen causing Bacterial Kidney Disease, BKD). In the case of ERM, the amplified target was a sequence stretch of the gene *yruI/yruR* encoding the quorum sensing system which controls the expression of virulence genes. In the case of BKD, a sequence stretch of the gene encoding the major soluble antigen protein (p57) in *R. salmoninarum* was amplified. This protein indicates an active infection because it is the predominant cell surface-associated and secreted protein by the bacterium.

The newly established LAMP assays for ERM and BKD enabled amplification of a stretch of each target gene at a temperature of 63°C in less than one hour, with no need of thermal cycling. Assays are carried out with a reaction mix containing four specific primers, the sample and *Bst* DNA polymerase. Amplification products were detected by visual inspection, agarose gel electrophoresis, and in real-time using a turbidimeter. Assays specificity were demonstrated using DNAs from other related bacteria yielding no amplification product, and by restriction analysis with *HphI* and *EcoRV* enzymes producing a specific bands´ pattern of the amplified products.

SUMMARY

Compared to regular PCR-based detection methods, the developed LAMP assays were consistently faster and ten-fold more sensitive. A safe detection of the specific sequence stretches with high specificity and efficiency was possible using DNA isolated both from bacterial extracts and from clinical fish specimens. These findings showed that LAMP assays are more sensitive than other detection methods such as time consuming culture methods and PCR assays.

In conclusion, for the first time LAMP assays developed and optimised to detect *Y. ruckeri* and *R. salmoninarum* were introduced as diagnostic tools. In comparison with the performance of already established diagnostic methods, LAMP assays are superior in sensitivity, rapidness, specificity, and cost-efficiency. Both assays are highly appropriate for application in field inquiries to monitor the spread of ERM and BKD.

6 Zusammenfassung

Entwicklung von Testsystemen auf der Basis der "Loop Mediated Isothermal Amplification (LAMP)" Methode zum Nachweis von *Yersinia ruckeri*, dem Erreger der Rotmaulseuche (ERM) und von *Renibacterium salmoninarum*, dem Erreger der bakteriellen Nierenkrankheit (BKD)

„Loop-mediated isothermal amplification (LAMP)“ ist eine neuartige Technik Gensequenzen zu amplifizieren, die als leicht anwendbare und schnell durchzuführende Methode immer mehr Verbreitung beim Nachweis und der Erkennung mibrobiell bedingter Erkrankungen findet.

Ein schneller und präziser Nachweis der hochansteckenden Bakterien *Yersinia ruckeri* und *Renibacterium salmoninarum* ist von großer Bedeutung für die Eindämmung der von ihnen verursachten Krankheiten Rotmaulseuche (enteric redmouth disease, ERM) und bakterielle Nierenerkrankung (bacterial kidney disease, BKD). Darüberhinaus wird ein leicht durchzuführender Test benötigt, falls eine Diagnostik unter den Bedingungen von Felduntersuchungen erfolgen soll. Die Methode besteht aus einem einzigen Reaktionsschritt, der in einem 1,5 mL Reaktionsgefäß erfolgt und für den lediglich ein temperierbares Wasserbad benötigt wird. In den Experimenten der vorliegenden Arbeit werden LAMP Tests zum Nachweis von *Y. ruckeri* und *R. salmoninarum* entwickelt und optimiert. Im Falle von ERM wurde ein Sequenzabschnitt des Gens *yruI/yruR* amplifiziert das, die Expression der Virulenzgenen kontrolliert. Im Falle der BKD wurden Sequenzabschnitte des Genes, das das "major soluble antigen protein (p57)" von *R. Salmoninarum* kodiert, vervielfältigt. Dieses Protein ist ein hervorragender Marker für eine aktive Infektion, der überwiegend auf der Zelloberfläche der Bakterien auftritt bzw. von diesen sezerniert wird. Die neu etablierte LAMP Methode für ERM und BKD ermöglicht die Vervielfältigung von Genabschnitten bei einer Temperatur von 63 °C in weniger als einer Stunde und ohne die bei PCR Reaktionen übliche Abfolge von Temperatur-Zeit-Zyklen. Die Tests wurden mit einem Reaktionsansatz, der vier Oligonukleotidprimer, die Probe und *Bst* DNA Polymerase enthielt, durchgeführt.

Entstandene Amplifikationsprodukte konnten visuell durch eine Farbänderung des Reaktionsansatzes, in der Agargelelektrophorese sowie in Echtzeit mithilfe eines Turbidimeters identifiziert werden. Die Spezifitätskontrolle der Tests wurde zum einen dadurch dokumentiert, dass die Verwendung der DNA anderer Erreger kein Amplifikationsprodukt ergab, zum anderen aufgrund der spezifischen Bandenmuster, die nach Spaltung der Amplifikationsprodukte durch die Restriktionsenzyme *HphI* und *EcoRV* auftraten. Im Vergleich zu den üblichen PCR Methoden lieferte die hier entwickelte LAMP Methode den schnelleren und zehnfach sensitiveren Erregernachweis. Die spezifischen Genabschnitte konnten sowohl bei DNA, isoliert aus Bakterienkulturen als auch bei DNA, isoliert aus klinisch erkrankten Fischen vervielfältigt werden. Diese Befunde zeigen, dass LAMP Tests wesentlich sensitiver sind als zeitaufwendige Kulturmethoden oder herkömmliche PCR Techniken. Somit wurden in der vorliegenden Arbeit auf LAMP basierende Testsysteme für ERM und BKD entwickelt und zum erstenmal in die Diagnostik eingeführt. Es zeigt sich, dass die LAMP Technik bezüglich Sensitivität, Schnelligkeit, Spezifität und Preis/Leistungs-Verhältnis den herkömmlichen Nachweismethoden überlegen ist. Beide Testsysteme sind, aus den bereits genannten Gründen, für den Einsatz in Felduntersuchungen, mit deren Hilfe die Ausbreitung von ERM und BKD überwacht werden soll, besonders gut geeignet.

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